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NOVEL INSIGHTS INTO REGULATION OF PHOTOSYNTHETIC LIGHT REACTIONS

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TABLE OF CONTENTS

ABBREVIATIONS

ATP	Adenosine triphosphate
CET	Cyclic electron transport around PSI
Cyt b_6f	Cytochrome b_6f complex
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DTT	1,4-Dithiothreitol
F'	Chlorophyll fluorescence under actinic light illumination
Fd	Ferredoxin
Fm	Maximal chlorophyll fluorescence in dark acclimated leaf
Fm'	Maximal chlorophyll fluorescence under actinic light illumination
FNR	Ferredoxin- $NADP^+$ reductase
Fo	Minimal chlorophyll fluorescence in dark acclimated leaf
Fv	Variable fluorescence, (Fm – Fo)
GL	Growth light intensity
HA	High intensity actinic light
HL	High light intensity
LA	Low intensity actinic light
LET	Linear electron transfer
LHCI	Light harvesting complex I
LHCII	Light harvesting complex II
LL	Low light intensity
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced $NADP^+$
NDH	NAD(P)H dehydrogenase-like complex
NPQ	Non-photochemical quenching of excitation energy
OEC	Oxygen evolving complex

P680	Reaction center Chlorophyll of PSII
P700	Reaction center Chlorophyll of PSI
PBCP	PSII core phosphatase
PC	Plastocyanin
PGR5	Proton gradient regulation 5
Pm	Maximal absorbance change at 830 nm induced by full oxidation of P700
PQ	Plastoquinone
PQH_2	Plastoquinol
PSI	Photosystem I
PSII	Photosystem II
qE	Energy-dependent quenching
qI	Photoinhibition-dependent quenching
qT	State transitions-dependent quenching
STN8	State transition kinase 8
TAP38	Thylakoid-associated phosphatase 38
WT	Wild-type

ABSTRACT

In oxygen-evolving photosynthesis, the balanced excitation of photosystem (PS) I and PSII complexes is required for safe and efficient utilization of light energy. During the course of evolution, plants have acquired dynamic regulatory mechanisms to control the excitation energy transfer and distribution as well as the photosynthetic electron transport. Concerted action of the regulatory mechanisms is required for the optimization of photosynthetic efficiency under changing light conditions.

Short-term changes in light intensity alter the phosphorylation pattern of the PSII core and its light harvesting antenna (LHCII) proteins. Reversible phosphorylation of the LHCII proteins mediates the excitation energy distribution between the two photosystems in the so called state transitions, which are known to be dependent on the redox status of the plastoquinone pool. In this study, the analysis of the kinase mutants (*stn7* and *stn8*) and the phosphatase mutants (*pph1/tap38* and *pbcp*) revealed that the PPH1/TAP38 phosphatase is required for the dephosphorylation of LHCII in high light (HL). In *pph1/tap38* mutant, both the PSII core and LHCII proteins are simultaneously phosphorylated upon transfer to high light, which lead to increased excitation energy distribution towards PSI, thus mimicking the effect of the state 2 light. It is also shown that the PGR5 protein, which is essential for the generation of a transthylakoid proton-gradient, is likely to be involved in the regulation of the thylakoid protein phosphorylation upon increasing light intensities. Indeed, a close cooperation between the redox and proton-gradient dependent regulatory mechanisms is required for maintaining functionality of the photosynthetic machinery.

Novel information on the effects of PSII photoinhibition on the dissipation of excitation energy in the thylakoid membrane is likewise provided. It is also demonstrated that the proton gradient-dependent and PGR5-mediated control of electron transfer via the Cyt b_6f complex together with controlled photoinhibition of PSII limit the electron flow from PSII to PSI, thereby providing protection for the PSI complex against photodamage. Further, PSII photoinhibition is shown to lead to the phosphorylation-independent loss of thylakoid membrane lateral heterogeneity, thus allowing the oxidized P700 to act as an energy quencher. In addition, the PsbS-dependent non-photochemical quenching of excess excitation energy is revealed to enhance the spillover of the excitation energy towards PSI at low lumenal pH. Collectively, the evidence provided in this thesis has highly improved our knowledge concerning photoprotective mechanisms inside the plant cell.

TIIVISTELMÄ

Fotosynteesin valoreaktiot muuttavat valoenergian kemialliseen muotoon. Reaktiot perustuvat kahteen perättäiseen valoenergiaa hyväksikäyttävään proteiinikompleksiin (valoreaktiot I ja II) ja niiden väliseen elektroninsiirtoketjuun. Jotta yhteyttämisen valoreaktiot voivat toimia tehokkaasti ilman haitallisia sivureaktioita, kaikkien osareaktioiden on tapahduttava juuri oikeassa paikassa ja oikeaan aikaan. Evoluution kuluessa kasvit ovat kehittäneet joukon säätelymekanismeja, joiden turvin valoreaktiot pystyvät toimimaan muuttuvissa valo-olosuhteissa tuhoamatta fotosynteettistä koneistoa.

Valoreaktio II:n reaktiokeskusproteiinien ja valoenergiaa keräävän haavin proteiinien fosforylaatio säätelee valoreaktioiden toimintaa lyhyellä aikavälillä. Proteiinien fosforylaatiota säätelevät puolestaan sekä valon määrä että laatu, mutta kirkkaassa valossa tapahtuvan valohaaviproteiinien defosforylaation merkitys on pysynyt mysteerinä. Väitöskirjassani käytin hyväksi TAP38/PPH1 fosfataasimutanttia, joka ei pysty defosforylomaan valohaavin proteiineja. Osoitin, että valohaavin defosforylaatio korkeassa valossa takaa valoreaktioiden välisen viritystasapainon. Osoitin myös, että PGR5 proteiini liittyy valohaaviproteiinien defosforylaatioon. Saamani tulos osoitti, että valoreaktioiden säätelymekanismit toimivat keskinäisessä vuorovaikutus-suhteessa.

Mikäli säätelymekanismien kapasiteetti on riittämätön, valoreaktiot saattavat vaurioitua. Valorektio II vaurioituu helposti, mutta vaurio korjataan yleensä nopeasti. Tämän vuoksi valoreaktio II:n toiminta estyy ainoastaan tilanteessa, jossa korjausmekanismi ei toimi normaalisti. Valoreaktio I on yleensä suojattu valon aiheuttamalta vauriolta, mutta voi vaurioitua pysyvästi, mikäli elektronien virta valoreaktio II:lta ylittää valoreaktio I:n toimintakapasiteetin. Väitöskirjatyössäni osoitin, että valoreaktio II:n vaurioituminen suojaa valoreaktio I:stä pysyvältä vauriolta rajoittamalla elektroninsiirtoa. Valoreaktio II:n vaurioituminen ei ainoastaan vähennä elektroninsiirtoa, mutta myös aktivoi ylimääräisen viritysenergian haihduttamisen. Osoitin työssäni, että valoreaktio II:n vaurioituminen johtaa fotosynteettisten proteiinikompleksien uudelleen järjestäytymiseen viherhiukkasen sisäosien yhteyttävissä kalvoissa sallien tehokkaan viritysenergian haihduttamisen valorektio I-kompleksissa. Tässä väitöskirjassa esitetty todistusaineisto osoittaa lisäksi, että viherhiukkasen sisäosien matala pH aktivoi ylimääräisen energian haihduttamista valoreaktio I:ssä. Kokonaisuudessaan väitöskirjani pureutui fotosynteesin säätelyn perusmekanismeihin ja mekanismien välisiin vuorovaikutussuhteisiin. Tuottamani informaatio auttaa ymmärtämään, miten yhteyttämisen valosta riippuvat molekyylitason reaktiot sopeutuvat muuttuviin ympäristöolosuhteisiin sekä osaltaan myös auttaa ymmärtämään tapoja, joilla yhteyttämistä pystytään entistä tehokkaammin hyödyntämään ihmiskunnan hyväksi tulevaisuuden globaalien ongelmien ratkaisuissa.

1. INTRODUCTION

1.1 Chloroplast structure and its membrane system

Chloroplasts are specialized organelles, capable of carrying out photosynthesis in plant and algal cells (Figure 1). Each chloroplast is surrounded by an envelope with an outer and inner membrane and encloses the chloroplast stroma. Thylakoid membrane sacs in the soluble stroma comprise the inner membrane network and enclose a separate compartment, the thylakoid lumen. This photosynthetic unit harvests and converts light energy of sun into chemical energy through photosynthetic reactions. Photosynthetic light reactions occur in well-organized thylakoid membrane network. The chemical energy produced by the photosynthetic light reactions is stored in the form of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). These energy carriers are then used in the soluble stroma to reduce carbon dioxide into sugars by the enzymes of the Calvin-Benson-Bassham cycle.

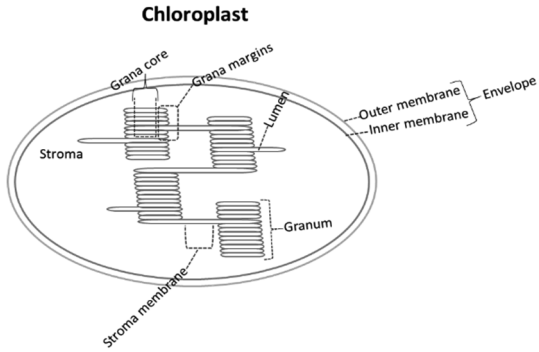

Figure 1. Sketch of the chloroplast structure showing the thylakoid network composed of highly stacked grana thylakoids and unstacked stroma thylakoids. Light-dependent reactions occur in the thylakoid membrane. Carbon fixation reactions take place in the soluble stroma.

Chloroplast thylakoids form stacks of membranes referred to as grana (singular: granum) (Mustárdy and Garab 2003). A granum can be further divided into a grana core, grana margins and the stroma-exposed end-membranes of grana (Albertsson 2001). Grana thylakoids are connected by the stroma-exposed, unstacked thylakoids, also called stroma lamellae (Arntzen 1978). Thylakoid membrane encloses a continuous soluble space called the thylakoid lumen (Shimoni et al., 2005).

The thylakoid membrane is embedded with four major integral multiprotein complexes (photosystem II (PSII), cytochrome b_6f complex (Cyt b_6f), photosystem I (PSI)), which harvest light-energy and mediate light-dependent electron transfer reactions of photosynthesis, and the ATP synthase (Figure 2). A minor thylakoid

membrane protein complex, the NDH-1 like complex, is dedicated to cyclic electron transfer around PSI (Peltier et al., 2016). The PSII complex together with its light-harvesting antenna complex (LHCII) organized as super- and mega complexes enriched in the grana thylakoid membranes (Kouril et al., 2012). The PSI complex with its light-harvesting antenna complex (LHCI) and the ATP synthase are localized in the grana margins and the stroma lamellae (Anderson 1989; Albertsson et al., 1990). The Cyt b_6f complex is likely to be more or less equally distributed in the stacked and unstacked regions of the thylakoid membrane network (Nevo et al., 2012).

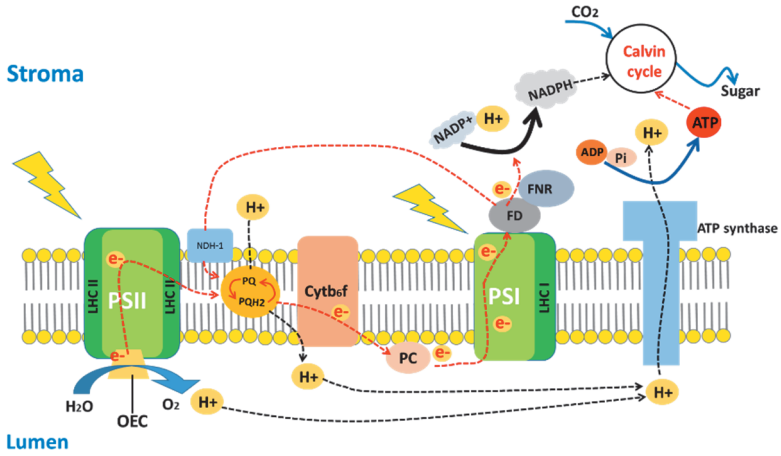

Figure 2. A simplified scheme including the linear pathway and the NDH-1 dependent cyclic pathway of the photosynthetic electron transfer chain. ADP = adenosine diphosphate; ATP = adenosine triphosphate; Cyt b_6f = cytochrome b_6f complex; Fd = ferredoxin; FNR = ferredoxin-NADP$^+$ reductase; LHCI = light harvesting complex I; LHCII = light harvesting complex II; NADPH = reduced nicotinamide adenine dinucleotide phosphate; NDH-1 = NAD(P)H dehydrogenase-like complex; OEC = oxygen evolving complex; PC = plastocyanin; PQ = plastoquinone; PQH_2 = plastoquinol; PSI = photosystem I; PSII = photosystem II.

1.2 Light harvesting and electron transport in the thylakoid membrane

In plants, LHCs of PSII and PSI harvest the light energy (Fork and Satoh 1986; Mullineaux and Emlyn-Jones 2005). The light energy harvested by antennae excites the reaction center chlorophyll molecules, P680 in PSII and P700 in PSI. The excited PSII reaction center chlorophyll molecule ($P680^*$) donates an electron to a plastoquinone (PQ) molecule. A PQ molecule, after receiving two electrons from two sequential excitations and charge separations in the reaction center of PSII plus two protons (H^+) from the soluble stroma, is converted to plastoquinol (PQH_2), which is then released from the PSII complex. In the next step, the Cyt b_6f complex oxidizes PQH_2 in the Q-cycle, which, in turn, leads to the net pumping of protons across the thylakoid membrane.

Plastocyanin (PC) a mobile lumenal electron carrier, then transfers the electrons from the Cyt b_6f complex to the P700 reaction center of the PSI, which is again excited by light. PSI finally transfers electrons to ferredoxin (Fd). Ferredoxin-$NADP^+$- reductase (FNR) mediates the transfer of electrons from reduced Fd to $NADP^+$, which results in the production of reducing power (NADPH).

The oxidized $P680^+$ is rapidly reduced by a tyrosine residue (Yz) of the D1 protein, thus returning to its ground state P680 and is again ready for excitation. $P680^+$ is the strongest biological oxidant known and it eventually withdraws electrons from the manganese-oxygen-calcium cluster of the oxygen evolving complex of PSII. The manganese-oxygen-calcium cluster, in turn, withdraws electrons from water molecules on the lumenal side of the thylakoid membrane and releases oxygen as a byproduct. Together, the photosynthetic water splitting and the electron transfer along the electron transport chain create a proton gradient across the thylakoid membrane, which drives ATP synthesis in the process of photophosphorylation. Thus, the linear electron transport (LET) where both PSII and PSI function in series, ultimately results in the production of NADPH and ATP. The LET pathway alone cannot satisfy the ATP/NADPH ratio to meet the environmental demands and hence, plants have developed alternative electron transport pathways to adjust the ratio of ATP and NADPH according to metabolic demands.

In cyclic electron transport (CET) around the PSI, electrons are transported from the accepter side of PSI to the PQ pool. This pathway promotes the proton translocation across the thylakoid membrane and results in the production of ATP without the net production of the reducing power (NADPH). In higher plants, two distinct pathways of the PSI CET have been proposed. One pathway is proposed to be mediated by the PROTON GRADIENT REGULATION 5 (PGR5) and the PGR5-LIKE PHOTOSYNTHETIC PHENOTYPE 1 (PGRL1) proteins (Munekage et al., 2002; DalCorso et al., 2008). Another pathway is mediated by the chloroplast NADH dehydrogenase-like (NDH-1) complex functioning also in chlororespiration (Peng and Shikanai 2011). As the cyclic PSI electron transfer is difficult to measure directly, the PGR5-dependent cyclic electron flow still remains elusive. As mentioned, both the putative PGR5 and the NDH-1 dependent PSI CET pathways are coupled to the generation of the transthylakoid proton gradient (Munekage et al., 2002; Peltier et al., 2016; Suorsa et al., 2012). Generation of the proton gradient across the thylakoid membrane is, in turn, coupled with downregulation of photochemistry by non-photochemical quenching (NPQ), the thermal dissipation of absorbed light energy from PSII antennae (Müller et al., 2001) and with the control of electron flow via the Cyt b_6f complex (Kramer and Evans 2011).

1.3 Major protein complexes in the thylakoid membrane

1.3.1 Structure and function of the PSII-LHCII complex

Photosystem II, a complex of proteins that never relax. The PSII complex utilizes absorbed light energy to catalyze the electron transfer from water molecules and through redox-active cofactors eventually to the PQ pool (Figure 3). The crystal structure of PSII has been determined at an atomic level of 3.8-1.9 Å (Guskov et al., 2009; Kawakami et al., 2011; Suga et al., 2015; Wei et al., 2016). Detailed studies of the PSII complex at an atomic level have enabled the analysis of protein subunits and cofactors of PSII. The most active PSII is the PSII-LHCII supercomplex, which is composed of the PSII core and LHCIIs and has a molecular weight of 1400 kDa (Caffarri et al., 2009).

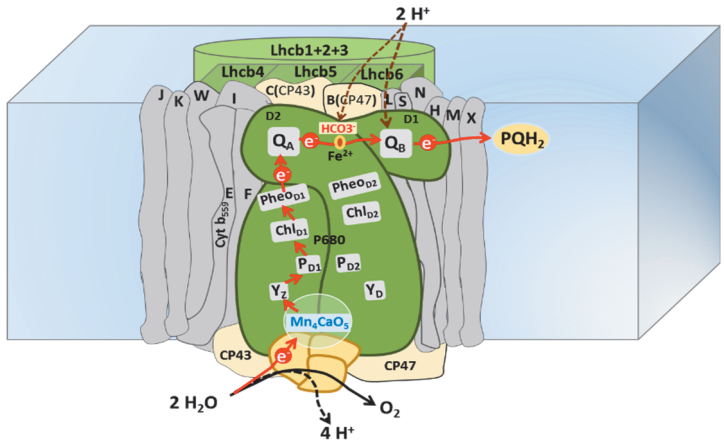

Figure 3. Schematic drawing of PSII-LHCII in higher plants.

Each monomeric PSII core complex is composed of almost 40 proteins (Shi and Schröder 2004; Shi et al., 2012). The PSII core proteins include two homologous protein subunits known as D1 and D2, which bind the reaction center chlorophyll P680 and other redox-active cofactors. Moreover, the PSII core complex includes the PsbI subunit (Loll et al., 2005), the heterodimeric cytochrome b_{559} (Dau et al., 2012) and two closely related chlorophyll binding proteins, called CP43 and CP47 (Dekker and Boekema 2005; Dang et al., 2008; Nosek et al., 2016). This reaction center part of PSII is surrounded by several low molecular weight subunits. The functionality of these subunits still remains elusive but each protein seems to increase the stability of the complex (Rokka et al., 2005).

The PSII core complex harbors several pigments (chlorophylls (a/b), carotenoids, pheophytins, quinones and hemes) and inorganic cofactors (Mn^{n+}, Ca^{2+}, Cl^-, HCO_3^- and

$Fe^{2+/3+}$) as essential drivers of electron transport from water to PQ (Umena 2011). In higher plants and green algae, the oxidation of water molecules occurs at the catalytic center, the manganese-oxygen-calcium cluster (Mn_4O_5Ca), which is localized on the lumenal side of PSII and is stabilized by extrinsic proteins (PsbO, PsbQ, PsbP and PsbR) (Ifuku et al., 2010; Kawakami et al., 2011; Ifuku and Noguchi 2016).

The PSII core is surrounded by the LHCII complex, which is composed of six different polypeptides. LHCII subunits belong to a super-gene family of the lhcb (light-harvesting complex) (Jansson 1999), which binds the pigments: chlorophyll a/b, carotenoids, and xanthophylls in different ratios. The absorbed light energy by LHCII is transferred to the PSII core, where it is converted into chemical energy. In plants and green algae, the LHCII is organized in two layers of protein complexes, i.e., (*i*) the outer, most abundant major LHCII antenna and (*ii*) the inner, less abundant minor LHCII antenna (Dekker and Boekema 2005). In green plants, there are three major LHCII proteins, encoded by three genes: lhcb1, lhcb2 and lhcb3, which form homo- and heterodimers. Three minor monomeric LHCII proteins, also called CP29, CP26 and CP24, are encoded by the lhcb4, lhcb5 and lhcb6 genes, respectively (Jansson 1994). Single particle electron microscopy analysis has revealed that a variable number of LHCII proteins can associate with the dimeric PSII core complex to form so-called PSII-LHCII supercomplexes. The LHCII antenna is composed of two to three LHCII monomers (CP29, CP26 and CP24) which are bound directly to the PSII core and either strongly and/or moderately bound LHCII trimers at the periphery of PSII-LHCII supercomplex (Boekema et al., 1998; Minagawa 2011; Kouril et al., 2012; Wei et al., 2016).

1.3.2 Photodamage and repair-cycle of the PSII-LHCII supercomplex

Although light is required for photosynthesis, light is also dangerous to the photosynthetic apparatus (Aro et al., 1993; Tyystjärvi and Aro 1996; Takahashi et al., 2010; Takahashi and Badger 2011). When plants are exposed to excess light conditions, the PSII complex may be damaged. Photodamage is targeted mainly to the reaction center protein D1. After photodamage, PSII activity is restored efficiently by a repair cycle in the thylakoid membrane.

The PSII repair cycle involves multiple steps; (*i*) the phosphorylation of the PSII core protein subunits by the STN8 kinase, damage and dissociation of the PSII-LHCII supercomplex and monomerization of the PSII core dimer in the grana stacks, (*ii*) the lateral migration of the photodamaged PSII monomer from the grana stacks to the stroma lamellae, (*iii*) dephosphorylation of the PSII core proteins by the PBCP phosphatase and the proteolytic degradation of the photodamaged D1, (*iv*) the

replacement of the photodamaged D1 with a newly-synthesized D1 protein and the reassembly of the PSII core monomer and (*v*) the migration of the newly synthesized PSII core monomers from stroma lamellae back to grana stacks, and dimerization and reformation of the PSII-LHCII supercomplex (Aro et al., 1993; Lindahl et al., 1996; van Wijk et al., 1996; Baena-Gonzáles et al., 1999; Zhang et al., 1999; Haussuhl et al., 2001; Aro et al., 2005; Tikkanen et al., 2008b; Järvi et al., 2015; Theis and Schroda 2016). In PSII photoinhibition studies, the antibiotic lincomycin is often used to inhibit protein translation and thus the PSII repair cycle in chloroplasts (Tyystjärvi et al., 1992).

1.3.3 Reversible phosphorylation of the PSII-LHCII proteins

Several PSII core and LHCII proteins from thylakoid membranes undergo light- and redox-dependent reversible phosphorylation-dephosphorylation cycles (Rintamäki et al., 1997, 2000; Aro and Ohad 2003; Tikkanen and Aro 2012). Indeed, the PSII core proteins (D1, D2, CP43 and PsbH) undergo reversible phosphorylation (Vener 2007). The LHCII trimers, as mentioned above, are composed of three polypeptides (Lhcb1, Lhcb2 and Lhcb3) in higher plants. Of these, only Lhcb1 and Lhcb2 undergo reversible phosphorylation cycles (Bennett 1979). Out of three monomeric Lhcb proteins (CP29, CP26 and CP24), only the CP29 is a phosphoprotein (Betterle et al., 2015).

Reversible phosphorylation of the PSII-LHCII proteins is regulated by the kinases STN7 and STN8, and by the phosphatases PPH1/TAP38 and PBCP, whose action is dynamic and redox regulated (Shapiguzov et al., 2010). The so called state transitions are a regulatory mechanism that works on a minute time scale (5-20 min) in order to regulate the excitation energy distribution between the two photosystems upon changes in the light quality/quantity (Bennett 1979; Allen et al., 1981; Trotta et al., 2016). State transitions depend on reversible phosphorylation of LHCII by interplay between the STN7 kinase and the PPH1/TAP38 phosphatase. The reversible phosphorylation of the PSII core proteins affects the ultrastructure of the thylakoid membrane and thereby regulates the PSII repair cycle (Baena-Gonzalez et al., 1999; Baena-González and Aro 2002; Tikkanen et al., 2006; Goral et al., 2010; Herbstova et al., 2012). The STN7 kinase phosphorylates not only the LHCII proteins, but also, to a minimum level, the PSII core proteins D1, D2 and CP43 (Bonardi et al., 2005), and only in low light intensity (LL), while the STN8 kinase primarily phosphorylates the PSII core proteins in high light intensity (HL) (Vainonen et al., 2005). Dephosphorylation of the LHCII phosphoproteins is mainly mediated by the phosphatase PPH1/TAP38 (Shapiguzov et al., 2010), whereas the dephosphorylation of the PSII core phosphoproteins is operated by the PBCP phosphatase (Samol et al., 2012).

1.3.4 Structure and function of the PSI-LHCI complex

PSI complex efficiently mediates the light-dependent electron transport from plastocyanin (PC) to ferredoxin (Fd) (Figure 4). Electrons from reduced Fd are transferred to FNR, which reduces $NADP^+$ to NADPH. The structure of plant PSI-LHCI complex includes 15 core protein subunits (PsaA-L and PsaN-P), four Lhca antennae proteins (Lhca1-4), approximately 173 chlorophylls and 15 carotenoids (Jansson 1999; Amunts et al., 2010; Busch and Hippler 2011; Schöttler et al., 2011; Mazor et al., 2015). Of the 15 intrinsic subunits forming the PSI core complex, the two largest protein subunits (PsaA and PsaB) form a heterodimeric reaction center. The heterodimeric PSI core binds 80 chlorophylls and the majority of the electron transport cofactors: P700 (chlorophyll dimer), A_0 (chlorophyll a), A_1 (phylloquinone), and F_X, iron-sulfur [4Fe-4S] center. Two iron-sulfur [4Fe-4S] centers (F_A and F_B), terminal electron transport cofactors, are bound to the PsaC subunit. PsaC, together with the PsaD and PsaE protein subunits, form a binding site for Fd on the stromal side of the PSI core complex. PC binds to the lumenal subunits of PSI, PsaF and PsaN. The PsaN subunit, extrinsically bound on the lumenal surface of the PSI complex is also needed for efficient oxidation of PC (Haldrup et al., 1999, 2000).

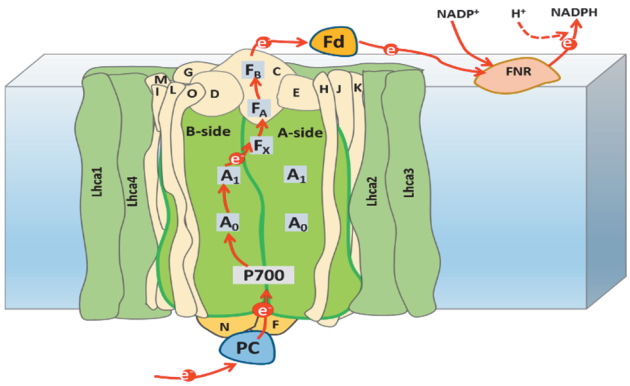

Figure 4. Schematic representation of PSI-LHCI complex of higher plants.

LHCI mainly transfers light energy to PSI. LHCI is composed of four distinct polypeptides (Lhca1-4), organized in two heterodimers: Lhca1-Lhca4 and Lhca2-Lhca3. These two dimers are bound together and form a crescent-shaped "LHCI belt" that docks to the side of the PsaF, PsaG and PsaJ subunits of the PSI core complex (Ben Shem et al., 2003). The other side opposite to the LHCI belt, with PsaH, PsaI, PsaL and PsaO subunits, is open, at least under constant growth light conditions (Amunts et al., 2010). As mentioned, the phosphorylation status of LHCII regulates the excitation energy distribution between PSII and PSI during state transitions. The docking site of

LHCII on PSI is composed of three protein subunits (PsaH, PsaL and PsaI) (Lunde et al., 2000; Zhang and Scheller 2004). It has been shown that at least two proteins, namely PsaH and PsaL, are crucial for transferring the excitation energy from phosphorylated LHCII to PSI. It has also been found that lack of the PsaI subunit destabilizes the binding of the PsaH and PsaL subunits to the PSI complex (Plochinger et al., 2016).

1.3.5 Photodamage of the PSI-LHCI complex

In sharp contrast to PSII, PSI is generally resistant to light-induced damage. Photoinhibition of PSI has been observed in cold-sensitive plants such as cucumber, under low temperatures and the reaction depends on the electron transfer from PSII to PSI (Powles et al., 1983; Sonoike 1996). At normal growth light conditions, the photosynthetic electron flow from PSII through the Cyt b_6f complex to PSI does not exceed the capacity of PSI electron acceptors and the PSI complex remains stable (Munekage et al., 2002). PSI complex is damaged, when the electron flow from PSII exceeds the capacity of electron carriers on the acceptor side of PSI. The subsequent recovery of photodamaged PSI is rather slow (Sonoike 1995; Munekage et al., 2002). When the electron transfer is inhibited from PSII to PSI in the presence of DCMU the PSI complex is protected efficiently against light induced oxidative damage (Sonoike 1995). It has been shown that in *pgr5* mutant plants that lack the PGR5-dependent photosynthetic control of the Cyt b_6f complex and induction of NPQ, PSI is highly sensitive to the HL shifts (Tiwari et al., 2016).

1.3.6 Cytochrome b_6f complex

The membrane-embedded Cyt b_6f complex is positioned between PSII and PSI and it functions both in LET and CET. The Cyt b_6f complex mediates electron transfer between the PSII and PSI reaction center complexes by oxidizing PQH_2 and reducing PC (Kurisu et al., 2003; Allen 2004; Smith et al., 2004). The photosynthetic electron transport via Cyt b_6f complex is coupled with proton translocation across the thylakoid membrane and generates a proton gradient across the thylakoid membrane that is utilized by ATP synthase to synthesize ATP. In the thylakoid membrane, a gradual increase in the pH gradient across the thylakoid membrane concomitantly reduces electron flow rate through the Cyt b_6f complex. The exact molecular mechanism of such an important regulation mechanism in protection of PSI against photodamage still remains elusive, but the PGR5 protein has been demonstrated to have a crucial role in this process (Munegage et al., 2002; Tiwari et al., 2016). On the other hand, it is well known that the Cyt b_6f complex holds a key role in state transition. Indeed, the STN7 kinase responsible for phosphorylation of LHCII is activated upon binding of PQH2 to the Q_0 site in Cyt b_6f

complex (Zito et al., 1999). It has been shown that several Cyt b_6f mutants from higher plants lack LHCII phosphorylation (Coughlan et al., 1988; Gal et al., 1988).

1.3.7 ATP synthase

The chloroplast ATP synthase catalyzes the formation of ATP from adenosine diphosphate (ADP) and phosphate (Pi) at the expense of transmembrane proton gradient generated by light-dependent electron flow (McCarty et al., 2000). The ATP synthase is constituted with two structurally and functionally distinct moieties: the membrane embedded ion-translocating F_0 complex and the peripheral, stroma exposed F_1 portion, which carries the catalytic sites.

1.4 Regulation of excitation energy by non-photochemical quenching

Plants in their natural habitats are constantly subjected to changes in their environments, such as fluctuations in light intensity. Plants require light for photosynthesis, but excess light energy is potentially harmful to photosynthetic machinery. The absorbed light energy exceeding the capacity of photochemistry can lead to photo-oxidative damage of the photosynthetic machinery. Excess absorbed light energy can lead to over-reduction of photosystems and subsequent generation of triplet state chlorophylls (^{3}Chl), which in turn transfer energy to ground-state O_2 and thus generate extremely reactive singlet oxygen species ($^1O_2$) (Aro et al., 1993; Roach and Krieger-Liszkay 2014). Highly reactive singlet oxygen can cause damage to the pigments, lipids and proteins in the photosynthetic thylakoid membrane. This photo-oxidative damage to the photosynthetic apparatus ultimately reduces plant growth and fitness. To counter the potential light-induced damage, photosynthetic organisms have evolved numerous mechanisms that help to optimize the absorption of light energy. These include the non-photochemical quenching (NPQ), one photoprotective mechanism that dissipates excess excitation energy harmlessly as heat (Niyogi 1999; Müller et al., 2001). NPQ has been divided into at least three different components: (*i*) the energy dependent quenching (qE), (*ii*) the state transition dependent quenching (qT), and (*iii*) the photoinhibition dependent quenching (qI).

The major component of NPQ is qE, which is induced by a low thylakoid lumen pH that is generated by the electron transport particularly in HL. Induced qE results in dissipation of excess absorbed light energy as heat in the LHCII (Müller et al., 2001). The second component of NPQ is qT. In qT, phosphorylated LHCII collects light energy for PSI, thereby reducing the amount of excess excitation energy in PSII (Tikkanen et al., 2008a; Minagawa 2011). The third component of NPQ, qI, is related to the

photoinhibition of PSII. In qI, excess excitation energy decreases because the PSII core protein D1 is photodamaged and thereby subjected to a repair cycle (Tikkanen et al., 2008b; Theis and Schroda 2016).

1.4.1 Energy dependent quenching (qE)

Photosynthetic electron transport is associated with the pumping of protons across the thylakoid membrane, from the soluble stroma to the thylakoid lumen. This proton flux causes a transmembrane proton gradient which becomes higher under increasing light intensities. It has been established that the proton gradient is necessary for qE induction, since qE cannot be induced when the development of the proton gradient is blocked in the presence of the uncoupler nigericin (Müller et al., 2001). In the *pgr5* mutant plants, which have been reported to be defective in the PSI CET, only a poor induction of NPQ takes place (Munekage et al., 2002). The proton gradient across the thylakoid membrane plays two significant roles in qE (Niyogi et al., 1998; Holt et al., 2004). First, it activates the xanthophyll cycle. In the xanthophyll cycle, violaxanthin de-epoxidase (VDE), an enzyme bound on the luminal side of thylakoid membrane, is activated when the luminal pH is decreased at HL. The active enzyme catalyzes the formation of zeaxanthin from violaxanthin in a de-epodixation reaction (Arnoux et al., 2009; Jahns et al., 2009; Jahns and Holzwarth 2012; Li et al., 2013). Second, the low lumenal pH at HL also leads to protonation of the PSII protein, PsbS, which belongs to the LHCII superfamily (Li et al., 2000). Protonation of the PsbS protein together with zeaxanthin cause a conformational change in LHCII, which results in dissipation of excess absorbed light energy harmlessly into heat (Bonente et al., 2008; Horton et al., 2008; Kiss et al., 2008; Jahns et al., 2009; Kereïche et al., 2010; Correa-Galvis et al., 2016). Indeed, also the *npq4* mutant, lacking the PsbS protein, shows a substantial defect in NPQ capacity (Niyogi et al., 2005).

1.4.2 State transitions related quenching (qT)

Differences in the light absorption properties of PSII and PSI, together with variations in light conditions, can lead to unequal excitation of the two photosystems. qT is a rapid, short-term physiological acclimation to changes in light conditions in order to balance the absorbed excess excitation energy between PSI and PSII. The qT component was characterized independently by two research groups (Bonaventura and Mayers 1969; Murata 1969). The historical explanation of state transitions is as follows. Under state two light, when PSII is selectively over excited relative to PSI, the PQ pool becomes more reduced. This condition favors the binding of reduced plastoquinone (PQH_2) to the Q_0 site in Cyt b_6f complex, which, in turn, leads to the phosphorylation of LHCII by activating a protein kinase STN7 in Arabidopsis or STT7 in

Chlamydomonas (Vener et al., 1997; Zito et al., 1999). Upon phosphorylation, the mobile portion of LHCII antenna complex dissociates from PSII and migrates along the thylakoid membrane and binds to PSI, thereby re-equilibrating the absorption cross sections of the light-harvesting complexes of PSII and PSI, and redistributing the absorbed excess excitation energy in favor of PSI (state two). This transition is reversible. Indeed, when PSI becomes more excited than PSII, the PQ pool is oxidized. These conditions inactivate the STN7 kinase and the thylakoid membrane-bound phosphatase PPH1/TAP38 dephosphorylates LHCII. Upon dephosphorylation, LHCII returns to PSII from PSI and thereby redistributes the excess absorbed light energy in favor of PSII (state one).

Phosphorylation/dephosphorylation cycles of LHCII proteins dependent on the redox status of the PQ pool has thus been argued to balance the relative distribution of excess excitation energy between the two photosystems under changing light conditions. These conclusions are, however, based on experiments by applying different light qualities, which specifically excite either PSI or PSII. Such light conditions are rare in nature, and more recent research and opinion articles have provided novel insights into the *in vivo* role of LHCII phosphorylation in plants (Grieco et al., 2012; Tikkanen et al., 2012; Tikkanen and Aro 2012; Grieco et al., 2015).

1.5 pH-induced changes in the thylakoid membrane

Photosynthetic light reactions are coupled to the translocation of protons across the thylakoid membrane from the soluble stroma to the thylakoid lumen that generates a transmembrane proton gradient. Indeed, in darkness, the lumenal pH is around 7.0, and in light the lumenal pH is in between 5.8 - 6.5 (Kramer et al., 1999; Tikhonov 2013). pH is known to regulate the thylakoid architecture, and the electron flow rate through the Cyt b_6f and qE (Li et al., 2004; Kirchhoff et al., 2011). Moreover, proteolytic degradation of the D1 protein and the function of the OEC complex are dependent on pH (Kapri-Pardes et al., 2007; Hall et al., 2010; Yoshioka and Yamamoto 2011; Commet et al., 2012). In addition, low pH has been claimed to participate in the state transitions in isolated spinach thylakoids (Bergantino et al., 2003; Kereïche et al., 2010; Singh-Rawal et al., 2010; Jajoo et al., 2012).

2. AIMS OF THE STUDY

Comprehensive 3D structures of all four macromolecular protein complexes of the photosynthetic light reactions are now available. The function of these large protein complexes in a thylakoid network under changing environmental conditions has, however, remained rather elementary. In my thesis, the regulation of thylakoid proteins and protein complexes was addressed to gain information about structural-functional relationships in the thylakoid network.

The specific aims were:

1. To investigate the physiological role of reversible LHCII and PSII core protein phosphorylation upon exposure of plants to different light conditions and to investigate its implications in relative distribution of excitation energy between the two photosystems.

2. To investigate the role of the proton gradient and the PGR5 protein in electron transfer between photosystems, and the impact of controlled PSII photoinhibition on functional properties of PSI.

3. To investigate the consequences of photoinhibition of PSII on excitation energy distribution, and reorganization of pigment-protein complexes in the thylakoid membrane.

4. To investigate the putative mechanisms underlying low pH-dependent regulation of excitation energy distribution in thylakoids.

3. MATERIALS AND METHODS

3.1 Plant material, growth conditions and light treatments

Wild-type (WT) Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia and mutant plants (Table 1) were grown in a phytotron for five to six weeks at 120-130 µmol photons $m^{-2}s^{-1}$ with a 8-h light/16-h dark cycle (23^0C) and a relative humidity of 70%. OSRAM PowerStar HQIT 400/D Metal Halide Lamps were used as the light source. Details of Arabidopsis mutant lines and light and or chemical treatments (Table 2) are described in their respective papers.

Table 1. Arabidopsis mutant lines used in this thesis

MUTANT LINE	PAPER (S)	References
stn7	I & IV	Bellafiore et al., 2005
stn8	I & IV	Bonardi et al., 2005
stn7/8	III & IV	Bonardi et al., 2005
pph1/tap38	I	Shapiguzov et al., 2010 Pribil et al., 2010
Pbcp	I	Samol et al., 2012
pgr5	I & II	Munekage et al., 2002
npq4	IV	Niyogi et al., 2005

Table 2. Treatments (light and chemical) used in this study. GL= Growth light intensity, 120-130 µmol photons $m^{-2}s^{-1}$; LL= Low light intensity, 20 µmol photons $m^{-2}s^{-1}$; HL= High light intensity, 800-1000 µmol photons $m^{-2}s^{-1}$; DTT= 1,4-Dithiothreitol.

PAPER	LIGHT TREATMENTS	CHEMICAL TREATMENTS
PAPER I	GL, LL, HL, State 1 light & State 2 light	-
PAPER II	Dark, GL & HL	Lincomycin
PAPER III	Dark, GL & HL	Lincomycin
PAPER IV	Dark	DTT, Ascorbate, pH 7.5 & pH 5.5

3.2 Isolation of thylakoid membranes

Thylakoid membranes were isolated as described by Suorsa et al. (2004). The chlorophyll content was measured according to Porra et al. (1989).

3.3 Separation of thylakoid proteins and protein complexes

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate thylakoid proteins according to molecular mass (Laemmli 1970). Large pore blue native polyacrylamide gel electrophoresis (lpBN-PAGE), in turn, was

used to separate the native protein complexes (Strecker et al., 2010). For lpBN-PAGE (Paper I) and SDS-PAGE (Paper III), either n-dodecyl β-D-maltoside (DM) (Sigma) or digitonin (Calbiochem) was used as a solubilization agent. A low concentration of mild non-ionic detergent, such as DM or digitonin, maintained the protein complexes in their native form. Of these, digitonin preferentially solubilized protein complexes from the grana margins and stroma lamellae whilst DM solubilized protein complexes from the entire thylakoid membrane network. The thylakoids were solubilized for lpBN-PAGE (Paper I) and SDS-PAGE (Paper III) according to Järvi et al. (2011). BN-PAGE gel was stained with Commassie Brilliant Blue (Paper I), whereas SDS-PAGE gel was stained with SYPRO Ruby Protein Gel Stain (Paper III).

3.4 Identification of proteins

After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and the membrane was blocked with 5% (w/v) fatty acid-free bovine serum albumin (Sigma-Aldrich) or 5% (w/v) milk (non-fat dry milk, Bio-Rad). The proteins were detected with specific antibodies. The even loading of gels was confirmed by Coomassie Brilliant Blue G-250 (Bio-Rad) staining.

3.5 Biophysical measurements

The fluorescence emission spectra at 77 K were recorded by using an Ocean Optics QE Pro Spectrometer (Papers I & III) or a diode array spectrophotometer (S2000; Ocean Optics) equipped with a reflectance probe (Paper IV) and normalized as described in the respective papers. In order to investigate the mechanisms involved in the low pH induced alterations in the isolated thylakoid membrane, thylakoids were suspended in the pH 5.5 or 7.5 medium in the presence or absence of DTT (1,4-Dithiothreitol) and ascorbate prior to recording the fluorescence emission spectra (Paper IV). DTT and ascorbate were used in this experiment to inhibit violaxanthin de-epoxidation to zeaxanthin (Srivastava and Zeiger 1995) and to convert violaxanthin to zeaxanthin (Pfündel and Strasser 1988; Havaux and Gruszecki 1993), respectively.

The amounts of functional PSI and PSII reaction centers were estimated using Dual-PAM-100 (Walz, Effeltrich, Germany). The redox state of PSI was measured using red actinic light (625 nm) of 58 and 533 µmol photons $m^{-2}s^{-1}$ (Paper II & III). The photochemical efficiency of PSII (Fv/Fm) and the fluorescence parameters (Fo, Fm, F′ and Fm′) were determined as described in Paper III.

4. RESULTS

4.1 Phosphorylation pattern of thylakoid proteins under steady state and dynamic light conditions

Under changing natural light conditions, thylakoid membrane proteins (PSII core and LHCII proteins) undergo phosphorylation/dephosphorylation cycles. Reversible phosphorylation of the PSII-LHCII proteins is regulated by the kinases STN7 and STN8, and by their counteracting phosphatases PPH1/TAP38 and PBCP, respectively, whose action is dynamic and redox regulated (Shapiguzov et al., 2010). In this study, the individual physiological roles of above mentioned enzymes in the regulation of reversible thylakoid membrane protein phosphorylation under different light conditions were investigated.

I first analyzed the phosphorylation levels of the PSII core and LHCII proteins in Arabidopsis WT as well as in kinase mutants (*stn7* and *stn8*) and phosphatase mutants (*pph1/tap38* and *pbcp*) acclimatized to standard GL conditions (Paper I). The *pgr5* mutant, defect in PGR5-dependent photosynthetic control at Cyt b_6f complex, was also included in my study in order to investigate the physiological role of transmembrane proton gradient in thylakoid membrane protein phosphorylation and in absorbed light energy distribution in the thylakoids. The protein phosphorylation levels/changes were monitored by immunoblotting with an anti-phosphothreonine antibody.

In WT plants, both the PSII core and LHCII proteins were moderately phosphorylated in standard GL conditions. The *stn7* mutant, lacking the STN7 kinase and containing a functional STN8 kinase, completely failed to phosphorylate LHCII proteins, but phosphorylated the PSII core proteins more strongly than those of control WT plants. The *stn8* mutant, with a functional STN7 kinase, showed very low phosphorylation levels of D1 and D2, whilst the phosphorylation levels of CP43 and LHCII proteins were not significantly different from those in WT. The *pbcp* mutant showed slightly higher phosphorylation levels of the PSII core proteins compared with WT plants, whereas the phosphorylation level of the LHCII proteins was not significantly different from that of the WT plants. Interestingly, in both the *pph1*/*tap38* and *pgr5* mutant plants, the phosphorylation levels of the PSII core and its LHCII phosphoproteins were very similar to those in WT.

Next, I focused on the dynamic regulation of the reversible phosphorylation of PSII core and LHCII proteins by using illumination sets of changing light intensities (Paper I). To that end, WT and the above mentioned mutant plants were shifted from standard GL to LL and then to HL, followed by another shift to LL and to HL with duration intervals

of 20 min. The duration of various illumination periods was selected to allow the phosphorylation and dephosphorylation events to occur. Another set of plants were subjected to standard GL and then shifted to HL for 1 and 2 hours.

In WT plants upon the shift to LL, phosphorylation levels of the PSII core proteins always decreased while the phosphorylation levels of LHCII proteins increased. Noteworthy is, that exactly the opposite phosphorylation changes were observed when WT plants were subjected to HL. The *pbcp* mutant showed similar phosphorylation changes as WT in the light shifts. Interestingly, the *pph1*/*tap38* and *pgr5* mutant plants behaved differently. Upon the shift to HL, neither of the *pph1/tap38* and *pgr5* mutant plants dephosphorylated the LHCII proteins and allowed both the PSII core and LHCII phosphoproteins strongly phosphorylated (Paper I). The similar strong and simultaneous phosphorylation of both the PSII core and LHCII phosphoproteins was observed in WT plants only when transferred from GL to the state 2 light (Paper I).

4.2 Sustained phosphorylation of LHCII proteins drives excitation energy towards PSI

In order to investigate the effects of missing LHCII dephosphorylation of the *pph1*/*tap38* and *pgr5* mutant plants upon the shift of plants to HL, WT plants and mutants were shifted to HL for 1 and 2 h and subsequently submitted to the measurement of 77 K fluorescence emission spectra (Paper I). Previous studies had shown no difference in the distribution of excitation energy between PSII and PSI in the thylakoid membrane upon light shifts of WT and the kinase mutants, despite distinct changes in the phosphorylation levels of PSII core and LHCII proteins (Tikkanen et al., 2010). Similarly, in my experiments the WT as well as the kinase mutants (*stn7* and *stn8*) and *pbcp* mutants showed no distinct changes in the relative distribution of excitation energy in favour of PSI upon the shift to HL, despite distinct changes in the phosphorylation levels of thylakoid membrane proteins. On the other hand, the 77 K fluorescence emission spectra from the thylakoid membranes of both the *pph1*/*tap38* and *pgr5* mutants showed a strong increase in PSI excitation, which coincided with a simultaneous increase in both PSII core and LHCII protein phosphorylation levels upon the shift of plants to HL. Accordingly, the *pph1*/*tap38* and *pgr5* mutants, upon the shift to HL, closely resembled WT under state 2 light with respect to both phosphorylation pattern of thylakoid membrane proteins and the relative distribution of excitation energy between PSII and PSI.

4.3 Major rearrangements of thylakoid protein complexes in high light enhance excitation energy transfer towards PSI

Under HL conditions, a simultaneous increase in both PSII core and LHCII protein phosphorylation levels in the *pph1/tap38* mutant lacking LHCII dephosphorylation was shown to drive excitation energy towards PSI (Paper I). To elucidate the molecular mechanism of increased excitation energy in favour of PSI at HL, I performed BN-PAGE analysis of the thylakoid membrane protein complexes after exposure of the WT and *pph1/tap38* mutant plants to HL for 1 h. Thylakoid membranes were isolated and solubilized with DM or with digitonin prior to BN-PAGE analysis. After DM-treatment, no clear differences were observed in the PSII-LHCII complexes between WT and *pph1/tap38*, when plants from GL and HL were compared. BN-gels of digitonin solubilized thylakoids revealed a difference between WT and *pph1/tap38* by indicating that the so-called state transition complex (i.e., LHCII-PSI-LHCI complex) (Pesaresi et al., 2009) was stable in *pph1/tap38* upon a shift to HL, but disappeared from WT. Additionally, a larger amount of thylakoid megacomplexes, containing both photosystems PSII and PSI with their LHCII and LHCI, respectively, was observed in the *pph1/tap38* mutant plant compared to WT upon the shift to HL.

4.4 Photoinhibition of PSII is an ultimate mechanism for protecting PSI in excess light

It has been known already for decades that PSII is very susceptible to light (Kok 1956; Jones and Kok 1966; Kyle et al., 1984; Baena-Gonzalez and Aro 2002). In contrast to PSII centers, the PSI centers are very tolerant against photoinhibition at excess light but extremely sensitive to uncontrolled electron transfer from PSII (Sonoike and Terashima 1994; Sonoike 2011). Here, I tested a hypothesis whether the controlled PSII turnover could function in the protection of PSI against oxidative photodamage (Paper II). To that end, fully developed detached leaves from the WT plants and *pgr5* mutant plants that are extremely sensitive to photoinhibition of PSI, were studied. Leaves were incubated with or without lincomycin overnight in darkness followed by transfer to GL for 2 h. Lincomycin is an inhibitor which prevents the translation of the chloroplast encoded proteins. Particularly the translation of the "rapidly-turning-over" PSII reaction center protein D1, required for PSII repair, is inhibited in the presence of lincomycin. Further, it was investigated how the remaining active PSII centers (Fm) influenced the amount of active PSI centers (Pm). In the absence of lincomycin neither of the Fm and Pm parameters were affected in WT plants nor in the *pgr5* mutant plants, when plants were shifted from darkness to GL. On the contrary, in the presence of lincomycin the same light shift decreased the Fm value to about 80% of the initial value

in both WT and *pgr5* mutant plants, whereas the Pm value remained unchanged in WT as well as in the *pgr5* mutant plants.

To understand better the consequences of PSII photoinhibition on the amount of functional PSI, detached leaves were subsequently subjected to HL up to 2 h in the presence/absence of lincomycin (Paper II). In the absence of lincomycin the Fm value decreased to about 75% of the initial value in both WT and the *pgr5* plants. In WT, the Pm value was not affected, while the Pm value in the *pgr5* mutant showed a decrease in response to the HL-treatment. When the HL-treatment was applied in the presence of lincomycin, a drastic decrease was observed in the Fm value of both WT and the *pgr5* mutant. However, the Pm value remained unchanged in the HL-treated WT plants in the presence of lincomycin. In contrast, a decrease was observed in the Pm value in the HL-treated *pgr5* mutant in the presence of lincomycin. Interestingly, while prolonging the HL illumination from 30 min to two hours led to a further decline in the Pm value in the *pgr5* mutant plants in the absence of lincomycin. Such enhanced PSI photodamage was not observed in the presence of lincomycin. Therefore, when the PSII repair cycle was blocked by lincomycin treatment in the *pgr5* mutant plants, the sustained down-regulation of the PSII activity likely protected PSI activity against photooxidative damage.

Next, I monitored the degradation of the PSII reaction center protein D1 during different illumination levels by western blots (Paper II). During the course of HL treatment, the content of D1 protein gradually decreased both in WT and *pgr5* in the presence of lincomycin, thus being in line with changes in the Fm values. Notably, the degradation of the D1 protein was much faster in the *pgr5* mutant compared with the WT plants.

4.5 P700 oxidation is essential in protection of PSI against photoinhibition

As already mentioned, the PGR5-dependent photosynthetic control at the Cyt b_6f complex plays a major role in the protection of PSI activity against photooxidative damage under HL (Munekage et al., 2002; Tiwari et al., 2016). Indeed, when the PQH_2 oxidation rates by the Cyt b_6f complex are slowed down, the suppression of the electron flow from Cyt b_6f to P700 and concomitant P700 oxidation prevent photoinhibition of PSI under HL. The *pgr5* mutant that lacks the PGR5-mediated and transmembrane proton gradient-dependent control of photosynthetic electron transport through the Cyt b_6f complex and NPQ induction, cannot keep P700 oxidized, and is thus extremely sensitive to PSI photoinhibition (Suorsa et al., 2012).

I investigated the effects of the transmembrane proton gradient-dependent photosynthetic control of the Cyt b_6f complex on P700 oxidation in Paper II. To that end, I measured P700 oxidation levels under low or high intensity actinic light (LA or HA, respectively) from the detached, fully developed leaves of WT and the *pgr5* mutant, which were pre-illuminated in the presence/absence of lincomycin as described in Paper II. It has been reported previously that P700 is reduced in the dark, but is oxidized to $P700^+$ by PSI photochemistry upon the shift to light (Munekage et al., 2002). In the absence of lincomycin, upon the shift from darkness to LA, almost no oxidation of P700 was observed in WT or the *pgr5* mutant plants. Upon subsequent shift from LA to HA illumination, in the WT plants the oxidation level of P700 increased rapidly, whereas in the *pgr5* mutant, the level of P700 oxidation remained low.

In the presence of lincomycin, when the leaves were measured under LA illumination, the oxidation level of P700 gradually increased with an increase in HL pre-illumination time in both WT and the *pgr5* mutant. Oxidation level of P700 upon LA illumination showed inverse proportionality compared with Fm (for the Fm measurements, see Section 4.4) both in WT and the *pgr5* mutant plants in the presence of lincomycin. When P700 oxidation was measured upon subsequent shift from LA to HA illumination, the WT plants in the presence of lincomycin showed a maximum level of P700 oxidation independently of pre-illumination. Indeed, the WT plants showed similar P700 oxidation under HA both in the presence and absence of lincomycin. By comparison, the oxidation level of P700 in the *pgr5* mutant plants in the absence of lincomycin did not respond to the actinic light intensity, but light intensity independent oxidation was observed when the HL pre-illumination time with lincomycin was prolonged.

4.6 PSII photoinhibition decreases lateral heterogeneity of thylakoid membrane and thus increases excitation energy towards PSI

Photoinhibition of PSII is associated with a strong quenching of chlorophyll fluorescence, which can be measured as a decrease in the Fv/Fm value. The parameter Fv is calculated as Fm-Fo, with Fo representing the minimal fluorescence level observed in the dark-acclimated plants. To examine changes that are associated with strong PSII photoinhibition, the Fv/Fm ratio was measured from detached Arabidopsis leaves. Leaves were incubated in lincomycin solution in the dark overnight, followed by a shift to HL for 1 and 2h. After dark-acclimation, the Fv/Fm ratio gradually decreased with increasing HL illumination time in the presence of lincomycin (Paper

III). Decline in the Fv/Fm value was shown to originate from both the decreased Fm value and the increased Fo value.

The effect of photoinhibition-induced injury to the energy distribution was assessed next. To that end, 77 K fluorescence emission spectra of thylakoid membranes were analyzed. First, a red-shift of the PSII fluorescence emission peaks at 685 nm and 695 nm pointed out, indicating that a part of the LHCII antenna (emitting at 680 nm) was detached from PSII (Paper III). This partial detachment of LHCII not only red shift, but also increase the fluorescence emitted by the PSII associated fluorescence bands. Nevertheless, a strong decrease in the relative PSII to PSI fluorescence emission upon the shift to HL in the presence of lincomycin was observed and this fluorescence quenching from PSII occurred despite the partial detachment of LHCII, emitting at 680 nm, and thus also increasing the emission from nearby 685 nm and 695 nm peaks. This indicated that the photoinhibition-induced quenching of chlorophyll fluorescence is associated with increased PSI fluorescence at 77 K. To further study the role of PSI in excitation energy dissipation, the proportion of oxidized P700 (Y(ND)) was measured using both LA and HA. In paper II, it is shown that in non-photoinhibited leaves P700 was oxidized only upon the onset of HA. However, in the strongly photoinhibited leaves P700 was oxidized already in LA, which likely resulted due to the decreased PSII/PSI ratio (Papers II & III).

Next, the effect of PSII photoinhibition on dissipation of absorbed excitation energy into heat was followed by measuring the F′ and Fm′ values using either LA or HA. In photoinhibited leaves, upon the onset of the actinic light the F′ and Fm′ values decreased below the Fo and Fm values, respectively (Paper III), which indicated that severe photoinhibition of PSII did not prevent the thermal dissipation of excitation energy. Further, the quenching of Fm′ was not significantly increased in photoinhibited leaves upon shift to high actinic light intensity. Indeed, the PSII photoinhibition-induced dissipation of excitation energy as heat was dependent on the presence of light, but not on light intensity.

Both the PSII photoinhibition-repair cycle and the reduction status of the electron transfer chain are linked to the phosphorylation status of the thylakoid membrane proteins. To that end, the phosphorylation levels of the PSII core proteins (CP43, D2 and D1) in relation to their amounts were monitored by immunoblots and SYPRO Ruby Protein Gel Stain from photoinhibited Arabidopsis leaves (Paper III). The content of D1 protein decreased during HL exposure in the presence of lincomycin while the amounts of D2 and CP43 remained quite stable. The remaining PSII core proteins gradually dephosphorylated upon the photoinhibitory light conditions. Dephosphorylation of the PSII core proteins likely originated from the inactivation of

the STN8 kinase by the oxidized electron transfer chain. As expected, also the STN7 kinase was inactivated and the LHCII proteins dephosphorylated immediately upon the shift to HL.

To further study the molecular mechanism behind the increased relative excitation of PSI following severe PSII photoinhibition, I investigated the photoinhibition-induced reorganization of the pigment-protein complexes. To reach this goal, isolated thylakoid membranes were solubilized either by digitonin or DM followed by the separation of the thylakoid proteins by denaturing SDS-PAGE (Paper III). During the course of HL exposure, in the presence of lincomycin, a gradual release of PSII proteins from the grana thylakoids to the stroma lamellae was observed. Photoinhibition-induced reorganization was independent of thylakoid protein phosphorylation evidenced by similar results obtained from WT and the double mutant *stn7 stn8* (deficient of the thylakoid protein phosphorylation) plants.

4.7 *In vitro* experiments with isolated thylakoids demonstrate energy spillover from PSII to PSI at low pH

To uncover the mechanisms involved in low pH-associated changes in the distribution of excitation energy between the two photosystems (PSII and PSI) in thylakoids, an *in vitro* approach was taken in Paper IV. Total thylakoid membranes isolated from WT Arabidopsis plants and its kinase mutants (*stn7, stn8* and double mutant *stn7 stn8*) and the *npq4* mutant, lacking the PsbS protein were incubated in buffers of pH 7.5 and pH 5.5 in the dark for 30 min on ice. Samples were subsequently submitted to the analysis of RT and 77 K fluorescence emission spectra, and BN-PAGE analysis. Fluorescence spectra revealed that incubation of isolated thylakoid membranes from the WT plants in buffer of pH 5.5, the RT fluorescence emission from PSII was quenched and the 77 K PSI/PSII fluorescence ratio (F730/F680) significantly increased in comparison to the WT thylakoids incubated in buffer of pH 7.5. Isolated thylakoid membranes from the kinase mutants of WT Arabidopsis (*stn7, stn8* and double mutant *stn7 stn8*) also showed an increase in the F730/F680 fluorescence ratio at pH 5.5 compared with all mutant thylakoids treated with pH 7.5. In contrast to WT plants and its kinase mutant thylakoids, no pH induced change in the F730/F680 ratio was observed in the *npq4* mutant thylakoids, which lacks the PsbS protein. The BN-PAGE analysis of the thylakoid membrane pigment-protein complexes revealed no major low pH-associated changes in the composition or re-organization of the pigment protein complexes in the thylakoid membranes. Further, the 77 K fluorescence excitation spectra of the WT thylakoid membrane (excited at wavelengths of 400-540 nm) incubated at pH 7.5 and

5.5 showed no significant difference in the absorption cross sections of both photosystems (PSII and PSI).

Next, I focused on the involvement of the xanthophyll cycle pigments as probable candidates to explain the pH-induced changes in the properties of fluorescence emission of isolated thylakoids (Paper IV). To that end, the fluorescence emission spectra from WT thylakoids were recorded again at low pH both in the absence and presence of DTT (prevents zeaxanthin formation) and ascorbate (promotes zeaxanthin formation). In the presence of DTT, a similar increase in the F730/F680 fluorescence ratio was observed as in the control. The measurements with and without ascorbate showed no significant difference in the spectra.

5. DISCUSSION

5.1 PSII-LHCII protein phosphorylation is dynamically regulated by the STN7 and STN8 kinases and the PPH1/TAP38 and PBCP phosphatase

Multisubunit protein complexes governing the photosynthetic light reactions are embedded in the thylakoid membrane network of the chloroplast. Of these, the components of the LHCII complex are the most abundant membrane proteins in nature. It has been known for more than 30 years ago that part of the thylakoid proteins, including a set of LHCII and PSII subunits, undergoes reversible phosphorylation cycles according to the quality of light, whereas the reversible phosphorylation events induced by changes in the quantity of light have remained much less investigated (Tikkanen and Aro 2012). In the past decades, knowledge has accumulated revealing that reversible phosphorylation, which takes place in an opposing manner in the PSII and LHCII subunits, plays a key role in the optimization of the light-harvesting and energy-transduction reactions (Tikkanen and Aro 2014). The precise regulation of the thylakoid membrane protein phosphorylation is especially important in fluctuating light conditions (Grieco et al., 2012). In higher plants, both the PSII core and LHCII proteins undergo light intensity dependent reversible phosphorylation controlled by the kinases STN7 and STN8, and their respective counteracting phosphatases PPH1/TAP38 and PBCP. Identification and characterization of two kinase mutant plants (*stn7* and *stn8*) and two phosphatase mutant plants (*pph1/tap38* and *pbcp*) enabled us to investigate the physiological significance of thylakoid protein phosphorylation under changing light intensities (Paper I).

It is known that the protein kinase STN7 predominantly mediates LHCII protein phosphorylation in LL and the reversible phosphorylation of LHCII is mainly involved in redistribution of excitation energy between two photosystems in the thylakoids during a short-term acclimation response to changes in light known as state transitions. The STN7 kinase is activated in light when reduced plastoquinone (PQH_2) binds to the so-called Q_O site in Cyt b_6f complex (Rochaix 2007; Shapiguzov et al., 2016). Further, it has been proposed that the STN7 kinase activity is inhibited in HL by the ferredoxin-thioredoxin system (Rintamäki et al., 2000; Nikkanen and Rintamäki 2014). The STN8 kinase mainly phosphorylates the PSII core proteins under HL (Vainonen et al., 2005; Bonardi et al., 2005). These two kinases, STN7 and STN8 show partial substrate overlapping as observed by the comparison of the protein phosphorylation pattern of *stn7* and *stn8* with the double mutant *stn7 stn8* (Paper I). The phosphorylation of the

PSII core proteins is thought to play a major role in the PSII repair process (Baena-González et al., 1999; Tikkanen et al., 2008b). The most active PSII complexes, the PSII-LHCII supercomplexes, are localized in the appressed regions (grana core) of the thylakoid membrane network, while the degradation and *de novo* synthesis of the D1 protein occur in the non-appressed regions (stroma lamellae) (Aro et al., 2005). The phosphorylation-dependent lateral migration of the damaged PSII core from the stacked grana to unstacked stroma lamellae, which is likely to originate from alterations in the thylakoid membrane architecture, facilitates the proteolysis of the damaged D1 protein (Aro et al., 1993; Baena-González and Aro 2002; Tikkanen et al., 2008b; Fristedt et al., 2009; Goral et al., 2010; Herbstova et al., 2012). In stroma lamellae the D1 protein is dephosphorylated to enable the proteolytic degradation of the protein. Indeed, the phosphorylated form of the D1 protein is highly protective from proteolytic degradation (Koivuniemi et al., 1995).

In previous studies (Shapiguzov et al., 2010; Pribil et al., 2010), it has been shown that the PPH1/TAP38 phosphatase is responsible for dephosphorylation of the LHCII phosphoproteins under state 1 and also in dark conditions (when the STN7 kinase is inactive). Moreover, the PPH1/TAP38 phosphatase dephosphorylates LHCII phosphoproteins also in HL (Paper I). Indeed, the pph1/*tap38* mutant plants, upon exposure to HL, did not dephosphorylate the LHCII phosphoproteins and showed a hyper simultaneous phosphorylation of both the PSII core and the LHCII proteins. It has been shown earlier that the PBCP phosphatase dephosphorylates the PSII core proteins in the traditional state 1 conditions and also in photoinhibitory illuminations (Puthiyaveetil et al., 2014). Interestingly, the PSII core proteins were shown here to be dephophorylated in the *pbcb* mutant plants upon the shift of plants to LL (Paper I). This result clearly indicates that there is still an undiscovered PSII core protein phosphatase that is responsible for the dephosphorylation of PSII core phosphoproteins under weak illumination. Genome-wide predictions have shown that the chloroplast might contain several so-far uncharacterized phosphatases (Schliebner et al., 2008).

The thylakoid lumen cyclophilin CYP38/TLP40 has been observed to function as a negative regulator of the protein phosphatase responsible for dephosphorylation of the PSII core proteins (Fulgosi et al., 1998; Vener et al., 1999). It is, however, not known whether CYP38/TLP40 regulates the function of the PBCP phosphatase or of another so far unknown thylakoid-bound phosphatase. There is indirect evidence suggesting that the CYP38 protein might regulate the PBCP phosphatase. Indeed, plants overexpressing PBCP (*oepbcp*) and the *cyp38* plants share a similar thylakoid protein phosphorylation pattern – both PSII core and LHCII proteins are dephosphorylated in the mutant plants compared with WT under white light condition (Samol et al., 2012).

Further, the constant LHCII dephosphorylation in the *oepbcp* and *cyp38* plants has been shown to reduce the capacity of state transitions measured in artificial light conditions. There are two major possibilities to explain the highly dephosphorylated LHCII in the *oepbcp* and in *cyp38* plants: either LHCII is a substrate of the PSII core phosphatase or the high amount of active phosphatase in the mutant plants induces a loss of the phosphatase specificity (Samol et al., 2012).

5.2 Physiological significance of the thylakoid protein phosphorylation in high light

Plants in their natural environments often experience great variations in light conditions, particularly in the intensity of light. As mentioned, the PSII core and LHCII proteins undergo reversible phosphorylation under changing light intensities (Paper I). When plants are shifted from GL to LL, the phosphorylation levels of PSII core phosphoproteins decreases while the phosphorylation levels of LHCII phosphoproteins concomitantly increases. Exactly the opposite phosphorylation changes occur when plants are exposed to HL. This drastically differs from the traditionally known phenomenon, i.e., state transitions induced by artificial state lights which selectively excite either PSI (state 1 light) or PSII (state 2 light) (Allen et al., 1981; Rochaix 2007). State 2 light leads to strong and concomitant phosphorylation of both the PSII core and LHCII phosphoproteins, whereas state 1 light leads to dephosphorylation of PSII core and LHCII phosphoproteins.

It is shown in Paper I, that the reversible but opposite PSII core and LHCII protein phosphorylation is a key factor in maintaining the functionality of the photosynthetic machinery under changing illumination conditions. Indeed, I show that the relative distribution of excess excitation energy between PSII and PSI remains nearly unchanged despite the shifts in the WT, in the kinase mutant plants (*stn7* and *stn8*), and in *pbcp* mutant plant between two different light conditions (HL and LL) (Paper I; Tikkanen et al., 2010). Instead, when the traditional state lights were applied, a strong imbalance appeared in the relative distribution of excess excitation energy between PSI and PSII in thylakoids (Paper I; Tikkanen et al., 2010).

Interestingly, in both the *pph1*/*tap38* and *pgr5* mutant plants, PSII core and LHCII phosphoproteins strongly phosphorylated upon shift from GL to HL, which was reflected as a strongly increased excitation energy spilling in favour of PSI as compared to PSII (Paper I). The behavior of HL-shifted *pph1*/*tap38* and *pgr5* mutant plants thus resembled those of the WT plants under state 2 light conditions, with respect to both the thylakoid protein phosphorylation and distribution of excitation energy in the thylakoids (Paper I). As the *pgr5* plants (defect in the formation of proton gradient

across the thylakoid membrane at HL (Suorsa et al., 2012) have been shown to possess almost similar levels of STN7 protein kinase and PPH1/TAP38 protein phosphatase as WT (Paper I). It was concluded that the *pgr5* plants have the full phosphorylation/dephosphorylation capacity, but the regulatory mechanisms needed to activate the dephosphorylation pathway does not work in HL. In the *pgr5* plants, PSI photoinhibition is combined to low NPQ in HL, which together lead to reduction of the PQ pool and oxidation of PSI electron acceptors. This resembles the condition that is traditionally induced in WT by state 2 light where the STN7 and STN8 kinases phosphorylate their respective substrates and the PPH1/TAP38 and PBCP phosphatases cannot compete with the kinase activity, and dephosphorylate LHCII and the PSII core proteins, respectively (Tikkanen et al., 2012; Rochaix 2013). Indeed, it is conceivable that strong phosphorylation of PSII core and LHCII proteins is not a sole force that drives state transitions, but rather one of the components driving them to take place. The mechanism of state transitions is activated only when strong concomitant phosphorylation of both the PSII core and the LHCII phosphoproteins is occurring under conditions that enhance excitation pressure toward PSII but keep the PSI reducing side oxidised. In natural white light conditions, redox-dependent but opposite PSII core and LHCII protein phosphorylation, and the regulation of transmembrane proton gradient, interact and together maintain the proper excitation of the two photosystems.

5.3 Thylakoid membrane megacomplexes facilitate energy transfer to PSI in high light conditions

Thylakoid membrane harbors a high number of megacomplexes (Pesaresi et al., 2009; Järvi et al., 2011). Recently, the composition of the thylakoid membrane-embedded megacomplexes in non-appressed regions of thylakoid membranes (grana margins, stroma lamellae) has been shown to undergo redox-dependent changes, and their organization is mainly dependent on the phosphorylation status of LHCII proteins (Suorsa et al., 2015). Further, phosphorylation dependent alterations in the megacomplexes play a major role in balancing the distribution of excess excitation energy between PSII and PSI under changing light conditions (Suorsa et al., 2015). In line with this, the energy spillover towards the PSI in *pph1/tap38* mutant plants in HL conditions (Paper I) was shown to be linked to high amounts of the LHCII-PSI megacomplex (Pesaresi et al., 2009) and to the megacomplex containing both photosystems (PSII-LHCII-and PSI-LHCI) (Järvi et al., 2011; Suorsa et al., 2015) in non-appressed regions of thylakoid membranes. The functional role of the phosphorylation-dependent megacomplexes remains to be verified, but so far the LHCII-PSI megacomplex and PSI-PSII megacomplex have been suggested to be linked

to state transitions and quenching of excess absorbed light energy, respectively (Pesaresi et al., 2009; Yokono et al., 2015).

5.4 Photoinhibition of PSII as a photoprotection mechanism

Light-induced oxidative damage to PSII is a response to excess absorbed light energy, which is accompanied by induction of excess energy dissipation as heat (Müller et al., 2001; Tikkanen et al., 2008a; Theis and Schroda 2016; Tiwari et al., 2016). Indeed, most of the chlorophyll binding pigment protein complexes remain unaffected in photoinhibited leaves and concomitantly the captured light energy needs to be dissipated safely (Powles 1984). The most studied mechanisms of excess absorbed light energy dissipation are dependent either on the acidification of the thylakoid lumen (Niyogi et al., 2005; Paper IV) or the reduction status of the electron transfer chain (Allen et al., 1981; Paper I). However, both the maintenance of lumenal acidification and the reduction of electron transfer chain become gradually unmanageable in the course of progressing PSII photoinhibition (Paper II). Thus, the photoinhibition-induced thermal dissipation needs to be independent of the photoinhibitory reactions caused by the high energy status of the photosynthetic light reactions.

Despite the importance of the regulatory flexibility of the photosynthetic light reactions and active research in the field during the past decades, the mechanisms which allow the photosynthetic light reactions to tolerate the constantly changing environmental conditions have remained elusive. In paper III, the physiological role and mechanisms resulting from PSII photoinhibition were revealed. Because of the rapid repair cycle of PSII, the *in vivo* investigation of PSII photoinhibition requires the use of inhibitors (lincomycin or chloramphenicol) in order to arrest the chloroplast translation machinery (i.e., the repair machinery). Nevertheless, the inhibition of PSII in natural conditions is a response to many different stress conditions that chronically limit light utilization (Kok 1956). In Paper II and III, I demonstrated that if the PSII repair cycle is blocked prior to the shift of plants to HL, the photoinhibitory quenching originates from the loss of the lateral heterogeneity of the thylakoid membrane network (Paper III). This, in turn, likely results from the reorganization of higher order PSII-LHCII supercomplexes and also other protein complexes ordered in arrays in grana thylakoids, which takes place independently of the thylakoid protein phosphorylation (Paper III). Further, under such conditions the PSI complex is responsible for the dissipation of the light energy harvested by LHCII (Paper III).

5.5 Novel insights into protection of PSI against photodamage

PSI complex is generally a stable protein complex at physiological temperature conditions. However, it was shown recently that even in WT plants the PSI iron-sulphur clusters are sensitive to HL illumination (Tiwari et al., 2016). Protection of the PSI subunits from photodamage is highly important as both the biosynthesis and assembly of the protein components of PSI are slow processes (Schöttler et al., 2011). It has been known for decades that the PSI complex is protected against photodamage by antioxidant enzymes, which scavenge the reactive oxygen species (Asada 1999). More recently, it has become evident that the PGR5-dependent control of Cyt b_6f is critically important to protect PSI from irreversible photooxidative damage (Suorsa et al., 2012). Indeed, the formation of a strong proton gradient across the thylakoid membrane plays a major role in controlling the rate of electron flow from PSII through Cyt b_6f complex to PSI, which otherwise would exceed the capacity of electron carriers on the acceptor side of PSI and lead to PSI photoinhibition. However, a strong proton gradient is dangerous under prolonged HL illumination when the transient protonation of the thylakoid lumen should gradually become relaxed (Davis et al., 2016). The results obtained showed that the operation of the PGR5-dependent photosynthetic control of Cyt b_6f in short periods, upon a sudden shift to excess light intensity, is important in order to limit an abrupt electron flow to PSI (Paper II). Sonoike (2011) initially proposed a possibility that photoinhibition of PSII could, in fact, protect PSI from photoinhibition. To study this mechanism in detail, the consequences of decreasing the amount of functional PSII centers on the activity of PSI reaction centers was assessed in WT and the *pgr5* mutant plants (Paper II). It was demonstrated that under prolonged periods of HL illumination, the down-regulation of PSII activity (PSII photoinhibition) played a major role in preventing excess electron flow to PSI and thereby rescued PSI from photodamage (Paper II). To that end, the photodamage of PSII cannot be simply considered as a fault in photosynthesis, but also as a protection system of PSI.

5.6 PsbS protein plays a role in driving energy towards PSI in low pH

Despite many scientific studies, there are still many open questions related to the pH induced changes in isolated thylakoid membranes. Previously, low pH has been suggested to drive the state transitions (Singh-Rawal et al., 2010; Jajoo et al., 2012). However, in Paper IV it was shown that the traditional state transitions (i.e., phosphorylated LHCII proteins play a major role in the distribution of excitation energy from PSII to PSI in the thylakoids) are not involved in the low pH dependent distribution

of the excitation energy between PSII and PSI in isolated thylakoid membranes (Paper IV). Instead, we concluded from experiments with several photosynthesis regulation mutants, that the protonated PsbS protein subunit at low lumenal pH regulates the energy spillover (Paper IV). A decrease in the thylakoid lumen pH at HL has been shown to induce PsbS- and xanthophyll cycle-dependent dissipation of excess excitation energy as heat (Bergantino et al., 2003). Acidification of the thylakoid lumen results in the activation of an enzyme called violaxanthin de-epoxidase (VDE) in the lumen (Demming-Adams 1990) and also leads to the protonation of the lumen-exposed domain of the PsbS protein. Activated VDE catalyzes the de-epoxidation of violaxanthin to zeaxanthin in the xanthophyll cycle (Bugos and Yamamoto 1996). An accumulation of zeaxanthin and protonation of PsbS triggers conformational changes in the LHCII complex, which, in turn, results in thermal dissipation of excess excitation energy (qE) as heat (Kiss et al., 2008). However, experiments with DTT (the inhibitor of violaxanthin de-epoxidation) and ascorbate (stimulate zeaxanthin formation) provided evidence that zeaxanthin cycle dependent NPQ is not involved in pH induced modifications of excitation energy distribution between the two photosystems (PSII and PSI) (Paper IV).

5.7 Co-operation of various regulation mechanisms in optimizing the photosynthetic light reactions

A number of different regulation mechanisms participate in optimization of photosynthetic light reactions in varying environmental conditions. In my thesis, I aimed to understand how the regulatory mechanisms co-operate, from protecting the photosynthetic apparatus against light-induced damage and in keeping the photosynthetic apparatus efficient despite changes in environmental conditions. It has become evident that dynamic re-organizations of the pigment protein complexes in the thylakoid membrane are a driving force for maintenance of the activity of the photosynthetic apparatus in ever-changing environmental conditions. It is worth mentioning that in constant light conditions, whether they are of low, medium or high intensity, the photosynthetic apparatus easily acclimates. However, the fluctuations in environmental conditions are a big challenge and strict regulation of photosynthetic light reactions is vital to overcome potentially highly damaging effects. Figure 5 summarizes our current understanding on the co-operation of the major regulatory mechanisms of the photosynthetic apparatus in the thylakoid membrane upon sudden changes in light intensity.

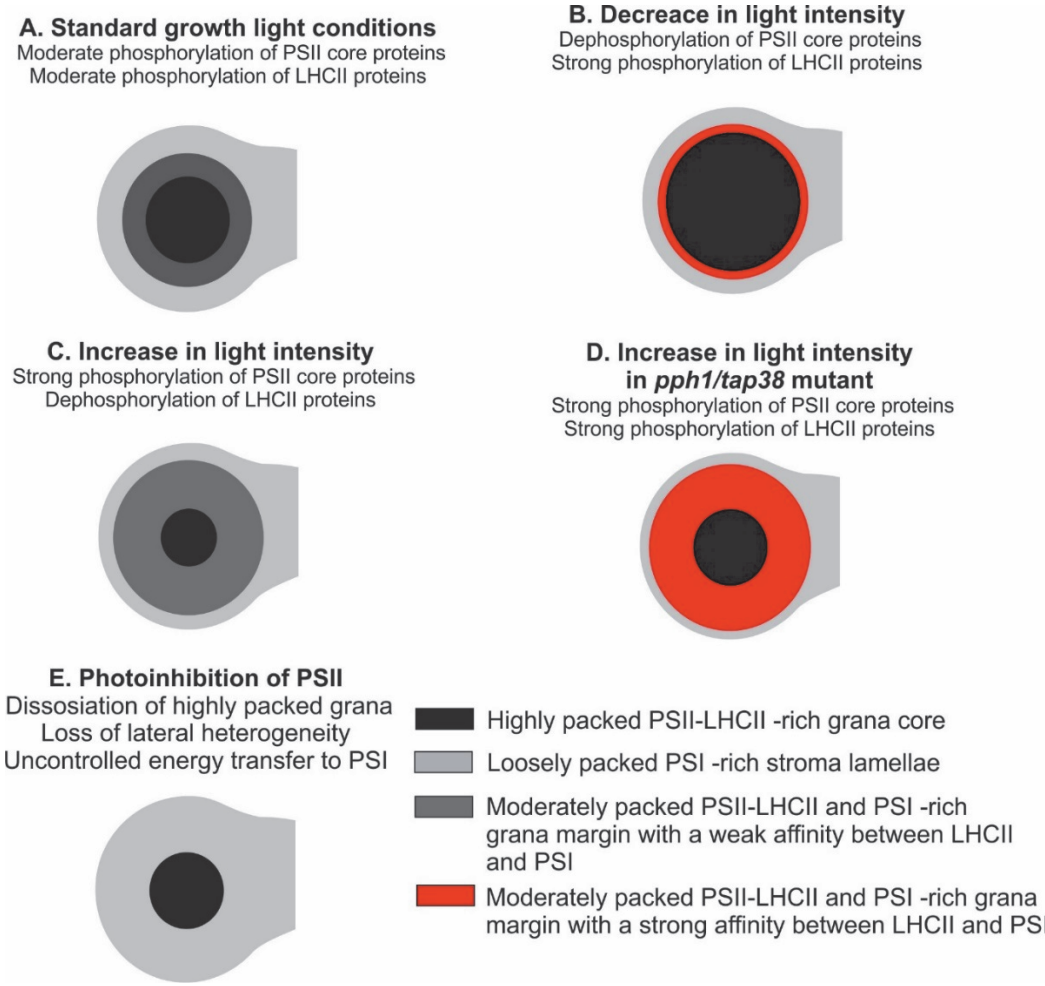

Figure 5. A simplified scheme of the regulatory mechanisms which control the transfer and distribution of excitation energy in order to optimize the photosynthetic efficiency under changing light conditions (modified from Mekala et al., 2015 (Paper I)).

A. In the standard growth light conditions (constant light of low, medium or high intensity) both PSII core and LHCII complex proteins of thylakoid membranes are moderately phosphorylated. Moderately phosphorylated PSII-LHCII proteins provides fluidity for the thylakoid membranes and is a prerequisite for sufficient excitation energy transfer from LHCII to PSI, thus allowing proper turnover of PSII and an optimal oxidation state of the electron transfer chain, respectively. Indeed, the moderate phosphorylation of thylakoid proteins inhibits the damage of the photosynthetic apparatus upon sudden increases in light intensity.

B. PSII core phosphoproteins undergo dephosphorylation upon a decrease in light intensity. This, in turn, induces a dense packing of the PSII-LHCII supercomplexes and thereby increases the light absorption efficiency. Such high-order PSII-LHCII complexes create a physical barrier between PSI and LHCII while the increased LHCII phosphorylation enhances the excitation energy transfer from LHCII in favour of PSI.

Concomitantly, these two opposite events balance the relative distribution of excitation energy between PSII and PSI.

C. Upon an increase in light intensity, the PSII core proteins are strongly phosphorylated and loosen the thylakoid architecture, thus releasing the segregation between PSII-LHCII and PSI. This, in turn, results in the transfer of excess excitation energy from PSII-LHCII to PSI, but the concomitant dephosphorylation of the LHCII proteins by the PPH1/TAP38 phosphatase takes place.

D. In *pph1/tap38* mutant plants (deficient in dephosphorylation of the LHCII proteins) and *pgr5* mutants (impaired in regulation of LHCII phosphorylation in HL), both the PSII core and LHCII proteins are simultaneously phosphorylated upon a shift to high light. The hyperphosphorylation of the PSII core and LHCII complex proteins leads to the release of the high amount of PSII-LHCII from the grana thylakoids to the PSI-enriched grana margin, thus establishing a strong connection between the LHCII complex and PSI. The concomitant increase in the excitation energy flow towards PSI under such conditions mimics the effect of the state 2 light. Low luminal pH drives excitation energy towards PSI in darkness (Paper IV).

E. The PSII photoinhibition induces the unpacking of the highly organized PSII-LHCII supercomplexes in the grana membranes, and thus leads to the loss of lateral heterogeneity of the thylakoid membrane. This Loss leads to uncontrolled excitation energy flow towards PSI and subsequent dissipation of excitation energy by PSI.

6. CONCLUSIONS

The data presented here demonstrate that the dynamic network of regulatory mechanisms controls the excitation energy transfer and distribution in order to optimize the photosynthetic efficiency under changing light conditions (Figure 5). The following findings were revealed as part of the thesis work:

- The LHCII dephosphorylation in HL is dependent on the PPH1/TAP38 phosphatase, which prevents concomitant high phosphorylation of both the PSII core and LHCII proteins upon increase in light intensity. The opposite phosphorylation of the PSII core and LHCII proteins prevents the increased relative excitation of PSI, which occurs when plants are illuminated with traditional state 2 light.

- The PGR5 protein, which is essential for generation of a transthylakoid proton gradient upon increase in light intensity and for the protection of the PSI complex, is likewise required for dephosphorylation of LHCII in HL.

- The PSII photoinhibition-repair cycle, which regulates the amount of active PSII centers in the thylakoid membrane, interacts with the PGR5-dependent control of electron flow and thus protects the PSI complex from photodamage.

- PSII photoinhibition-related quenching of excitation energy is due to the loss of lateral heterogeneity of the thylakoid membrane, thus allowing the PSI complex to act as a strong energy quencher. This process is independent of phosphorylation of thylakoid proteins.

- The PsbS protein plays an important role in enhancing the energy spillover to PSI at low lumenal pH.

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