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DIVERSITY IN PHOSPHORYLATION OF THYLAKOID MEMBRANE PROTEINS IN CHLOROPLASTS

Azfar Ali Bajwa

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Azfar Ali Bajwa

University of Turku

Faculty of Technology
Department of Life Technologies
Molecular Plant Biology
Doctoral programme in Technology

Supervised by

Prof. Eva-Mari Aro
Department of Life Technologies
University of Turku, Finland

Dr. Andrea Trotta
Department of Life Technologies
University of Turku, Finland

Dr. Caterina Gerotto
Università Politecnica delle Marche, Italy

Reviewed by

Dr. Monica Meijon Vidal
Department of Organisms and Systems
Biology
University of Oviedo, Spain

Dr. Szilvia Z Toth
Biological Research Centre Szeged
Institute of Plant Biology, Hungary

Opponent

Prof. Agnieszka Mostowska
Department of Plants Anatomy and
Cytology
University of Warsaw, Poland

UNIVERSITY OF TURKU
Faculty of Technology
Department of Life Technologies
Molecular Plant Biology
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ABSTRACT

Photosynthesis is the most fundamental process for life on Earth. Sunlight is highly variable and fluctuates both in intensity and duration, which induces short and long-term responses in photosynthetic organisms. Photosynthetic organisms have evolved several processes to cope with environmental stresses, including light fluctuations. Many thylakoid properties and functions are regulated by diverse protein phosphorylations, ranging from thylakoid ultrastructural changes and the optimization of excitation energy between the two photosystems via Light-harvesting complexes (LHCs) to signaling mechanisms for long-term regulation, maintaining proteostasis, and thereby ultimately regulating photosynthesis via photoprotection mechanisms.

My PhD research is divided into three major projects. The first one gives evolutionary insights into the two main thylakoid kinases, STN7 and STN8 in *Physcomitrium patens* (previously *Physcomitrella patens*), and their target proteins. *Physcomitrium patens* differs from angiosperms in their photoprotection strategy via LHCII phosphorylations (LHCB6 and LHCBM) and in formation of a Photosystem (PS)I-supercomplex, PSI-large, depending on LHCBM phosphorylation.

The second project focuses on angiosperm model species *Arabidopsis thaliana* and deals with protein phosphorylation-related changes in thylakoid architecture. The study reveals that the phosphorylation dynamics of LHCII and CURT1B respond co-operatively to fluctuating light intensities. The findings also suggest that CURT1B phosphorylation contributes to the fine-tuning of thylakoid membrane structure and function in response to light conditions.

The third project delves into the potential association between calcium signaling and induction of photoprotective mechanisms, by providing a novel screening tool to identify calcium-dependent chloroplast proteins. Calcium-transient dependent phosphorylation of essential proteins involved in the repair of PSII, including THF1, HCF136, and FTSH protease were disclosed. Additionally, the study proposes a potential link of calcium in PSI-Fd-FNR interaction. Moreover, this study not only identifies the new phosphorylation targets but also presents opportunities for exploring the intricate interplay between calcium signaling and protein phosphorylation processes. Taken together, my PhD research helps in understanding the regulation of photosynthesis, provides new tools for photosynthesis research and will thereby contribute to engineering photosynthetically resilient organisms to cope with changing environmental conditions for improving the production of food, feed and renewable energy.

KEYWORDS: Photosynthesis, Physcomitrium patens, PSI-large, phosphorylations, thylakoid curvature, calcium signaling

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TIIVISTELMÄ

Fotosynteesi ylläpitää elämää maapallolla muuttamalla auringon valoenergiaa kemialliseksi energiaksi. Auringonvalo vaihtelee sekä voimakkuudeltaan että kestoltaan, mikä saa aikaan lyhyt- ja pitkäaikaisia vasteita fotosynteettisissä organismeissa. Fotosynteettiset organismit ovat kehittäneet useita prosesseja selviytyäkseen ympäristönstresseistä, mukaan lukien voimakkaat valon vaihtelut. Kasvien fotosynteesi tapahtuu viherhiukkasten sisäisessä kalvosysteemissä, tylakoideissa, joiden ominaisuuksia ja toimintoja säätelevät erilaiset proteiinien fosforylaatiot. Valohaaviproteiinien (LHCII) fosforylaatio muuttaa tylakoidin hienorakennetta ja optimoi viritysenergian jakautumista kahden valoreaktion (PSII ja PSI) välillä, mutta vaikuttaa myös signaalimekanismien kautta proteostaasin ylläpitämiseen ja siten viime kädessä fotosynteesin pitkänajan säätelyyn.

Tohtoritutkimukseni on jaettu kolmeen osaprojektiin. Ensimmäinen pureutuu kahdessa tärkeimmässä tylakoidikinaasissa (STN7 ja STN8) ja niiden kohdeproteiineissa evoluution aikana tapahtuneisiin muutoksiin. Tutkimus osoitti sammalen, evolutiivisesti vanhimman maakasvin, eroavan valosuojausstrategiassaan siemenkasveista, ja muokkaavan LHCB6 ja LHCBM proteiinien fosforylaatioiden ja PSI-superkompleksien muodostumisen kautta viritysenergian jakautumista PSII:n ja PSI:n välillä.

Toinen osaprojekti käsittelee siemenkasvien malliorganismin, lituruohon (*Arabidopsis thaliana*), tylakoidiproteiinien fosforylaation aiheuttamia muutoksia tylakoidiverkoston hienorakenteessa, ja paljastaa LHCII:n ja CURT1B:n fosforylaatiodynamiikan yhteistyön vaikuttavan rakenteen ja toiminnan väliseen hienosäätöön, toimien vasteena valo-olosuhteiden muutoksille.

Kolmannessa osaprojektissa tarkastellaan mahdollista kalsium-signaloinnin ja kasvin proteiinifosforylaatioon perustuvan valosuojausmekanismin välistä yhteyttä, käyttämällä hyväksi seulontaan kehitettyä uutta työkalua. Tutkimuksessa kuvattiin PSII:n korjaamiseen välttämättä tarvittavien proteiinien (THF1, HCF136 ja FTSH-proteaasi) kalsiumista riippuvainen fosforylaatio. Lisäksi tutkimustulokset viittaavat kalsiumin toimintaan PSI-Fd-FNR vuorovaikutuksessa sekä erittäin monimutkaiseen vuorovaikutukseen kalsium-signaloinnin ja proteiinien fosforylaatioprosessien välillä.

Yhteenvetona totean, että väitöskirjatutkimukseni auttaa ymmärtämään fotosynteesin säätelyä, tarjoaa uusia työkaluja fotosynteesin tutkimukseen ja myötävaikuttaa siten uusien, fotosynteettisesti kestävien ja tehokkaiden organismien suunnitteluun ja tuottamiseen. Tämä puolestaan edesauttaa selviytymistämme muuttuvissa ympäristöolosuhteissa ruoan, rehun ja uusiutuvan energian tuotannon parantamiseksi.

ASIASANAT: Fotosynteesi, Physcomitrium patens, PSI-large, fosforylaatiot, tylakoidikaarevuus, kalsiumsignalointi

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Abbreviations

2D	Two-dimensional
2-PG	2-phosphoglycolate
3-PGA	3-phospho-glycerate
ATP	Adenosine triphosphate
CAS	Calcium Sensor protein
CaM	Calmodulin
CBB	Calvin Benson Bassham cycle
CET	Cyclic electron transport
Chl	Chlorophyll
CL	Control Light
CP	Chlorophyll-binding protein
CRX	Calredoxin
CSP	Calcium-binding proteins
CURT1	CURVATURE THYLAKOID1
Cyt	Cytochrome
Cyt b_6f	Cytochrome b_6f
DHAP	Dihydroxyacetone-3-phosphate
DIGE	Differential in gel electrophoresis
ETC	Electron transfer chain
Fd	Ferredoxin
Fd-TRX	Ferredoxin-dependent thioredoxin system
FL	Fluctuating light
FNR	Ferredoxin-NAD(P)H-oxidoreductase
FR	Far-red
GAP	Glyceraldehyde-3-phosphate
Gya	Giga years ago
H_2O_2	Hydrogen peroxide
HCaTC	High $[Ca^{2+}]$ treated chloroplasts
HGT	Horizontal gene transfer
HL	High light
IEF	Isoelectric focusing

LCaTC	Low [Ca^{2+}] treated chloroplasts
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LET	Linear electron transport
LHC	Light harvesting complex
LL	Low light
L-LHCII	Loosely bound LHCII
LP	Loose pellet
lpBN-PAGE	Large pore blue-native polyacrylamide gel electrophoresis
LUCA	Last universal common ancestor
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NADP$^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NDH	NAD(P)H dehydrogenase-like complex
NPQ	Non-photochemical quenching
NTRC	NADPH-dependent chloroplast thioredoxin reductase system
OEC	Oxygen evolving complex
P-Thr	Phosphothreonine
PAGE	Polyacrylamide gel electrophoresis
PBCP	PSII CORE PHOSPHATASE
PC	Plastocyanin
PCR	Polymerase chain reaction
PGR5	Proton gradient regulation 5
PGRL1	Pgr5-like photosynthetic phenotype 1
Pheo	Pheophytin
PMF	Proton motive force
PQ	Plastoquinone
PQH2	Plastoquinol
PS	Photosystem
PSAL	Photosystem I subunit L
PSB33	Photosystem II protein 33
PTM	Post-translational modifications
RC	Reaction center
RNAi	RNA interference
ROS	Reactive oxygen species
RuBP	Ribulose 1,5-bisphosphate
SC	Supercomplex
STN7	STATE TRANSITION7 kinase
STN8	STATE TRANSITION8 kinase
SDS	Sodium dodecyl-sulfate
TAP38	THYLAKOID ASSOCIATED PHOSPHATASE of 38kD

UV	Ultraviolet
WT	Wild Type
β-DM	n-dodecyl β-D-maltoside

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I   *Trotta A, *Bajwa AA, Mancini I, Paakkarinen V, Pribil M, Aro E-M. The Role of Phosphorylation Dynamics of CURVATURE THYLAKOID 1B in Plant Thylakoid Membranes. *Plant Physiol*, 2019; 181: 1615–1631.

II   Gerotto C, Trotta A, Bajwa AA, Mancini I, Morosinotto T, Aro E-M. Thylakoid Protein Phosphorylation Dynamics in a Moss Mutant Lacking SERINE/THREONINE PROTEIN KINASE STN8. *Plant Physiol*, 2019; 180: 1582–1597.

III Gerotto C, Trotta A, Bajwa AA, Morosinotto T, Aro EM. Role of Serine/Threonine Protein Kinase Stn7 in the Formation of Two Distinct Photosystem I Supercomplexes in Physcomitrium patens. *Plant Physiol*, 2022; Jun 23: kiac294.

IV   Bajwa AA, Trotta A, Nurmi M, Aro E-M. Calcium-induced Modulations of Chloroplast (Phospho-)proteins in Arabidopsis thaliana. (Manuscript), 2024.

*Authors contributed equally to the manuscript

1   Introduction

The planet Earth is precisely at the right spot in the Universe for life to exist. Sun, via massive fusion reactions, releases energy in the form of solar radiation. The Earth receives energy in the form of light (photon) and heat from the sun. The solar energy received by Earth is the essential ingredient to sustain life on Earth. The energy enters the Earth's ecosystem via photosynthetic organisms i.e. anoxygenic photosynthetic bacteria and oxygenic cyanobacteria, algae, and plants. These organisms convert solar energy into chemical energy (organic compounds), via specialized pigment-protein complexes localized at the membranes, in a process of photosynthetic light reactions. Thylakoid pigment-protein complexes (LHCII, LHCI) harvest light energy, which is trapped in the two photosystems (PSII and PSI) and eventually by the reaction center chlorophylls RCP680 and RCP700, respectively. Collected light energy excites the RC chlorophylls leading to charge separation in both PSII and PSI, and transfer of an electron from both photosystems to the electron transfer chain (ETC). The electrons excited by PSI can take different routes, depending on environmental and metabolic conditions, but are primarily used as reducing power to fix atmospheric CO_2 to organic compounds. Photosynthetic organisms have occupied various ecological niches and are fundamental for providing the source of primary energy in the ecosystem. Therefore, it is essential to extensively understand the regulation of photosynthetic energy and electron transfer mechanisms. This is necessary to ensure and preserve the Earth's ecosystem and to increase the possibilities of a sustainable environment in the presently increasing imbalances of ecosystems due to global warming. Understanding the regulatory mechanism also helps create sustainable solutions in bioenergy, crop production, food, and feed.

1.1   Origin of oxygenic photosynthesis

Anoxygenic photosynthesis pre-dates the origin of oxygenic photosynthesis (Hohmann-Marriott & Blankenship, 2011). The possible invention of oxygenic photosynthesis, in cyanobacteria, is evidenced by free molecular oxygen presence in a 2.4 billion-year-old fossil (Hohmann-Marriott & Blankenship, 2011). The increase in atmospheric oxygen started between 2.9 to 2.4 Gya followed by a great

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oxygenation event at around 2.4 to 2.0 Gya, and later stayed stable till 0.54 Gya (Holland, 2006). The oxygen levels are at about 21 percent in the atmosphere, since the advent of the land plants, at around 0.470 Gya (Gensel, 2008; Gray et al., 1985), with remarkable increases up to 35% found at about 0.360 to 0.300 Gya-coinciding with the emergence of vascular plants (Berner, 1999), consequently, increasing the carbon burial (Berner, 1999). Oxygen production (increase) is considered the critical event in the course of evolution, which also led to the formation of the ozone layer in the stratosphere of Earth, thus protecting against unwanted solar radiation (Cavalier-Smith, 2006) and thus allowing land plants to succeed. Moreover, it was critical for land plants to evolve photoprotection/regulatory mechanisms to cope with land environments.

The core components of photosynthesis have undergone horizontal gene transfer (HGT) during the course of evolution (Raymond, 2002). Cyanobacteria have two photosystems (PSI and PSII), PSII being the one with an Mn-containing oxygen evolving complex (OEC), as compared to primitive phototrophic anoxygenic prokaryotes which have either one of the photosystems, without the OEC (Blankenship et al., 2007). According to the concept of evolution, a simple life form leads to a complex one. Due to HGT, it has been assumed that the origin of oxygenic photosynthesis is based on either the gene selective loss or the fusion mechanism (Hohmann-Marriott & Blankenship, 2011). However, the origin of oxygenic photosynthesis is still debated (Cardona, 2019; Sánchez-Baracaldo & Cardona, 2020), but most researchers think that its origin is ancestral to cyanobacteria (Martin et al., 2018). Overall, there are still missing gaps in the origin of photosynthesis.

1.2   Origin of chloroplast and first land plant

First appearance of photosynthetic eukaryotic organisms occurred at around 2 to 2.1 Gya (Bhattacharya & Medlin, 1998). However, the first evidence of a eukaryotic organism comes from a rock 2.7 Gya (Brocks, 1999). The origin of plastids (chloroplast) is known to have occurred via endosymbiosis, when a proteoeukaryote engulfed a cyanobacterium (Hohmann-Marriott & Blankenship, 2011). Further endosymbiosis events are the basis of the origin of glaucophyte, red and green algae, retaining the same primary chloroplast. The closest relatives of land plants are the streptophyte green algae (charophyta), and this is considered to be the origin of all embryophytes that include bryophytes (non-vascular) and tracheophytes (vascular) (Buschmann & Holzinger, 2020). The origin of the first land plants is estimated to be around the middle Cambrian-early Ordovician interval at about 0.470 Gya (Buschmann & Holzinger, 2020; Morris et al., 2018). This is the time when the shift from the aquatic to terrestrial (land) took place, and the first land plants, bryophytes, diverged from the tracheophytes in the early phases of terrestrialisation. Thereafter,

different adaptation mechanisms evolved to overcome harsher land conditions such as desiccation, UV light, high light, temperatures and changed nutrient supply (Buschmann & Holzinger, 2020). Adaptations required changes in morphology as well as in cellular and molecular regulatory processes.

Most importantly, the land plants developed complex signaling networks in response to environmental stresses. Such complex protein networks between different cell compartments were already present in the streptophyte algae, but the function in algae is mostly different from that in land plants (Fürst-Jansen et al., 2020). Because of bryophytes' unique position in evolution, between the charophyta and the tracheophytes (Rensing et al., 2008), their study will contribute to the understanding of regulatory mechanisms during transition of life from oceans to land.

1.3 Moss Physcomitrium patens as a representative of early land plants

The model organism for bryophytes is the moss *Physcomitrium patens* (previously *Physcomitrella patens*) (hereafter Physcomitrium)(Rensing et al., 2020), which belongs to the family of Funariaceae (Lang et al., 2008). It was the first bryophyte to have its whole genome sequenced (Rensing et al., 2008, 2020), and soon the genomes of the hornworts were sequenced (Zhang et al., 2020), complementing the Physcomitrium genome. The comparative genomic studies showed that gene loss among the bryophytes was more common than gene gain throughout evolution, while Physcomitrium had more gene gain than loss (Zhang et al., 2020). Physcomitrium in comparison to algae, has gained genes to survive on land but lost genes necessary for aquatic environments, eventually enabling it to cope with new types of stresses such as heat, drought, light, and less water (water is considered necessary for its reproduction). These factors also make the bryophyte genomes highly dynamic and make Physcomitrium an appealing research material.

Physcomitrium is a very amenable organism for research work. It is efficient in homologous recombination and transient RNAi, reproduces vegetatively, is easy to collect and culture, and has a predominantly haploid life cycle. The moss can regenerate from a spore (a haploid spore), which forms protonema tissue comprised of filamentous cells. Protonema grows by tip growth of apical cells. Subapical cells may also divide a few times and form branches. A switch from 2D to 3D growth gives rise to a bud, which then differentiates into a gametophyte. Following fertilization of the gametophyte, a sporophyte is formed, which is the only diploid stage of this plant. Meiosis within the sporophyte then produces spores (Cove, 2000; Rensing et al., 2020).

During my research work in the lab, I used juvenile protonema phase of the plant, which grows as a single cell thick layer of tissue. There are only two cell types in this tissue: chloronema and caulonema. Chloronema grows more slowly than caulonema, with a higher number of chloroplasts and a perpendicular wall. Caulonema grows faster and has slanting walls and fewer chloroplasts (Cove, 2000; Rensing et al., 2020).

As the last common ancestor of all land plants, the genome of bryophytes differs from that of aquatic algae and therefore provides interesting possibilities to investigate the genes enabling life on land. Terrestrial life also led to the evolution of specific signaling cascades, for example based on plant hormones and photoreceptors, to develop resilience to different biotic and abiotic stresses (Rensing et al., 2008; Waters, 2003). Adaptation and acclimation of land plants to environmental stress involves the synthesis and accumulation of protective pigments such as carotenoids and flavonoids, as well as various osmoprotectants, the elaboration of plant transport capabilities, and a number of biochemical adaptations of proteins as well as DNA replication and repair mechanisms.

1.4   Photosynthesis of land plants

1.4.1   Energy trapping by pigments within the chloroplast

Light energy is captured by photosynthetic eukaryotes in chloroplasts. The chloroplast is composed of the soluble stroma compartment and lipid bilayer membranes, which comprise two envelope membranes surrounding the chloroplast and an extensive inner membrane system, called thylakoid, and enclosing another soluble chloroplast compartment, the lumen (Figure 1A). Chloroplast has its own DNA, like that of bacteria, which provides a template for protein synthesis via chloroplast-localized protein synthesis machinery. The chloroplast encoded proteins are not capitalized in their abbreviations in this thesis. Proteins essential for photosynthetic light reactions are embedded in the thylakoid membrane, and provide a platform where the light energy is captured and photosynthetic light reactions take place. Thylakoid trans-membrane proteins form large pigment-protein complexes, which work in synergy to collect, transfer and convert solar energy to chemical energy. The two large pigment protein complexes, PSII and PSI, comprise the reaction center (RC) complexes responsible for energy conversion. Additionally, a great number of other proteins, protein complexes, pigments and various other factors reside either embedded in the thylakoid membrane or more loosely associated either on the stromal or lumenal side of the thylakoid membrane, and participate in completion of the light reactions. Typically, the thylakoid membrane in chloroplasts of angiosperms, such as

Arabidopsis thaliana (hereafter Arabidopsis) comprises stacked and non-stacked regions, also called appressed grana and non-appressed stroma regions, respectively (Figure 1). The distribution of the transmembrane protein complexes in different thylakoid regions occurs in a non-random manner, the PSII mainly accumulating in appressed grana membranes, while PSI and ATP synthase in the non-appressed regions of the thylakoid membrane, whereas the Cytochrome b_6f (Cyt b_6f) complex is evenly distributed throughout the membrane (Figure 1) (Anderson, 2012; Tikkanen et al., 2008b).
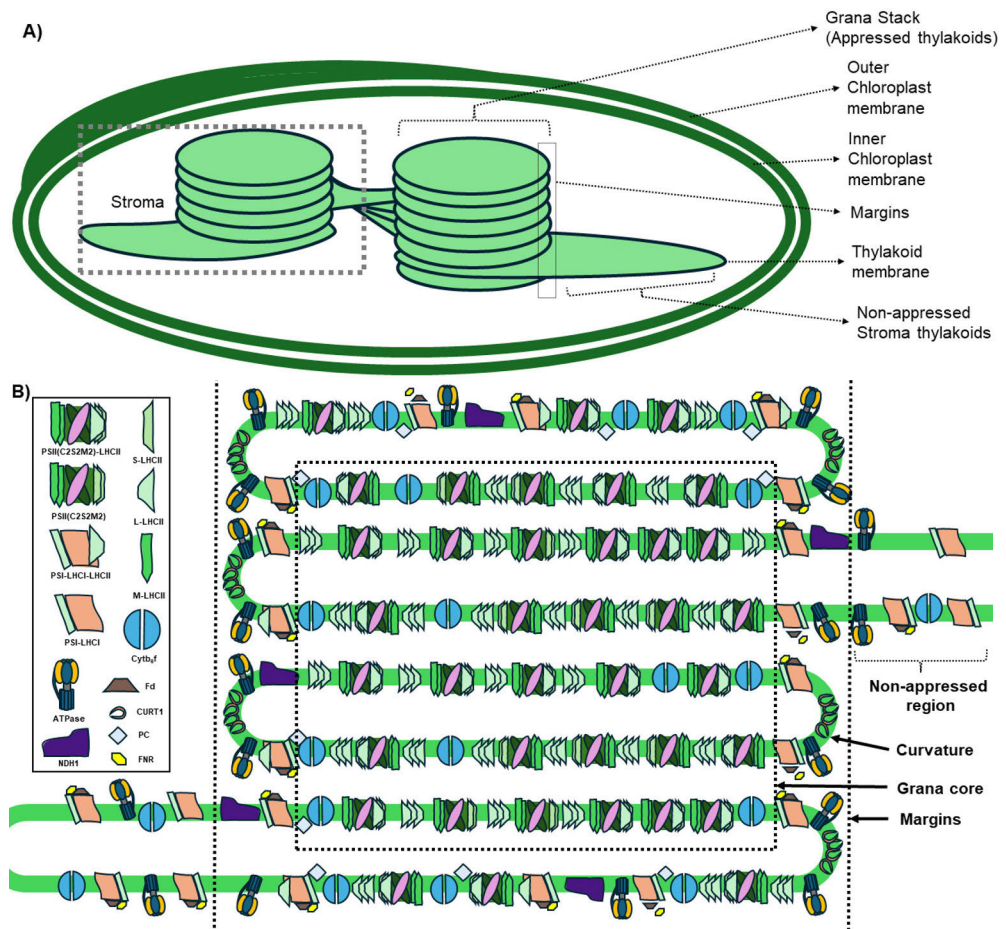
Figure 1. A simplified scheme of thylakoid ultrastructure and heterogeneous distribution of photosynthetic protein complexes in chloroplasts of angiosperms. **A.** Typical thylakoid membrane organisation with stacked grana and non-appressed stroma thylakoids. **B.** Heterogeneous distribution of thylakoid protein complexes between the stacked grana membranes and non-appressed stroma membranes.

The capture of light and its energy conversion take place in photosynthetic pigments, whose orientation in the thylakoid membrane is strictly determined by thylakoid proteins and lipids. All photoautotrophic organisms contain chlorophyll (Chl), and the special Chl a pair in PSII and PSI complexes function as reaction center Chl P680 and P700, respectively. However, the vast majority of Chl molecules are associated with inner and outer light-harvesting complexes that collet the light energy and transfer it to the P680 and P700 RC molecules to be used in chemical work (photochemistry).

The concept of energy capture and electron transfer is based on different models and theories (Blankenship, 2014). The widely accepted general phenomenon is that the energy is captured from photons hitting the antenna complexes on the photosynthetic organisms, followed by its transfer to neighboring Chl molecules until captured into a trap (RC) (Blankenship, 2014). In this context, a photosynthetic unit concept was developed based on Emerson and Arnold experiments (Emerson et al., 1957; Nickelsen, 2015). A photosynthetic unit comprises a collection of pigments, among which the excitation energy could "travel" before being trapped or stabilized by a limiting enzyme, the RC. The energy that is absorbed by a pigment molecule inside the thylakoid membrane, is used to excite the neighboring pigment to get it into an excited state, referred as exciton transfer. The energy is absorbed according to the absorption maximum of the molecule. For example, the energy is trapped and then funneled into a reaction center (P680 at PSII RC) via exciton transfer and the excited state is then quenched by photochemistry. When there is intense light, besides photochemistry, some of the energy is released as fluorescence (approximately 2 to 10%) while some is dissipated as heat or internal conversion, referred as non-photochemical quenching (NPQ) (Blankenship, 2014).

1.4.2   Major thylakoid protein complexes

The structure and function of the PSII and PSI core complexes, as well as the $Cytb_6f$ complex, have remained relatively conserved over the course of evolution. Their sequential order of function in linear electron transport (LET) is presented in Figure 2. More diversity exists in the light-harvesting systems between photosynthetic organisms to adjust their photosynthesis to variable light conditions and ecological niches (Allen et al., 2011; Crepin & Caffarri, 2018). Some structural aspects of the major photosynthetic protein complexes of the thylakoid membrane are introduced below.
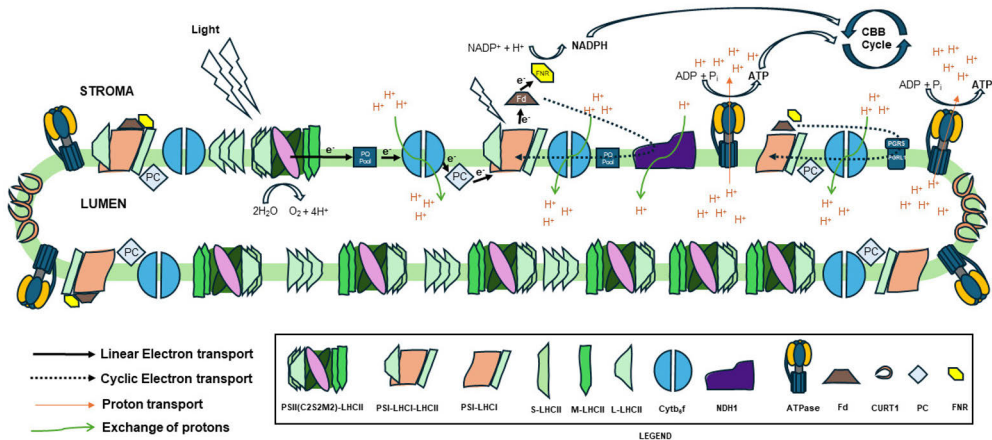
Figure 2. Simplified scheme of the main electron transfer components and pathways in angiosperm thylakoids. In addition to the linear electron transport, two cyclic pathways around PSI are shown, one via the NDH1 complex and the other involving the PGRL1 and PGR5 proteins.

1.4.2.1   Photosystem II (PSII)

Photosystem II is a multisubunit protein complex comprising over 20 different proteins, according to the spinach PSII-LHCII structure (Wei et al., 2016). There are four major intrinsic subunits (D1, D2, CP43 and CP47) that show 77 to 85 % similarities in their amino acid sequences to the cyanobacterial PSII. Among the four major intrinsic subunits, D1 and D2 form the reaction center (RC) complex of PSII. The RC Chl a of PSII occupies a central position and is named as P680 (Chl pigment with maximum absorption at 680 nm). Light energy from the PSII internal and peripheral antennae complexes is funneled to the P680 reaction center, where the primary charge separation takes place and photochemistry reactions are initiated.

CP43 and CP47 connect to the PSII RC complex and form the core antenna complex together with other 12 low molecular weight intrinsic proteins. The PSII core interactions with the peripheral antenna are reinforced by the PSBW, PsbH and PsbZ (Wei et al., 2016). The three extrinsic subunits PSBO, PSBP, and PSBQ surround the core on the lumenal side and interact with the lumenal loops of the CP43 and D1 proteins. This region at the lumenal side is essential in coordinating the functions of PSII core and the oxygen-evolving complex (Mn_4CaO_5 complex).

1.4.2.2   Photosystem I (PSI)

PSI consists of more than 19 protein subunits. The RC complex of PSI is a heterodimer, consisting of PsaA and PsaB. The stromal ridge, referred to the place on the PSI stroma exposed side where FNR may interact with Fd binding site,

constitutes of the PsaC, PSAD and PSAE proteins (Marco et al., 2019). The PSI LHC belt is attached to the PSI via PSAG and PSAK (Amunts et al., 2010; Caspy et al., 2021). The L-LHCII peripheral antenna attaches to PSI, at a docking site comprised of PSAH, PSAL, PSAO and PsaI, opposite to the LHCI belt (Galka et al., 2012; Lunde et al., 2000; Mazor et al., 2015). When PSII is more excited than PSI, there is an additional energy transfer to the LHCI antenna of PSI in a process called state transition (Benson et al., 2015).

1.4.2.3   Cytochrome b_6f

Cytochrome b_6f (Cytb_6f) is a multiprotein complex and is functional as a dimer. The monomer of the Cytb_6f is composed of four major proteins PetA, PetB, PETC, PetD and four minor proteins (PetG, PETL, PETM, PetN). One of the structural features includes the core helical bundle, which is composed of the transmembrane helices of the PetB and PetD subunits (Malone et al., 2021). This central core binds two b type haemes (b_n and b_p) and a c' haeme type c (haeme c_n). The central core is further surrounded by the transmembrane helices of minor Cytb_6f proteins. PetA and PETC protrude out from the lumenal side. In these domains at the lumenal side, the PETA binds the c-type haeme and PETC binds the 2Fe-2S cluster. Cytb_6f additionally attaches the Chl-a and β-carotene pigments connected to PetD. Overall, the dimeric complex is stabilized by the domain swapping of the PETC at the lumenal side (p side), interlinking the two monomers, thereby further strengthening the interaction between the PetB and PetD (Malone et al., 2021). The cavity is formed between the two monomers, where the internal pool of PQ/PQH_2 is present. Here, in this cavity, at the lumenal side of the complex is the Q_p site (a site for PQH_2 oxidation), and at the stromal side (the n side) resides the Q_n site (a site for PQ reduction) (Kurisu et al., 2003; Malone et al., 2019; Stroebel et al., 2003). Moreover, the Cytb_6f dimer is known to bind to PSI at a site where LHCA1 is located. This interaction is weak, because Cytb_6f binds from its short side, and these complexes are difficult to isolate (Yadav et al., 2017).

1.4.2.4   ATP synthase

ATP synthase is a multiprotein complex embedded partially in the membrane. Chloroplast ATP synthase comprises two major subcomplexes CF_o and CF_1. The α (AtpA) and β (AtpB) subunits comprise of a hexagonal ($α_3β_3$) CF_1, the soluble hydrophilic head at the stromal side. The catalytic and regulatory nucleotide binding sites are present at the αβ interfaces. The catalytic cycle of the ATP synthase is defined by different conformational states based on the number of nucleotides bound (Leyva et al., 2003). The γ (ATPC1 and ATPC2, two paralogs) and ε (AtpE) subunits

of CF_1, form the central stalk and is attached to c ring of CF_o. The δ (ATPD) is part of the CF_1 domain and is essential in regulating ATP synthase by thiol modulation. The CF_o is the other domain which is embedded inside the thylakoid membrane, comprised of the transmembrane a (AtpI) and c (AtpH) protein subunits, while subunits b (AtpF) and b' (ATPG) make up the peripheral stalk (Blankenship, 2014; Hahn et al., 2018).

From the functional point of view, the ATP synthase is conceptualized as two parts called the stator and rotor, based on an electric motor. ATP is synthesized in the CF_1 head powered by the rotary motor ring of 14 C subunits, and having a glutamate amino acid to donate a proton. The ring of c subunits is tightly associated with the gamma subunit, but loosely with others, which allows the rotary movement. The a subunit from c-ring translocates protons. The central stalk provides the CF_o rotor's torque to the catalytic CF_1 head. The b, b' and δ (ATPD) form the stator, connecting the CF_o to the CF_1 head (Blankenship, 2014; Hahn et al., 2018).

1.4.2.5   Light-harvesting antenna complexes

In eukaryotes, the Chl and carotenoid pigments and other cofactors are associated with the light-harvesting antenna complexes. About 35 Chls and 10 β-carotenes are associated with the D1, D2, CP43 and CP47 proteins of the PSII core monomer (Shen, 2015; Wei et al., 2016), while around 175 chlorophylls and 32 carotenoids are associated with the PsaA and PsaB proteins of the PSI core complex (Amunts et al., 2010; Mazor et al., 2015). Additionally, the associated LHC proteins with three transmembrane domains (Green & Durnford, 1996) harvest light energy for PSII and PSI, the LHCB and LHCA proteins, respectively.

In Arabidopsis PSII, 15 different isoforms of LHCII (LHCB) proteins have been identified as minor and major antenna proteins (Jansson, 1999; Klimmek et al., 2006; Rantala et al., 2020). The minor antenna (monomeric) proteins LHCB4, LHCB5 and LHCB6 connect the peripheral antenna proteins, LHCB1, LHCB2 and LHCB3, as trimers to the PSII core complex. The peripheral antenna proteins bind to the minor antenna proteins either strongly, moderately or loosely (Caffarri et al., 2009). LHCB5 (CP26) links the LHCB1/LHCB2 heterotrimer strongly to PSII (S-LHCII), while LHCB4 and LHCB6 link the LHCB1/LHCB3 heterotrimers (M-LHCII) moderately to the PSII core complex (Caffarri et al., 2009). I talk more about the Loosely bound L-LHCII trimers and their movements between the PSI and PSII in the later sections.

As to the PSI complex, six different isoforms of LHC proteins (LHCA) are found in Arabidopsis (Jansson, 1999; Klimmek et al., 2006; Rantala et al., 2020). The peripheral antenna of PSI constitutes of LHCA1, LHCA2, LHCA3, and LHCA4 proteins, which form functional heteromeres of LHCA1/4 and LHCA2/3. These

proteins are attached to the PSI core complex as a large belt on one side. The LHCA5 and LHCA6 proteins substitute LHCA2 and LHCA4 in the complex formed by PSI to the NDH1, serving the cyclic electron transport around PSI (Wientjes & Croce, 2011).

There are some distinct differences in Physcomitrium as compared to Arabidopsis. Physcomitrium has an additional LHCB (LHCB9) (Alboresi et al., 2008) but LHCA4 and LHCA6 are missing, and the major LHCB proteins in Physcomitrium comprise the LHCBM isoforms, instead of LHCB1,2 and 3 in Arabidopsis.

1.5   Photosynthetic electron transfer pathways

The main electron transfer pathways are shown in Figure 2. The LET, involving the PSII, $Cytb_6f$ and PSI complexes, produces reducing power, NADPH, which is primarily used to fuel CO_2 fixation. Vectorial electron transfer over the thylakoid membrane concomitantly pumps protons from chloroplast stroma to thylakoid lumen, contributing, together with lumenal PSII driven water splitting, to the generation of the proton motive force (PMF) (Figure 2). The ATP synthase uses PMF to produce ATP, which is also essential to fix CO_2 and produce carbohydrates, via the CBB cycle and beyond in metabolism (Blankenship, 2014; Stirbet et al., 2020). Nevertheless, depending on environmental conditions and plant stress situation, also other electron transfer pathways, apart from LET and PSI CET, take place in chloroplasts.

1.5.1   Linear electron transport (LET)

The first photochemistry reactions start at the RC P680 of PSII, where P680 Chl a is excited by capture of the photon harvested by the pigments bound to LHCII. The excited ($P680^*$) reaction center is capable of transferring the electron to the first (primary) electron acceptor, the pheophytin (Pheo, another pigment molecule), allowing the charge separation, $P680^+$ $Pheo^-$ in PSII RC. Reduced Pheo can rapidly transfer the electron to the first stable electron acceptor, the Q_A plastoquinone molecule in the Q_A pocket of the D2 protein, which then donates the electron to the Q_B plastoquinone in the D1 protein (the Q_B pocket). Contrary to Q_A, which is a single electron carrier, Q_B requires two electrons to become fully reduced in the Q_B pocket of the D1 protein and becomes a plastoquinole (PQH_2) after catching two protons from stroma. Fully reduced QBH_2 then leaves the Q_B pocket in D1 and releases as PQH_2 to the PQ pool residing in the thylakoid lipid bilayer. PQH_2 now becomes part of the internal PQ pool present in the cavity of the $Cytb_6f$. Meanwhile, the oxidized P680 ($P680^+$) is left with a high redox potential (highly

oxidizing molecule). As a strong oxidant, P680$^+$ receives electrons, by co-operation with the Mn$_4$CaO$_5$ complex, through 4 photoacts from TyrZ of the D1 protein, eventually leading to splitting of two water molecules and release of an O$_2$ molecule. Thereby, two water molecules are split at the OEC complex, to extract four electrons for reducing P680$^+$ four times, and concomitantly releasing four protons to the lumen which contribute to the formation of PMF (Blankenship, 2014; Joliot, 2003; Stirbet et al., 2020).

The Cytb$_6$f then continues the electron transfer from the pool of PQH$_2$ in the Cytb$_6$f cavity to reduce soluble plastocyanin (PC) in the lumen. The plastocyanin reduces the oxidized PSI RC (P700$^+$), resulting from charge separation and phylloquinone reduction in PSI. The electron is then transferred via three 4Fe-4S clusters (Fx, F$_A$ and F$_B$) to Ferredoxin (Fd)(Brettel & Leibl, 2001). Finally, at the stromal side of PSI, the electrons are transferred to NADP$^+$ to be reduced into NADPH, catalyzed by the enzyme leaf-type ferredoxin NAD(P)H oxidoreductase (FNR) (Mulo, 2011).

1.5.2 Cyclic electron transport (CET)

In cyclic electron transport (CET) around PSI, the electrons excited in PSI return back to the PQ-pool, instead of producing NADPH (Figure 2). From the PQ pool, the electrons flow through Cytb$_6$f and PC back to PSI RC (Yamori & Shikanai, 2016). The CET pathway thus produces ATP, which is essential for sufficient ATP production for CO$_2$ fixation and other metabolic needs as well as for efficient generation of PMF for photoprotection of both photosystems. The ATP/NADPH ratio increases when the CET pathway is active, as the same electron from the acceptor side of PSI reaches back to PSI, concomitantly pumping two protons from stroma to thylakoid lumen, and thus enhancing ATP production via the ATP synthase (Allen, 2003; Nawrocki et al., 2019). Under different metabolic stress conditions, LET and CET are regulated to adjust the optimal ratio of ATP/NADPH.

PROTON GRADIENT REGULATION 5 (PGR5)/ PGR5-LIKE PHOTOSYNTHETIC PHENOTYPE1 (PGRL1)-dependent pathway is the most predominant pathway suggested for CET (Yamori & Shikanai, 2016). PGR5 is soluble (Munekage et al., 2004) and PGRL1 is a thylakoid membrane protein (DalCorso et al., 2008). The electron transfer through this route is suggested to be facilitated by the formation of supercomplexes. It is suggested that PGRL1 and PGR5 form a complex to facilitate electron transfer. Other multi-protein super-complexes including PSI-LHCI-LHCII-FNR-Cytb$_6$f-PGRL1 have also been reported (Iwai et al., 2010). In *Chlamydomonas reinhardtii*, some of these multi-protein complexes include the CAS protein (calcium sensing protein) (DalCorso et al., 2008; Munekage et al., 2004; Terashima et al., 2012)

In the NAD(P)H dehydrogenase complex (NDH1) -dependent CET pathway, the electron flow from PSI is mediated by the NDH1 supercomplex formed with multiple PSI (up to 6) complexes (Yadav et al., 2017). NDH1 is also a high efficiency proton pump and increases the production of ATP by two-fold, as it is able to pump approximately two protons per electron (Strand et al., 2017). Lastly, another Cytb$_6$f complex related pathway has been suggested in which the direct transfer of electron to Cytb$_6$f via Fd is proposed (Nawrocki et al., 2019) (not shown in Figure 2).

1.6 Key regulatory mechanisms of Photosynthetic light reactions via protein phosphorylation

Photosynthetic land plants are sessile and therefore constantly facing harsh fluctuating environmental conditions, boosting both abiotic and biotic stress that needs to be circumvented by various acclimation responses at leaf, cellular and molecular level. The abiotic factors include changes in light intensities and duration. Excess of light energy, more than that required to drive photosynthesis and cellular metabolism, is detrimental for photosynthesis if not addressed (Schöttler & Tóth, 2014). Excess light converts the Chl singlet states to triplet states, and thus (Gruber et al., 2015; Müller et al., 2001) generates an overwhelming oxidizing environment inside the cell (and chloroplast), ultimately leading to production of reactive oxygen species (ROS) (Krieger-Liszkay, 2005; Krieger-Liszkay et al., 2008). Stressors like ROS are harmful but also essential for the signaling mechanisms to initiate specific repair and regulatory processes (Noctor et al., 2018). If not addressed, these stresses lead to oxidative photodamage of the photosystems, referred to as photoinhibition (Tyystjärvi & Aro, 1996). Although PSI exhibits a notable resilience to photoinhibition, it is also prone to get damaged and recovers only slowly, in contrast to PSII (Lima-Melo et al., 2019; Tikkanen et al., 2014; Tiwari et al., 2016).

The plants have developed photoprotective mechanisms to deal with potential photoinhibition and photodamage, by generating short- and long-term acclimation responses. The photoprotective mechanisms usually follow the two major strategies: to reduce the absorption of light energy and to dissipate excess energy via thermal means (Ruban, 2016). Thermal dissipation involves the NPQ processes. The reduction in absorption of light energy involves dynamic changes in the composition or stoichiometry of thylakoid supercomplexes (SCs) or changes in the antenna size of PSII or PSI as well as the modulation of the thylakoid ultrastructure and movement of loosely bound LHCII towards PSI (state transition).

The most well-studied short-term stress responses generated via thylakoid protein reversible phosphorylations, comprise the L-LHCII and PSII core protein

phosphorylations (Grieco et al., 2016; Reiland et al., 2009; Tikkanen et al., 2014). The two important molecular mechanisms, particularly influenced by reversible phosphorylations of the LHCII and PSII core proteins, are the state transitions and the PSII repair cycle, respectively. These reversible protein phosphorylations are carried out by chloroplast kinases and counteracting phosphatases, regulated upstream by specific signaling mechanisms. Among the auxiliary proteins involved in the state transitions and the repair cycle of PSII are the kinases (STN7 and STN8), phosphatases (PBCP, TLP18.3, TAP38) and proteases (FTSH and DEG) (Lu, 2016). The proteases play an essential role in maintaining the proteostasis of the PSII core (Kato & Sakamoto, 2018; Li et al., 2017; Lu, 2016)

1.6.1   PSII repair

Damage of the PSII D1 protein is an unavoidable process at all light intensities but the rate of damage increases with an increase in light intensity (Tyystjärvi & Aro, 1996), even though the detoxification systems for ROS scavenging are functional; the process is initiated with an (oxidative) photodamage of PSII (particularly the D1 protein)(Kato et al., 2023), which is also sensed by the redox state of ETC (E. M. Aro et al., 1993; Tyystjärvi & Aro, 1996). The D1 protein was already several decades ago shown to have a high, yet light-dependent turnover rate implying that the damage is also rapidly repaired (Li et al., 2017; Mattoo et al., 1999). The first step of the PSII repair cycle, after the initial photoinhibition of PSII (damage to D1), is the monomerization of PSII dimer complexes in the grana. At high light, where both the damage and repair of PSII occur rapidly, the PSII core proteins are mostly phosphorylated. Due to the phosphorylation of PSII core, the phosphate groups create repulsion between the thylakoid stacks, helping the change in the diameter of grana (fluidity in the membrane) and allowing the access of damaged PSII core to non-appressed grana margin regions, where the repair machinery resides (Järvi et al., 2015). After dephosphorylation of the D1 protein by PBCP (Rintamäki et al., 1996), the PSII core is partially disassembled by detachment of CP43 and D1 is degraded by the FTSH proteases (Järvi et al., 2015; Kato & Sakamoto, 2018; Lu, 2016; Tikkanen et al., 2008a; Tikkanen & Aro, 2012). The newly synthesized D1 is then co-translationally inserted into the PSII subcomplex, before CP43 reattachment to the PSII monomer. At the end, in grana membranes, the OEC complex-related extrinsic proteins (PSBO, PSBP and PSBQ) are bound back, followed by the binding of the LHCIIs (Lu, 2016; Mulo et al., 2008). The changes in the thylakoid ultrastructure and adjustments in the grana diameter by the CURT1 proteins have been suggested to impact the PSII repair mechanism (Pribil et al., 2018; Yamamoto et al., 2013).

1.6.2 Balancing energy distribution between PSI and PSII (State transition)

In nature, plants face fluctuating light conditions due to solar angle, cloudy and shady conditions (Slattery et al., 2018). According to the variation in the light quality and quantity, the photosystems are differentially excited. In order to protect photosystems from excess excitation and to balance the energy distribution to PSII and PSI, plants respond in the short-term by reversible phosphorylation of specific LHCII proteins. In low light intensity, PSII is preferentially excited and the plants have adopted the strategy to balance the antenna size between the two photosystems and move the fraction of LHC to serve the other photosystem (PSI). However, the exact mechanism in regulating the energy balance is still unclear. There is an increase in the phosphorylation of PSII core and a decrease in that of LHCII upon shift to high light, while the opposite effect is observed in low light (Mekala et al., 2015; Rintamäki et al., 1997). This does not follow the classic state transition model, in which both PSII and LHCII are simultaneously either completely phosphorylated or completely dephosphorylated. Overall, multiple mechanisms are needed to regulate the function of the photosynthetic machinery, such as the redox dependent control (Suorsa et al., 2015). According to the classic state transition model (Bellafiore et al., 2005; Minagawa, 2011) under far red-light conditions, PSI is preferentially excited, and in this state (State1) the L-LHCII is associated with PSII. While in red light conditions, when PSII is preferentially excited, the L-LHCII is phosphorylated by the STN7 kinase (activated because of PQ pool reduction) (Zito et al., 1999), leading to partial detachment of P-L-LHCII from PSII and movement from grana to the margins (non-appressed) to serve PSI (State 2). State 1 is achieved again by L-LHCII dephosphorylation by TAP38 phosphatase (Mekala et al., 2015; Pribil et al., 2010). This is one of the ways how the stoichiometry of photosystem functions is changed to regulate the photosynthesis and photoprotection is achieved.

1.6.3 Changes in the Thylakoid Ultrastructure

Changes in light intensity have a capacity to influence the thylakoid membrane ultrastructure, facilitating the regulatory mechanisms of photosynthesis (Anderson et al., 2012; Koochak et al., 2019; Kowalewska et al., 2019). The movement of PSII or L-LHCII towards the non-appressed regions (margins) (Figure 1) is dependent on the light-dependent-phosphorylation of these components and this is a crucial step in regulating the energy balance and the PSII repair cycle (Pribil et al., 2018). Not only the PSII core/LHCII phosphorylations but also the abundances of evolutionarily conserved CURT1 proteins directly influence the structure and shape of the thylakoid membrane architecture (Armbruster et al., 2013). Also, the pigment-protein megacomplexes and their disassembly affect the energy balance between the

photosystems (Suorsa et al., 2015). Since the photosystem complexes are distributed in a non-random manner in the thylakoid membrane, as shown in Figure 1, it is proposed that the LHCII, PSII and PSI interact at one specific location in different stress conditions, to maintain the balance of energy. These regions comprise the thylakoid domains called the grana margins, where the grana and stroma membranes are connected (fret membranes) and where all different thylakoid complexes are present. Such thylakoid domains carry the best opportunity to interact and regulate photosynthesis (Rantala & Tikkanen, 2018). The PSII repair process is facilitated by changes in the grana diameter (Wood et al., 2019), smaller diameter allows easier access of stromal FTSH protease to degrade damaged D1 protein at grana margins (Kirchhoff, 2013; Pribil et al., 2014). CURT1 protein is considered critical in modulating the thylakoid ultrastructural changes and thus contributing to regulation of the PSII repair and state transitions.

1.6.4   Regulation via redox and calcium signaling mechanisms

Both short- and long-term acclimation relies on signaling networks that often induce a response in the target proteins via PTMs, carried out by specialized proteins such as kinases, phosphatases and thioredoxins (Grieco et al., 2016; Hochmal et al., 2015; Nikkanen et al., 2016; Nikkanen & Rintamäki, 2019; Serrato et al., 2013; Stael, 2019; Stael et al., 2012; Wang et al., 2016). Under different light stresses, photoinhibition of PSI and PSII imbalances the redox state inside the chloroplast, the ROS are sensed and responded via regulatory systems to achieve the photoprotection (Nikkanen et al., 2016; Nikkanen & Rintamäki, 2014, 2019; Noctor et al., 2018). One of the regulatory methods are exercised by specialized proteins called thioredoxins, which catalyze the disulfide/dithiol exchange reactions. There are two types of thioredoxin based-systems, ferredoxin-thioredoxin system (Fd-TRX) and the NADPH dependent chloroplast thioredoxin system (NTRC) (Buchanan, 2016; Serrato et al., 2004, 2013).

The activation of ATP synthase is dependent on light-induced reduction of thioredoxin-f (TRX-f), which reduces the cysteine residues in gamma subunit of ATP synthase (Fischer & Gräber, 1999; Schürmann & Buchanan, 2008). The ATP synthase is deactivated in darkness by oxidation of the disulfide bonds in the gamma subunit of ATP synthase, leading to conformational changes in ATP synthase (Kohzuma et al., 2017; Vallejos et al., 1983). It was suggested earlier that the conformational changes in the gamma subunit, induced by the Fd-TRX system, lowers the threshold of PMF to activate the ATP synthase, in light.

Calcium is another well-known regulator of chloroplast localized processes, including photosynthesis, apart from influencing the overall calcium signaling

pathways. The light-to-dark transition is known to change the calcium concentrations ($[Ca^{2+}]$), inside the chloroplast (Sai & Johnson, 2002). These changes are sensed by the calcium-binding proteins (CSP) such as Calmodulin (CaM) (Dodd et al., 2010) or the thylakoid localized calcium sensory protein (CAS) (Nomura et al., 2008), and information is relayed downstream to the target proteins. CAS and CaM are known to interact with NADK2 and STN8 kinases. CAS plays an essential role in regulating photosynthetic processes (Li et al., 2022), and is the target of the STN8 kinase for phosphorylation in increasing light intensities (Vainonen et al., 2008). Interestingly, the *in vitro* experiments have shown a crosstalk between phosphorylation and calcium signaling (Stael et al., 2012). Yet, it still remains unclear how the protein phosphorylation induced under the stress conditions is linked to the calcium-dependent kinases and sensory proteins inside the chloroplast.

Chloroplasts harbor several photosynthesis-related proteins associated with calcium (Hochmal et al., 2015; Navazio et al., 2020; Wang et al., 2019). PSAN and PSAH proteins of PSI are phosphorylated in a calcium-dependent manner (Stael et al., 2012). PSAN is known to bind to LHCA2 and LHCA3 and contributes to PSI-CET. In PSII OEC, calcium is a structural component of the metal cluster (Mn_4CaO_5 cluster) required for water oxidation (OEC) (Ferreira et al., 2004; Popelkova & Yocum, 2011). PSBO, an OEC stabilizing protein and NDA2, a mitochondrial ubiquinone (UQ) reductase, have calcium binding sites in their tertiary structures (Desplats et al., 2009; Murray & Barber, 2006), which may suggest functional roles. FTSH protease, which degrades the D1 protein of PSII, is phosphorylated in a calcium dependent manner (Stael et al., 2012).

1.7 Chloroplast kinases & phosphatases with well-known target proteins

The global chloroplast phosphoproteome has revealed a plethora of phosphorylation target proteins and identified some kinases and phosphatases (Baginsky, 2016; Baginsky & Gruissem, 2009; Grieco et al., 2016; Mergner et al., 2020; Reiland et al., 2009). Several phosphorylations of chloroplast proteins are now being identified, thanks to recent advances in quantitative phospho-proteomics, however the functional roles remain often to be elucidated (Mergner et al., 2020). Moreover, apart from chloroplast casein kinase II (cpCK2), considered as the central regulator of multiple processes inside the chloroplast (Baginsky & Gruissem, 2009; Reiland et al., 2009), there are still gaps in finding the upstream kinases responsible for these phosphorylations and their connection to upstream signaling pathways. The most well-studied photosynthesis-related kinase and phosphate pairs in chloroplasts are the STN7 and STN8 kinases and protein phosphatases PPH1 (TAP38) and PBCP, which have been assigned the functional roles in PSII and L-LHCII reversible

phosphorylations (Bayer et al., 2012; Rochaix et al., 2012). There are also other protein kinases and phosphates reported in chloroplast, which have been assigned a possible functional role but no confirmations exist so far (White-Gloria et al., 2018)

The STN7 kinase is known to phosphorylate the Thr residues of LHCB1 and LHCB2 at the N terminus (Figure 3) (Bellafiore et al., 2005), which is a redox-regulated process (Rintamäki et al., 2000). The phosphorylation of phosphosites in STN7 itself in different light conditions is critical in determining the kinase activity (Trotta et al., 2016). FNR, found as a new substrate for the STN7 kinase (Schönberg et al., 2017), is important in accepting the electron from the first PSI-stromal electron acceptor (Fd) and reducing $NADP^+$. The long-known LHCII phosphorylations, functioning in state transitions, are reversible by the counteracting TAP38 phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010).

STN8, on the contrary, is known to phosphorylate the core PSII proteins particularly under HL (Figure 3), which regulates the transfer of damaged PSII to stroma lamellae and being thereafter repaired. STN8 also phosphorylates CAS (Vainonen et al., 2008) as well as PGRL1 (Reiland et al., 2011). The CBB-related enzyme Rubisco Activase is phosphorylated by cpCK2 in darkness (Kim et al., 2016), and RbcL is phosphorylated by STN7/STN8 (Schönberg et al., 2017).
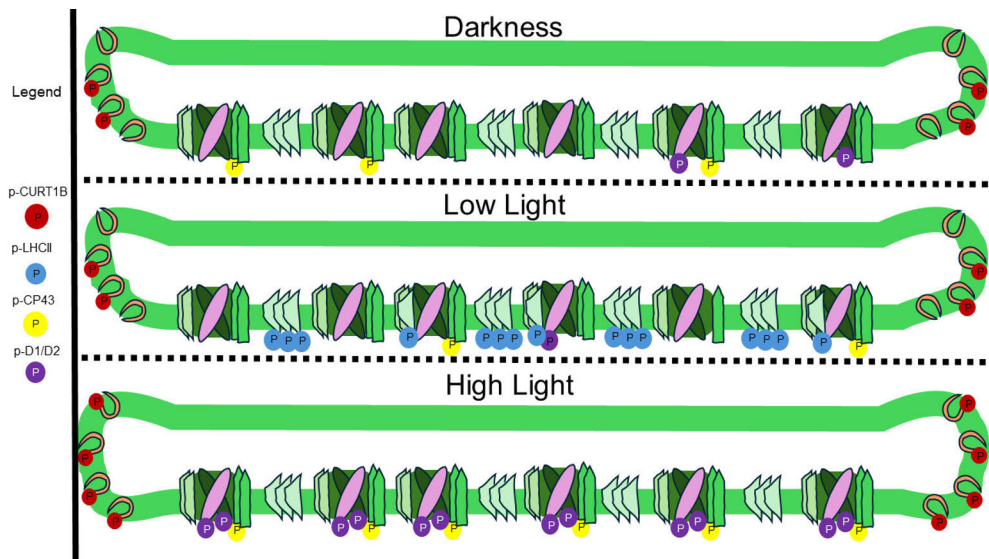
Figure 3. Simplified scheme of phosphorylation of the PSII core, LHCII, and CURT1B proteins in short-term transition of angiosperm leaves from darkness to low light and high light.

2   Aims of the Study

The STN7 and STN8 kinases, known to phosphorylate the LHCII and PSII core proteins in angiosperms and thereby being involved in photoprotection of photosynthesis, are the most well studied chloroplast kinases. Yet, by an evolutionary point of view, the diversity of their target proteins as well as the role of protein phosphorylations in various photoprotection mechanisms, which also induce changes in thylakoid ultrastructure, have remained elusive. Likewise, the connection of STN7 and STN8 phosphorylation events, and possibly also those of other chloroplast kinases, to the upstream signaling mechanisms needs further research and belong to the targets of my PhD research. Overall, to understand the regulation of photosynthesis carried out by protein phosphorylations, I focused on three specific aims in my PhD work, which are listed below:

i)   To generate and characterize Physcomitrium mutants devoid of the two STN7 kinase isoforms or the STN8 kinase, and to disclose the physiological roles of these kinases. Additionally, the analyses of the phosphorylation sites of target proteins and how the photoprotection is achieved in Physcomitrium, were also investigated.

ii)   To determine the responsive kinase and the role of phosphorylation dynamics of the CURT1B in Arabidopsis thylakoid membrane.

iii)   To shed light on calcium-induced dynamics of chloroplast protein phosphorylations in Arabidopsis.

3   Materials and Methods

3.1   Plant material and growth conditions

Physcomitrium genotypes (strains) were grown in minimal media (PPNO$_3$) at 24°C and a photoperiod of 16-h light/8-h dark with a light intensity of 45 μmol photons m^{-2}s^{-1}. Analyses of the acclimation capacity of Physcomitrium under different light treatments were performed on 11-days old culture grown in sterile petri dishes. Different Arabidopsis genotypes were grown in the phytotron at 23°C, under a photoperiod of 8h- light/16-h dark with a light intensity of 120 μmol photons m^{-2}s^{-1} and analyzed for different light treatments after growth of 32 days. An exception for growth was made in paper IV, with a growth period of 35 days under 150 μmol photons m^{-2}s^{-1}.

Table 1.   The species and strains used in the present thesis.

Strain	Species	Origin	Paper	Protein	Function
WT	Arabidopsis		I, II, III, IV	-	-
stn7	Arabidopsis	(Bellafiore et al., 2005)	I	STN7	LHCII phosphorylation
stn8	Arabidopsis	(Vainonen et al., 2005)	I, II	STN8	PSII core phosphorylation
psal	Arabidopsis	(Lunde et al., 2000)	I	PSAL and PSAH	Form the docking site for p-LHCII
tap38	Arabidopsis	(Pribil et al., 2010)	I	TAP38	Dephosphorylate LHCII
psb33	Arabidopsis	(Fristedt et al., 2015)	I	PSB33	Functional state transition
curt1a	Arabidopsis	(Armbruster et al., 2013)	I	CURT1A	Thylakoid membrane curvature
curt1b	Arabidopsis	(Armbruster et al., 2013)	I	CURT1B	Thylakoid membrane curvature
WT	Physcomitrium	Gransden 2004 Ecotype	II, III	-	-
stn8	Physcomitrium	II	II	-	-
stn7.1	Physcomitrium	III	III	-	-
stn7.2	Physcomitrium	III	III	-	-
stn7dKO	Physcomitrium	III	III	-	-

3.2   Light treatments

For Physcomitrium, the short term light treatments listed in Table 2 were given to 11-days old cultures after over-night dark-acclimation. For long term light-acclimation experiments, the 4 day old Physcomitrium protonemata were shifted to the long-LL, long HL, Long FL conditions and the growth was continued under a 16h light/8h dark photoperiod for seven days. The samples for different experiments were collected after 6 hours of the lights on in the growth chamber. For Arabidopsis in paper III, the light treatments were done after 16 hours of darkness (D1) and in paper IV, the samples were collected for the isolation of chloroplasts 5 hours after the lights were turned on.

Table 2.   The light treatments of plants in the present thesis.

	Treatment	LIGHT INTENSITY (μmol photons m^{-2} s^{-1})	Paper	Time (min)
SHORT TERM	CL	45	II, III	-
	LL	7-10	II	120
	HL	500	II	120
	660nm	40-45	III	120
	630nm	40-45	III	120
	FR	40-45	III	10
	LL1	20	I	120
	HL	1000	I	120
	LL2	20	I	120
	1hD	0	I	60
LONG TERM	LL	7-10	III	10080
	FL	25 and 800	II	5 min and 1 min
	HL	600	III	10080
	24h-HL	1100	II	1440

3.3   Protein analysis

Proteins analyses were made from thylakoids isolated from Arabidopsis leaves and Physcomitrium protonemata. Isolation of thylakoids from Arabidopsis in paper I was performed according to Suorsa et al. (2015) and from Physcomitrium thylakoids in papers II and III according to Gerotto et al., 2012, with minor modifications. In paper IV, the isolation of intact chloroplasts from Arabidopsis was performed first by

grinding the leaves and subjecting the suspension to percoll gradient (40-80%) centrifugation as described by (Lehtimäki et al., 2014; Nurmi, 2014; Zhang & Yuan, 1998). Isolated chloroplasts were resuspended in 50 mM Hepes-KOH pH 8.0, 330 mM sorbitol. The Chl content was determined according to the Porra et al., 1989.

3.3.1   Gel electrophoresis and Mass-spectrometry

Thylakoid extracts were used as starting material for gel electrophoresis and mass spectrometry (MS) analyses in papers I, II, III, and intact chloroplasts were used in paper IV. The SDS-PAGE, IpBN, 2D IpBN-SDS-PAGE and 2D IEF-SDS-PAGE were performed according to Järvi et al., 2011.

After denaturing SDS-PAGE, the proteins were blotted to PVDF membrane and immunodecorated with antibodies: CURT1B, CURT1A, D1, PSAB, CYTF, LHCB2, LHCB1, LHCB3, LHCB6, ATPF and FTSH1 protein specific antibodies, or the P-Thr specific antibody.

For IpBN-PAGE, the thylakoids were solubilized with 1% (w/v) β-DM at a final Chl concentration of 0.5µg-Chl/µL according to the protocol of Järvi et al., 2011. For 2D IpBN-SDS-PAGE, the lanes from the first dimension IpBN-PAGE were cut and solubilized with Laemmli buffer before loading on the top of the second dimension SDS–PAGE.

Differential-in-gel-electrophoresis (DIGE) in paper IV was performed with low and high calcium treated chloroplasts, using 2D IEF-SDS-PAGE for protein separation. Cy2 and Cy3 dyes were used to label the samples, followed by pooling. IEF (Iso-electric-focusing) was performed in first dimension followed by second dimension separation in SDS-PAGE.

Proteins separated in 2D gels were stained with silver nitrate in paper IV and with SYPRO Ruby (Invitrogen/Molecular Probes) in papers II and III to detect the total proteins. ProQ diamond staining was performed to visualize protein phophosphorylations according to the manufacturers (Invitrogen/Molecular Probes) instructions.

LC-MS/MS analysis was carried out (Papers I, II, III, IV) on the spots and bands excised from the gels, and subjected to in-gel- trypsin digestion protocol as described in Trotta et al., 2016, with some minor modifications in paper IV.

3.4   Thylakoid fractionation and solubilization

Fractionations of the thylakoid membrane were performed in papers I and III. Thylakoid solubilization for fractionations was performed with digitonin (DIG) detergent for Physcomitrium according to Pinnola et al., 2015 and for Arabidopsis thylakoids according to (Kyle et al., 1983, 1984), with slight modifications. For

solubilization of Physcomitrium thylakoids, the thylakoids equivalent of 0.4 mg/mL Chl, were mixed with 0.5% DIG (w/v, final concentration) and incubated at 4°C for 7 min with constant shaking. Unsolubilized material was removed by centrifugation for 5min at 4,000g, and the grana and grana margin fractions were collected together at 40,000g for 30 min, followed by pelleting the stroma-exposed membrane fraction at 100,000g for 90 min. The solubilization of Arabidopsis thylakoids (0.5 mg/mL Chl conc.) was performed with the final digitonin concentrations of 0.4% or 1 % (w/v) and by incubating for 8 min at 23°C with constant shaking. Solubilization was followed by removal of unsolubilized material by centrifugation (3 min, at 1,000 g). Later, the 10,000g (30 min) grana and 40,000g (30 min) grana margins fractions were collected. The stroma lamellae (tight pellet) and the curvature fraction (loose pellet, LP) were collected after 1h of centrifugation at 144,000 g.

4 Results

My PhD research focused on two evolutionarily distant plant species, Physcomitrium and Arabidopsis. The former represents the earliest group of land plants while Arabidopsis, as an angiosperm, represents the flowering plants. Particularly, the differences in phosphorylations of the thylakoid proteins and the architecture of the thylakoid network were compared between Physcomitrium and Arabidopsis. Papers II and III address the roles of the STN7 and STN8 kinases in Physcomitrium and reveal their unique roles and target proteins, compared to the well-established functions of STN7 and STN8 in Arabidopsis. The phosphorylation of the CURT1B protein, with respect to light-induced dynamics of the PSII core and LHCII proteins, was investigated in detail in Arabidopsis in Paper I, and Paper IV focuses on $[Ca^{2+}]$ dependent control of protein phosphorylation in Arabidopsis chloroplasts, and on the specific role of protein phosphorylation in targeting the protein for degradation.

4.1 Physcomitrium – distinct evolutionary features of thylakoid biochemical composition

4.1.1 Physcomitrium thylakoid protein complexes and their (phospho-)protein composition in comparison to angiosperms

A comparative analysis of native thylakoid protein complexes of the model species of bryophytes, Physcomitrium, with those of the model angiosperm species, Arabidopsis, was carried out first. For this purpose, the thylakoids were isolated and then solubilized using N-dodecyl β -D-maltoside (β-DM), a mild detergent, allowing us to examine the differences between the thylakoid protein complexes. The protein complexes were separated by IpBN-PAGE (Figure 1A, Supplemental Figure S1, paper II). IpBN-PAGE revealed a general pattern of protein complexes similar to Arabidopsis but also revealed the differences between the two species (Figure 1A, paper II).

In order to identify the native protein complexes comprised in the various green bands, the lpBN-PAGE lanes were separated into subunits by the second dimension of SDS–PAGE (Figure 1B, paper II) and stained with SYPRO Ruby (hereafter "Sypro"), a quantitative dye for total proteins. Sypro staining revealed a complete 2D map of thylakoid protein spots (Figure 1B, paper II), which were identified by MS (Figure S2, Table S1, paper II). The protein complexes separated in 1D BN-PAGE from Physcomitrium were assigned according to the identification of the protein spots in 2D BN-SDS-PAGE and then compared with detailed Arabidopsis maps (Rantala et al., 2017). Such comparison showed the PSI-NDH1 complex was present only in Arabidopsis, while two PSI supercomplexes, i.e. the green bands SC4 and SC6 were present only in Physcomitrium thylakoids (Figure 1A, 1B, Figure S3, paper II). The former is a Physcomitrium specific PSI supercomplex, hereafter called PSI-large (SC4). PSI-large comprised, in addition to the PSI core, also LHCA1-3, LHCBM, and the Physcomitrium specific LHCB9. PSI-large corresponds to a complex previously characterized in Physcomitrium thylakoids (Iwai et al., 2018; Pinnola et al., 2018). The other PSI supercomplex PSI-LHCI-LHCII (SC6), instead, included the PSI core, LHCI isoforms and LHCII trimers, in line with the well characterized "state transition" complex of Arabidopsis, visible when Arabidopsis thylakoids are solubilized with digitonin instead of β-DM (Rantala et al., 2017), and clearly lacks the LHCB9 protein.

The other Physcomitrium supercomplex bands SC1, SC2, SC3 and SC5 were identified as PSII supercomplexes C2S2M2, C2S2M, C2S2, and C2S, respectively based on MS identification. In addition to PSII core subunits, the smaller C2S(2) complex had several LHCBM isoforms and the bigger C2S2M(2) had also other LHCB isoforms (LHCB3, LHCB4, and LHCB6). Among other green bands from Physcomitrium, the LHCII trimers comprised LHCBM and LHCB3 while the LHCII monomer band additionally included LHCB4 (CP29), LHCB5 (CP26), and LHCB6 (CP24) proteins. LHCSR1 and LHCSR2 isoforms were also found in the monomer green band. PSBS protein was co-migrating with LHCB6.

To reveal differences between Physcomitrium and Arabidopsis thylakoid phosphoproteomes, the 2D gels were stained with ProQ Diamond, which detects the phosphorylated tyrosine, serine, or threonine residues of proteins (Figure 1C, paper II). Thr phosphorylations were also checked by anti-P-Thr antibody (Figure 2C, paper II). LHCBM subunits were detected phosphorylated by ProQ staining as well as by anti-P-Thr. The PSII complexes of Physcomitrium showed the phosphorylation of CP43 and D2 proteins, similar to that of Arabidopsis. On the opposite, the D1 protein phosphorylation was missing in Physcomitrium (Figure 1C, paper II). According to the MS data, we found only one phosphorylation in D1 protein, which however was not located at the N terminus, as found in Arabidopsis and in general

in angiosperms. Moreover, the LHCB6 protein was phosphorylated in Physcomitrium but not in Arabidopsis (Figure 2C, paper II).

4.1.2 Construction of STN8 and STN7 kinase knockout mutants

After taking an overview of the differences in thylakoid pigment-protein complexes and their phosphorylated subunits between Physcomitrium and Arabidopsis, I knocked out the genes encoding the STN7 and STN8 kinases in Physcomitrium, the kinases known for their involvement in photoprotection mechanisms in vascular plants like Arabidopsis (Bonardi et al., 2005; Mekala et al., 2015). It was of interest to solve how the model bryophyte Physcomitrium has developed the protein phosphorylation-dependent photosynthesis regulation and photoprotection mechanisms. Since homologous recombination is efficient in Physcomitrium, it was used to generate knockout mutants devoid of STN7 or STN8 kinase. In case of the STN7 kinase, a double knockout was carried out, since STN7 kinase is encoded by two genes (STN7.1, Pp3c4_25980; STN7.2, Pp3c26_5140) in Physcomitrium. The target genes were knocked out and substituted by an antibiotic resistance cassette. Stable antibiotic-resistant lines of mutants were obtained after two rounds of selection.

PCR was used to confirm the disruption of the desired gene locus with the insertion of the resistance cassette. The primer pairs were designed so that PCR amplification occurred only if the resistance cassette was present in the desired stn7.1, stn7.2 or stn8 gene locus, so the PCR products were amplified in the respective mutants but not in the WT (Figure 2A, paper II) (Figure 1A, paper III). Reverse transcription-PCR was done to verify the missing transcript of STN8 (Figure 2A, paper II) or STN7.1 and STN7.2 (Figure 1A, paper III). The bands of the transcripts were absent in mutants as compared to WT. The absence of protein peptides of the kinases STN7 (Figure S2 and Table S1, paper III) and STN8 (Table S4, paper II) was verified by MS.

4.1.3 STN7/STN8 Kinase-Dependent Phosphorylations in Physcomitrium

Characterization of the *stn7* and *stn8* mutants started by comparing them with WT Physcomitrium. As described above, in the WT four bands were detected with anti-P-Thr: phosphorylated CP43, D2, LHCBM and LHCB6. In *stn8* KO, D2 and CP43 phosphorylation was completely missing. LHCB6 phosphorylation intensity was affected compared to WT, while P-LHCBM signal was similar to that of WT (Figure 2C, paper II). Contrary to *stn8* KO, in the *stn7.1 stn7.2* double KO (*stn7*dKO) the

LHBM phosphorylation was undetectable (Figure 1B and Figure S3, paper III). Notably, the effects of STN7 depletion were evident only in the mutant depleted in both STN7 isoforms, while the single mutants *stn7.1* and *stn7.2* showed no visible phenotype.

Physcomitrium WT, *stn8* and *stn7*dKO were compared also at the level of thylakoid protein complexes, which revealed a similar thylakoid protein complex profile in WT and the *stn8* mutant (Figure 4, paper II). Instead, differences were observed in the thylakoid protein complex profile of the *stn7*dKO mutant with respect to PSI-large and PSI-LHCI-LHCII bands, which were almost undetectable in comparison to WT (Figure 1G, paper III).

4.1.4   Light-induced dynamics in phosphorylation of PSII core and LHCII proteins in Physcomitrium *stn7*dKO and *stn8* kinase mutants

Since the reversible phosphorylations by the STN7 and STN8 kinases of the L-LHCII and PSII core proteins, respectively, are key components in angiosperm photoprotection mechanisms under different light conditions (Grieco et al., 2016; Reiland et al., 2009; Tikkanen et al., 2014), the different Physcomitrium genotypes (WT, *stn7*dKO, *stn8*) were next subjected to both short-term and long-term light treatments (see methods). Additionally, specific light treatments related to state transitions (illumination by 630nm, 660nm, 730nm light) and to PSII repair cycle (uninterrupted 24h HL) were also applied to evaluate the responses of the mutants. The appearance of light-induced dynamics was examined at the levels of formation of the protein complexes, changes in protein abundances and in the phosphorylation status of the proteins.

4.1.4.1   Overall changes observed by immunoblotting with Anti-P-Thr antibody

The thylakoid extracts of light-treated Physcomitrium were separated by SDS-PAGE and immunoblotted with the anti-P-Thr antibody. This allowed us to see the light-induced thylakoid protein phosphorylation dynamics as summarized for *stn8* (Figure 3, paper II) and *stn7*dKO (Figure 2A, paper III).

At first, short term light treatments, differing either in the quality or in the intensity, were applied to WT Physcomitrium. Both exposure to red light (660 nm or 630 nm) and low intensity of white light (LL) induced LHCBM phosphorylation as compared to the dark-acclimated samples collected before the light treatment, with LL having the strongest effect (Figure S6, paper III). Conversely, treatment with FR light led to LHCBM dephosphorylation, similarly to HL. The

phosphorylation level of PSII core subunits CP43 and D2 was almost unaffected by the various treatments tested, while LHCB6 phosphorylation was enhanced the most by red light (Figure S6, paper III).

Next, the effects of short-term light treatments with different intensities of white light were investigated in the two kinase mutants. *stn8* KO, besides missing CP43 and D2 phosphorylation in all light conditions tested, showed a LHCBM phosphorylation dynamics similar to that of the WT, with stronger LHCBM phosphorylation in LL as compared to darkness and HL (Figure 3, paper II). In WT, LHCB6 phosphorylation increased in LL and HL, but this light-induced effect was diminished in *stn8* (Figure 3, paper II). In *stn7*dKO, LHCBM phosphorylation was completely missing in all light conditions, but CP43 and D2 phosphorylations were not affected by the light treatments (Figure 2A, paper III). However, the light-induced dynamics of LHCB6 phosphorylation changed also in the *stn7*dKO which displayed the highest LHCB6 phosphorylation in LL, at difference to both the WT and the *stn8*.

Then, the effects of long-term differential light treatments were investigated. The WT was characterized after seven days of acclimation to various light conditions (long-FL, long-LL and long-HL) (Figure 4, paper III). As in the short-term treatments, the phosphorylation of PSII core proteins D2 and CP43 showed negligible dynamics except for long-HL, a condition in which all signals detected with anti-P-Thr were drastically reduced compared to the other samples. The *stn7*dKO showed a similar behavior to WT, except for LHCBM phosphorylation which was missing in all conditions in *stn7dKO*. Also, the phosphorylation of LHCB6 was slightly pronounced in the long-LL condition in the *stn7*dKO as compared to WT.

Furthermore, the effects of the 24h-HL treatment relative to CL, were likewise tested (Figure S12, paper II). With heavily reduced phosphorylations of thylakoid proteins in general, all thylakoid phosphoproteins were clearly affected after 24 hours in both WT and *stn8*. In *stn8*, only a faint signal from LHB6 phosphorylation was visible.

4.1.4.2   Changes observed at the level of protein complexes

The effects of changing light conditions on the composition and abundances of various thylakoid protein complexes were investigated by IpBN-PAGE, followed by 2D-lpBN-SDS-PAGE to confirm the identity of the differentially accumulated green bands.

Upon short-term light intensity changes, in the WT the PSI–LHCI–LHCII band was visible only in the LL condition (Figure 4, 5A and S8, paper II) (Figure 3A, 3B and S8, paper III) and did not accumulate at all in the *stn7*dKO (Figure 3A, 3B paper

III). The other complex that did not accumulate in *stn7*dKO was PSI-large (SC4), shown to be present in WT in all light conditions (Figure 3A, 3B and S8, paper III). The *stn8* did not change the protein complexes profile upon differential short-term light treatments compared to the WT (Figure 4, 5A and S8, paper II).

Long-term light treatments of WT (Figure S9, paper III) showed similar patterns of thylakoid protein complexes, including the accumulation of PSI-large, in CL and Long-LL but PSI supercomplexes and PSII supercomplexes were not detectable in long-HL. PSI supercomplexes, PSI-large and PSI-LHCI-LHCII, were missing also when mosses were treated with 24h-HL (Figure 6C, paper II). The comparison of WT and *stn8* after 24h-HL showed, in addition to a similar lpBN profile, that the abundance of the D1 protein was unaffected in WT and *stn8*, which was checked by D1 immunodetection (Figure 6D and Figure S13A, paper II).

4.1.4.3 Protein phosphorylation levels (ProQ) analyzed from the 2nd dimension SDS-PAGE

The 2D-lpBN-SDS-PAGE from thylakoids from differentially light-treated plants also allowed to characterize the phosphorylation of thylakoid proteins by staining the gels with ProQ.

Overall, results from the 2D-lpBN-SDS-PAGE gels in the different light conditions and in different genotypes were in line with the results from anti-P-Thr blots, both in short term light changes as well as after prolonged light acclimations. In short-term light intensity changes, the *stn8* mutant showed, except for a distinct difference in missing the phospho-CP43 and phospho-D2 (Figure 5A and Figure S8, paper II), an otherwise similar protein pattern in all light conditions as compared to WT.

No major differences were observed for LHCBM phosphorylation between *stn8* and WT. The LHCB6 phosphorylation signal was lower in *stn8*, as compared to WT (Figure 5B and Figure S8, paper II), in line with the results with anti-P-Thr immunoblotting.

On the contrary, *stn7*dKO missed the accumulation of the PSI-large and PSI-LHCI-LHCII supercomplexes as well as most of the ProQ signals from LHCBM isoforms in the various complexes. Only a weak signal was detected from LHCBM in stn7dKO LHCII trimers and monomers compared to LL-treated WT (Figure 3C, paper III), which possibly originates from phosphorylated Ser residues (Table S1, paper III). The LHCB6 phospho-signals, observed in the *stn7*dKO P-Thr blot (Figure 2A, paper III), were likewise enhanced in ProQ staining (Figure 3B, paper III) in LL.

4.1.4.4   Protein and phosphorylation changes identified by mass-spectrometry

MS was used to identify the proteins and phosphorylations in the green bands of IpBN-PAGE and in the individual protein spots of the 2D IpBN-SDS-PAGE. The proteins were identified (Table S1, paper II and Table S1, paper III) and their phosphorylations are reported here for Physcomitrium (Table S2, paper II and Table S1, paper III). We focused on protein complexes PSI-large and PSI-LHCI-LHCII as well the LHCB monomer and the LHCBM spots that showed changes in light-induced dynamics in Physcomitrium (Table S1, paper III). Several phosphosites were identified in LHCBM isoforms. They include phosphorylated Thr at the N-terminus of the protein, the residue likely responsible for the anti-P-Thr signal, which was detected in several LHCBM isoforms in the LHCII monomer band. Also, in the PSI supercomplexes, we detected the same phosphorylation in a peptide common to LHCBM4/8 (both in PSI-large and PSI-LHCI-LHCII) and to LHCBM2 (only in PSI-LCHI-LHCII). Several others LHCBM Ser phosphorylations were also found and highlighted with yellow in Figure 7 (paper III). A common peptide to LHCBM3/6/9/10 was found to be phosphorylated on two Ser residues (Figure 7, Table S1, paper III). In addition to other phosphopeptides reported in Table S2, paper II, we reported also a new phosphopeptide "ATKKVSARPAAGGK" phosphorylated at Thr-48 of LHCB6 (Table S1, paper III), likely representing the N-terminus of the mature protein recognized by the anti-P-Thr-antibody.

4.2   Thylakoid ultrastructure – changes related to light-induced-dynamics of thylakoid biochemistry

4.2.1   Physcomitrium and comparison to Arabidopsis

Physcomitrium has an irregular thylakoid ultrastructure as mosses in general (Aro, 1982), and a relatively higher proportion of non-appressed membranes than in Arabidopsis (Figure 7, paper II). To assess the localization of protein complexes in the thylakoid membrane of Physcomitrium, the IpBN-PAGE followed by 2D-lpBN-SDS-PAGE analysis of the thylakoid subfractions was performed.

Analysis of the appressed and non-appressed (stroma-exposed) regions (Figures 5 and 6, Figure S12, paper III) revealed that grana fractions were enriched with PSII supercomplexes (i.e., SC1, SC2, SC3 and SC5) in both WT and *stn7*dKO. Conversely, the PSI supercomplexes, PSI-LHCI-LHCII and PSI-large, were detected in the stroma-exposed membranes of WT only and were missing from *stn7*dKO.

TEM pictures revealed a more frequent presence of two appressed membranes indicated by orange arrows (Figure 6B, paper II) in WT in comparison to the *stn8* mutant. Interestingly, no differences were observed between WT and *stn8* Physcomitrium in CL. Conversely, the long-term 24h-HL acclimation of Physcomitrium revealed 3 to 6 appressed membranes in WT and more than 10 in *stn8* (blue arrows in Figure 6B, paper II), indicating the PSII phosphorylation-induced decrease in thylakoid stacking.

4.2.2 Arabidopsis - CURT1 proteins are easily released from the thylakoid membrane by saponins

CURT1 proteins have an essential role in facilitating the formation of the highly curved domains in the thylakoid membrane, the curvature domains. Such domains reside in the edges of the grana but similarly also at the edges of the stroma-exposed thylakoids. In order to understand the role of CURT1 protein phosphorylation, and verification of its localization, we attempted to isolate the subfractions containing the thylakoids domains, as explained in materials and methods. Accordingly, the fractions corresponding to grana, margins and stroma membranes were collected. Apart from the strong stroma membranes pellet, a specific loose pellet was also collected (Figure 6A, paper I), as previously shown by (Puthiyaveetil et al., 2014), which here is called the curvature domain.

Investigating further the subfractions, Chl a was clearly shown to be accumulating in stroma membrane and grana margin fractions, with respect to the starting material, intact thylakoids (Figure 6B, paper I), indicating more of PSI and less of LHCII, as anticipated. Additionally, we wanted to see the differences in these margin and curvature domains at the protein level. For this purpose, the thylakoids and the subfractions were solubilized with either 0.4% or 1% digitonin and loaded on the Chl basis to SDS-PAGE, and subsequently immunoblotted with antibodies of CURT1B and CURT1A, PSAB representing PSI, CYTF representing $Cytb_6f$ complex, D1 representing PSII, and LHCB2 representing LHCII. Based on the role of CURT1 proteins in modulating thylakoid curvature, as anticipated, the CURT1B and CURT1A were dominant in the Curvature fraction i.e. the loose pellet. This enrichment is also because of the lower presence of the Chl-binding protein complexes in the curvature domain.

Comparing of thylakoid samples solubilized by 0.4% DIG with those solubilized by 1% DIG (Figure 6C, paper I), it appeared that the abundances of PSAB and LHCB2 were depleted in the curvature fraction (loose pellet) upon solubilization with 0.4% digitonin, while the opposite was observed for CURT1B and CURT1A. CYTF was mostly enriched in the curvature domain for both 0.4% and 1% DIG

samples. D1 and LHCB2 were more in the grana in 0.4% DIG, while they were found in similar amounts in all fractions with 1% DIG solubilization.

Further, the solubility of CURT proteins from the thylakoid membrane was tested with increasing concentrations of DIG in comparison to the thylakoid-bound protein complexes. The CURT1 proteins were shown to be differentially solubilized compared to other proteins, easily solubilized at just 0.4% and even at 0.25 % DIG, indicating that the curvature is a separate domain from grana margins (Figure 6D, paper I).

4.2.3 Phosphorylation of CURT1B and its relationship with light-induced dynamic phosphorylation of PSII core and LHCII proteins

The role and behavior of CURT1 proteins, their quantities and post-translational modifications (PTMs), in Arabidopsis plants exposed to fluctuating light were analyzed in paper I. The phosphorylation of CURT1B and its relationship with the phosphorylation of PSII core and LHCII proteins were on explicit focus. The phosphorylation dynamics were followed in response to light shifts (Figure 1A, paper I) using several mutants, including *stn7*, *stn8*, *tap38*, *psal*, and *psb33* (Figure 1C and 3B, paper I). The investigation aimed at improving the understanding of how the phosphorylation of these thylakoid proteins interact in modulating the appressed membranes. These dynamic processes enable efficient photosynthetic electron flow and optimal excitation of photosystems under changing natural conditions (Figure 1 and 3, paper I).

The phosphorylation dynamics of CURT1B, PSII core, and LHCII proteins were investigated in response to light shifts. In the wild type plants, the CURT1B phosphorylation clearly enhanced in shift to HL and 1hD light treatment. The dynamics of CURT1B phosphorylation was eliminated in *stn8*, although a baseline phosphorylation level was still detected. The baseline level of CURT1B phosphorylation in darkness was not affected in *stn7* and CURT1B phosphorylation increased when plants were shifted from darkness to low light. Interestingly, there were differences in the dynamics of CURT1B phosphorylation between *stn7* and the WT, particularly in the low-light phase. The *tap38* plants showed attenuated changes in CURT1B phosphorylation compared to the WT, apart after shifting from low light to darkness for one hour.

The *psb33* plants did not show any LHCII phosphorylation when exposed to LL conditions. In *psb33*, the shift to LL resulted in a decrease in phosphorylation of CURT1B, suggesting that PSB33 plays a key role in the sequence of events involved in thylakoid protein phosphorylation. However, the changes in the phosphorylation levels of CURT1B and PSII core proteins were less pronounced in *psb33* than in the

WT. Conversely, *psal* plants exhibited an increase in CURT1B phosphorylation when transferred to low light, similar to that observed in *stn7*. Furthermore, *psal* plants consistently showed high levels of LHCII phosphorylation under all tested light conditions. It is worth noting that the basal level of CURT1B phosphorylation was similar among the WT, *psb33*, and *psal*.

Next, the PSII core and LHCII protein phosphorylation dynamics were investigated in specific subfractions of Arabidopsis WT thylakoid membrane. The thylakoids, grana, grana margin, and stroma membranes, fractionated from plants exposed to fluctuating light, were loaded onto an SDS-PAGE gel and analyzed using an anti-P-Thr antibody (Figure 7B and 7C, paper I). The strongest phosphorylated LHCII signal was consistently observed throughout the LL phase across all sub-fractions (Figure 7C, paper I). The distribution of the p-LHCII signal among the sub-fractions was relatively similar, with a slight increase detected in the margin fraction under all light treatments. The dynamics of PSII core phosphorylation in the grana subfraction resembled those observed in thylakoids, showing a decrease after the LL period, followed by an increase after the HL period. During the HL phases, PSII core phosphorylation was explicitly enriched in the margin and, to less extent, stroma thylakoid fractions. This suggests the margin domain as the site for PSII repair.

4.3   Chloroplast calcium dependent proteins

The light-to-dark transitions are known to change the calcium concentrations ($[Ca^{2+}]$) inside chloroplasts (Sai & Johnson, 2002). In paper IV, I investigated changes in chloroplast (phospho)proteome as a response to changes in chloroplast $[Ca^{2+}]$, to find out possible links between changes in environmental light conditions and chloroplast protein phosphorylations. Intact chloroplasts were isolated (Figure 1, paper IV) and treated with 10 µM $CaCl_2$ (high $[Ca^{2+}]$, corresponding to the light-to-dark transient peak) or with 0.15 µM $CaCl_2$ (low $[Ca^{2+}]$, corresponding to the resting $[Ca^{2+}]$ in stroma). The low $[Ca^{2+}]$ treated chloroplasts (LCaTC) and the high $[Ca^{2+}]$ treated chloroplasts (HCaTC) proteomes were subjected to DIGE analysis (see methods) for initial screening of the proteins affected. Some of the protein spots were lower in abundance either in LCaTC or in HCaTC. These spots were excised from silver-stained 2D gels, manually mapped according to the DIGE results (Figure 2, paper IV), and identified by MS. Overall, 18 LCaTC (Table 2, paper IV) and 6 HCaTC (Table 1, paper IV) protein spots were identified, representing proteins in lower abundance, in comparison to the other $[Ca^{2+}]$ treatment of chloroplasts.

4.3.1 Chloroplast proteins changing in abundance by changes in [Ca^{2+}]

Lower abundance proteins identified in the LCaTC included proteins functionally related to PSII, like PSBQ-1. The proteins related to PSII repair included THF1 and HCF136, and the proteases FTSH5 and FTSH2 (Table 2, paper IV). The proteins related to ATP synthase complex comprised the ATPD and AtpF subunits. The proteins related to PSI-complex included PSAD1, PSAE-1, PSAE-2, PSAF and PSAG. The protein related to Cytb$_6$f complex comprised PETC. The protein related to CBB cycle were represented by Rubisco activase. The LHC antenna proteins LHCB6 and LHCA1 were found also to belong to this group. Other proteins included PRPL1, PPIase, APX4, CYP37, ATSTR14, RPL9, PRXQ and AtFKB16-4.

Lower abundance proteins identified in the HCaTC (Table 1, paper IV) included the FNR1 and FNR2 proteins functionally related to PSI, the AtpA subunit of the ATP synthase complex, and the CBB cycle proteins RBCS-1B and RBCS-1A.

4.3.2 Calcium-dependent phosphorylations of photosynthetic proteins

To evaluate the cross-talk between the protein phosphorylation and the calcium concentration, LCaTC and HCaTC were prepared and subjected to 2D-IEF in triplicate, without any dye labeling (Figure3, Figure S1 and Figure S2, paper IV). The same protein spots identified in Figure 2 (paper IV), and thus known to be affected in abundance by the calcium concentration, were excised and analyzed by MS. Only a few proteins were identified whose phosphorylations were dependent on changes in calcium concentration. These protein phosphorylations were picked up from the general list of Arabidopsis thylakoid phosphoproteins in Table S1 in paper IV, on the basis of their presence in, at least, two biological replicates, in either LCaTC or HCaTC. Phosphoproteins were further shortlisted in Table 3 of paper IV. The group of proteins specifically phosphorylated in LCaTC comprised FNR1, FNR2, AtpA, while FTSH5 was specifically phosphorylated in HCaTC (Table 3, paper IV).

4.3.2 Novel phosphosites

MS analysis revealed 16 phosphorylated proteins in two DIGE replicates (Table S1, paper IV). These proteins were the FTSH5, FTSH2, AtpA, CYP37, FNR1, FNR2, PRPL1, CYP26-2, APX4/TL9, THF1, LHCA1, ATPD, PSAE-1, RBCS3B, RBCS1A, and PSAG. A comprehensive examination of the data revealed a total of 88 phosphosites, 55 of which were entirely novel discoveries. Notably, when considering both replicates of the DIGE experiment, 46 phospho-sites were

identified, with 26 of them being novel findings (Table S1 for more details). Additionally, it was observed that 59 of the phosphosites identified in the DIGE gels were also detected during the analysis of LCaTC and HCaTC 2D-IEF-SDS-PAGE, without any dye labeling. This overlapping set of phosphosites indicates a degree of consistency and reliability between the two experimental approaches.

5 Discussion

## 5.1 Thylakoid protein phosphorylation from an evolutionary perspective

Photosynthetic organisms have developed photoprotective mechanisms to deal with the changing environmental conditions. When photosynthetic organisms transitioned from water to land during evolution, they faced new challenges and often harsher environmental conditions, for example in light and temperature, and were prone to drought among other stressors. As sessile organisms, opposite to cyanobacteria or algae, the land plants could not escape too intense or strongly fluctuating irradiation but had to deal with the sunlight they were exposed to. Sunshine would sometimes directly hit the plant, other times it is filtered through clouds or leaves and vegetation above, which is challenging for the photosynthetic apparatus, particularly under conditions when also other stressors are present. Accordingly, the land plants have developed several ways to protect themselves under otherwise hazardous conditions. Even though these photoprotective mechanisms have developed during evolution, the oxygen evolving photosynthetic ETC is rather conserved from prokaryotic cyanobacteria to early land plants (mosses) and advanced vascular plants (Arabidopsis). Indeed, the regulatory mechanisms of photosynthesis have undergone conspicuous evolution. In Introduction and Results sections, I already introduced the major evolutionary differences in photoprotective mechanisms between mosses and Arabidopsis. In addition to those mechanisms, and often necessary for the operation of the major regulation pathways, there are the thylakoid proteins PTMs (Järvi et al., 2017), which play an important role in regulation of many photoprotective mechanisms that require the reduction in absorption of light energy under harsh environmental conditions. Among the most well-studied PTMs regulating the photosynthesis process are the reversible phosphorylation's of the thylakoid proteins carried out by kinases and phosphatases (Rantala et al., 2020; Rantala & Tikkanen, 2018). The most well-studied kinases, responsible for the phosphorylation of subunits of LHCII and PSII, are the STN7 and STN8, respectively, in Arabidopsis (Bellafiore et al., 2005; Bonardi et al., 2005). However, the targets and physiological significance of thylakoid protein phosphorylation during the water-to-land

transition are poorly understood. In the quest to unlock these mysteries, a moss *Physcomitrium patens* (previously *Physcomitrella patens)*, a model species of bryophytes, was taken under investigation. By studying this moss, I contributed to understanding how these organisms evolved efficient survival mechanisms in the face of harsh sunlight and changing conditions.

My thesis (Papers II, III) investigates the targets and physiological significance of the STN7 and STN8 kinases in Physcomitrium, and compares them to respective phenomena in Arabidopsis. To this end, the overall profile of the thylakoid protein complexes, investigated side by side from both species (Figure 1A, paper II) revealed the PSI-large SC unique only for Physcomitrium and the PSI-NDH1 complex only for Arabidopsis. The structure of Physcomitrium PSI-large was recently published Sun et al. (2023) by making use of our biochemical characterization of the complex.

At the level of single thylakoid proteins, distinct differences are manifested in the Light Harvesting Complex (LHC) proteins when comparing Physcomitrium and Arabidopsis, whereas both the PSI and PSII core proteins appeared much more conserved (Allen et al., 2011; Bai et al., 2021; Müh & Zouni, 2020). The PSII antenna subunits in Physcomitrium include various LHCBM isoforms, which cannot be distinguished as LHCB1 or LHCB2 as in higher plants (Crepin & Caffarri, 2018), besides LHCB3, LHCB4, LHCB5 and LHCB6 (Alboresi et al., 2008) common to higher plants. In Physcomitrium, the PSI core associated LHCA proteins comprise LHCA1-3, while LHCA4 is notably absent (Alboresi et al., 2008; Busch et al., 2013). Furthermore, an intriguing LHC protein, denoted as Lhcb9, and sharing sequence homology with LHCB proteins, is distinctive only to Physcomitrium and is associated with PSI (Iwai et al., 2018; Pinnola et al., 2018). This unique LHCB9 protein is characterized by properties reminiscent of red Chl-like Lhca proteins (Alboresi et al., 2011; Iwai et al., 2015). Before delving into specific PTMs and the alterations at protein phosphorylation levels, it is important to recognize that the thylakoid ultrastructure of Physcomitrium markedly differs from that of Arabidopsis, a distinction visually represented in Figure 7 (Paper II). The presence of irregular grana stacking in mosses (Figure 4) may potentially introduce changes in the need to move PSII towards grana margins for repair as in Arabidopsis later in evolution, a phenomenon described in Järvi et al., 2015.
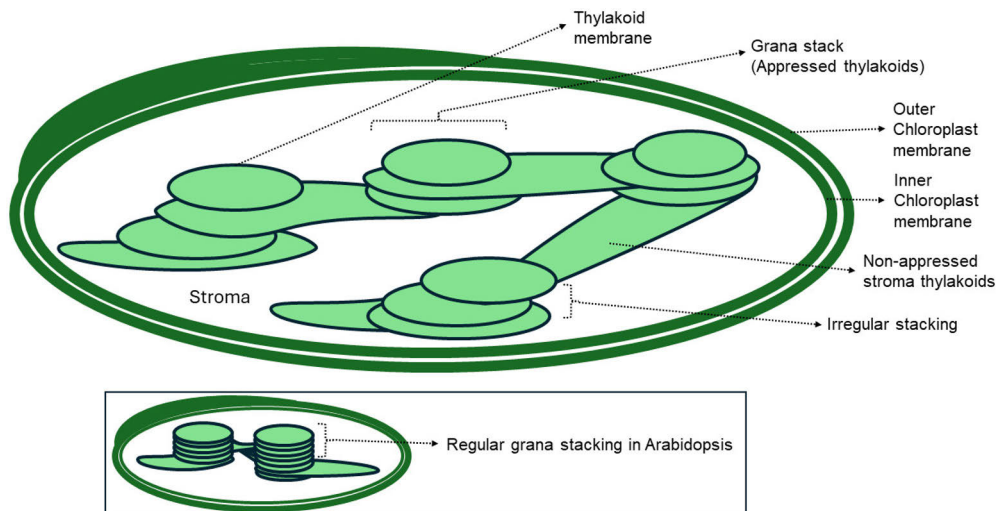
Figure 4. The thylakoid ultrastructure in Physcomitrium and the presence of irregular grana stacking.

Considering the differences in chloroplast ultrastructure and in apparent needs for photoprotection between Physcomitrium and Arabidopsis, we also observed distinct variation in STN7 and STN8 specificity between the two species. Notably, the complete absence of CP43 and D2 phosphorylation in the *stn8* KO mutant of Physcomitrium strongly implies their exclusive dependency on the STN8 kinase—a feature also identified in rice (*Oryza sativa*) (Nath et al., 2013). In contrast, in Arabidopsis these phosphorylation are also partially dependent on the activity of the STN7 kinase (Bonardi et al., 2005). Furthermore, our investigation on PSII core phosphorylation unveils a significant difference: the absence of phosphorylated D1 (P-D1) accumulation despite the conserved D1 N-terminal Thr residues present also in Physcomitrium (Figure S14, paper II). This variation is noteworthy, given that in higher plants, such as Arabidopsis, the phosphorylation of D1 is known to play a pivotal role in the repair cycle of PSII, delaying the degradation of damaged P-D1 at high light (HL) (Tikkanen et al., 2008a). Surprisingly, in Physcomitrium, prolonged exposure to uninterrupted HL illumination did not affect D1 protein degradation (Figure 6D, paper II) nor did it influence the integrity of PSII supercomplexes (Figure 6C, paper II). However, in the Physcomitrium *stn8* mutant, alterations in thylakoid ultrastructure were evident (Figure 6B, paper II). In context of these differences in PSII phosphorylation dynamics between Physcomitrium and Arabidopsis, it is plausible that the regulation mechanism involving D1 phosphorylation-controlled PSII repair evolved later in the course of plant evolution, requiring not only the STN8 kinase but also the participation of the STN7 kinase. From an evolutionary perspective,

these findings suggest that additional regulatory controls emerged later, shaping the regulation by PSII core D1 protein phosphorylation. Furthermore, the influence of the STN8 kinase on thylakoid architecture in Physcomitrium suggests that in this moss species the preliminary step of translocating damaged PSII complexes from grana to the thylakoid margins - a process documented for Arabidopsis (Järvi et al., 2015) may be less dependent on PSII core phosphorylation. The absence of P-D1 accumulation implies simplified PSII core phosphorylation dynamics in Physcomitrium compared to the more intricate mechanisms in higher plants.
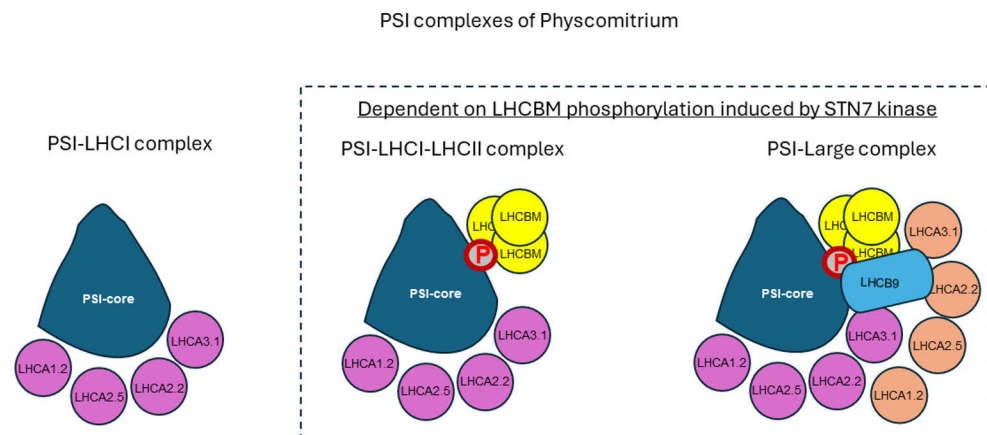
Phosphorylation of the LHC proteins, on the other hand, provides photoprotection against unequal distribution of excitation energy to PSII and PSI, and thereby protects the photosystems against oxidative damage. Such regulation involves dynamic changes in the composition or stoichiometry of thylakoid supercomplexes or changes in the antenna size of photosystems and the movement of loosely bound LHCII to serve PSI instead of PSII (state transition). It has been known already for decades that higher plants, like Arabidopsis, change the phosphorylation level of major LHCII proteins, LHCB1/LHCB2, when exposed to different white light intensities (Bonardi et al., 2005; Crepin & Caffarri, 2015; Martinsuo et al., 2003; Mekala et al., 2015; Rintamäki et al., 1997, 2000). LHCII phosphorylation changes are severely affected by the lack of the STN7 kinase, resulting in lack of LHCII phosphorylation and, consequently, the increase in the antenna size of PSI (Bellafiore et al., 2005; Bonardi et al., 2005; Rantala & Tikkanen, 2018).

In Physcomitrium *stn7*dKO mutant (paper III), the lack of STN7 clearly lead to the lack of anti-P-Thr immunodetection of LHCII trimer antenna subunits, the LHCBM isoforms (Figure 1B and S2, Table S1, paper III). Importantly, at the protein complex level, the lack of LHCBM phosphorylation prevents the formation of the canonical PSI-LHCI-LHCII state transition complex (Figure 1G, paper III). In fact, the formation of the state transition complex (PSI-LHCI-LHCII) is basically similar in Physcomitrium and in higher plants (Arabidopsis), despite the involvement of different LHCII protein paralogs, as also observed for Chlamydomonas reinhardtii (Huang et al., 2021). The state transition phenomenon, based on the PSI-LHCI-LHCII complex, seems to be conserved over the course of evolution from algae to higher plants. Furthermore, the structural features of LHCII trimers were found to be similar between the green algae and plants (Sun et al., 2023).

Interestingly, also the formation of the unique Physcomitrium PSI supercomplex, PSI-large (Figure 5), failed in the *stn7*dKO mutant, indicating that the lack of LHCBM phosphorylation in Physcomitrium prevents the assembly of the PSI-large SC. The formation of PSI-large was previously shown to rely on the presence of a specific antenna protein called LHCB9 (Iwai et al., 2018; Pinnola et al., 2018), which we also detected in PSI-large (Figure 1G, 3A and 6, Table S1,

paper III). Here, we revealed also the absolute requirement of LHCBM phosphorylation for PSI-large assembly, and the N-terminal regions of LHCBM4/8 are shown to harbor the Thr phosphorylation. As highlighted before, the LHCBMs, despite being the Physcomitrium "major" antenna subunits constituting the LHCII trimers (as in green algae), cannot be distinguished into LHCB1- and LHCB2-type proteins (Crepin & Caffarri, 2018). Our results show that the N-terminal region of Physcomitrium LHCBM proteins interacting both with PSI-LHCI-LHCII and PSI-large contain the residues (RRtVK) (Figure 7, paper III), identical to the conserved LHCB2 N-terminus in Arabidopsis, instead of the RKt typical of LHCB1 (Figure S14, paper III). The stability and assembly of PSI-large are distinct from those of the other PSI supercomplex, called PSI-LHCI-LHCII. Unlike PSI-LHCI-LHCII, the relative amount of the PSI-large remains almost unaffected by changes in light conditions (Figure 3, paper III). The phosphorylation of LHCBM and the interaction with additional light-harvesting complexes is likely to contribute to the stability of PSI-large. In line with our results, a very recent structural characterization of Physcomitrium PSI-large identified a phosphorylated LHCII trimer with one monomer harboring the phosphorylation at the N-terminus (Sun et al., 2023). This phospho-LHCBM was suggested to interact with the PSI core and the presence of several other antenna proteins was likewise indicated to differentiate the PSI-large from other PSI-LHCI-LHCII supercomplexes.

The accumulation of PSI-large in Physcomitrium is likely to be advantageous for efficient photosynthesis in different light regimes. PSI-large enhances photosynthesis at low light, when photosynthesis is light-limited, but is able in HL to dissipate excess energy as heat (Tiwari et al., 2016). PSI complexes with a larger antenna size are found also in various aquatic environments (Alboresi et al., 2017; Xu et al., 2020) but their composition differs from that of the PSI-large in Physcomitrium. The presence of different sizes of PSI complexes in Physcomitrium, the PSI-large, PSI-LHCI-LHCII and PSI-LHCI (Figure 5), enables optimal photosynthesis under changing light conditions, by adjusting the PSI antenna size and interaction with PSII according to the prevailing conditions.

Figure 5. The simplified version of Physcomitrium PSI-complexes and the effect of LHCBM phosphorylation induced by STN7 kinase on their complex formation. The formation of the complex shown here is a simplified version of the protein complex structure (PDB code: 7XQP).

5.2 Role of protein phosphorylation in modulation of thylakoid dynamics and ultrastructure according to environmental cues in Arabidopsis

Environmental stress factors, such as fluctuations in light intensity, are well known to influence the dynamic rearrangements of the thylakoid ultrastructure, which, in turn, play a pivotal role in regulating the processes governing photosynthesis (Anderson et al., 2012; Koochak et al., 2019; Kowalewska et al., 2019). These regulatory processes, particularly those related to photoprotection, are intricately linked to the dynamics of the thylakoid membrane (Fristedt et al., 2010; Pribil et al., 2018) and so far mostly studied in angiosperms such as Arabidopsis. Most importantly, the regulation of photosynthesis is influenced by the lateral heterogeneity of the thylakoid membrane (i.e. a non-random distribution of photosynthetic protein complexes) (Anderson et al., 2012). The structural attributes and conformation of the thylakoid membrane are also influenced by phosphorylation of the PSII core and LHCII proteins (Chow et al., 2005; Fristedt et al., 2010; Wood et al., 2019), and further impacted by the action of evolutionarily conserved CURT1 proteins, thereby contributing to the overall architectural features of the thylakoid membrane (Armbruster et al., 2013). Upon thylakoid fractionation, the CURT1B and CURT1A are found enriched in the LP fraction, which therefore has been assigned as curvature domain (Figure 6C, paper I) but, in fact, LP contains in addition to the curvature domain, also a huge amount of impurities (Trotta et al., submitted). Overall, the interplay of thylakoid ultrastructure, protein phosphorylation and various regulatory processes involving photoprotection still remains elusive.

My study (Paper I) investigates the phosphorylation dynamics of Arabidopsis CURT1B, a protein involved in thylakoid membrane curvature, and its impact on plant photosynthesis and possible connection to PSII repair. Dynamic regulation of CURT1B phosphorylation levels was interpreted from different phosphorylation levels in Arabidopsis leaves after darkness, low light and high light treatments for 2 hours (D, LL, HL) (Figure 1B, paper I). The photodamage of PSII under HL conditions leads to a net increase of the repair cycle of PSII. HL conditions also enhanced the CURT1B phosphorylation, while it remained unaffected when plants were transferred from darkness to LL. In HL, the CURT1B phosphorylation increased along with the PSII core protein phosphorylation, which is known to be linked to stacking of thylakoid membranes (Fristedt et al., 2010). Additionally, the *stn8* mutant of Arabidopsis completely lacked the CURT1B phosphorylation dynamics as well that of the PSII core protein phosphorylation, which suggests an association of CURT1B phosphorylation with the PSII repair and the control of CURT1B phosphorylation by STN8 kinase.

I hypothesize that increased CURT1B phosphorylation in the edges of grana margins may create repulsive forces that enlarge the local grana partition gaps, thereby facilitating the movement of damaged PSII to the grana margins (the frets connecting the grana membranes with stroma membranes) and its access to the PSII repair machinery in non-appressed thylakoid domains. Moreover, the perturbed LHCII phosphorylation in Arabidopsis *stn7* and *tap38* mutants coincided with the altered relationships between PSII core phosphorylation and CURT1B phosphorylation (Figure1, paper I), indicating an essential role of also LHCII phosphorylation in the dynamics of grana margins controlled by phosphorylation of CURT1B.

The interactive dynamics between PSII core, LHCII, and CURT1B phosphorylation in paper I show interlinked phosphorylation responses adjusting the thylakoid ultrastructure and providing photoprotection. The hypothetical scheme presented in Figure 8 (Paper I) highlights the possible roles of protein phosphorylations in photosynthetic regulatory processes, inducing changes in thylakoid ultrastructure in varying light intensities, based on results in paper I and a number of previous studies (Anderson et al., 2012; Armbruster et al., 2013; Bos et al., 2017; Chow et al., 2005; Dietzel et al., 2011; Fristedt et al., 2010; Grieco et al., 2012, 2015; Johnson & Wientjes, 2020; Puthiyaveetil et al., 2017; Schwarz et al., 2018; Suorsa et al., 2015; Tikkanen et al., 2008a; Tikkanen et al., 2008b; Tikkanen & Aro, 2014; Wientjes et al., 2013; Wood et al., 2019; Yamamoto et al., 2013; Yokono et al., 2015, 2019; Yoshioka-Nishimura & Yamamoto, 2014). In the dark period, grana stacking is facilitated by the interaction of non-phosphorylated LHCII domains and phosphorylated CP43. Transition from darkness to low light (LL) induces high LHCII phosphorylation, loosening the appressed grana stacks and

widening partition gaps. This promotes the interaction of phosphorylated LHCII with PSI, expanding the margin domain. Shifting from LL to high light (HL) leads to LHCII dephosphorylation and increased phosphorylation of PSII core proteins, decreasing the size of partition gaps. Simultaneously, increased CURT1B phosphorylation causes outward bending of the curvature, facilitating the migration of damaged PSII to the grana margins where the PSII repair machinery is located. The phosphorylation dynamics of LHCII and CURT1B are integrated to respond to fluctuating light intensities.

The change in thylakoid architecture and thus in the distribution, mobility and interaction of the photosystems, ultimately provides photoprotection to photosynthetic organisms by providing a balance between the energy distribution between photosystems and the level of PSII repair (Rantala et al., 2020). These findings suggest that CURT1B phosphorylation contributes to the fine-tuning of thylakoid membrane structure and function in response to light conditions. Understanding the signaling pathways and molecular mechanisms controlling CURT1B phosphorylation dynamics could provide insights into the broader regulatory network of thylakoid membrane dynamics and photosynthesis.

5.3 Regulation of photosynthesis via calcium dependent protein phosphorylation

Changes in environmental conditions have been documented to induce fluctuations in chloroplastic calcium levels (Sai & Johnson, 2002; Sello et al., 2018). These fluctuations are perceived by proteins such as Calcium-Binding Protein (CaM) and Calcium Sensor (CAS) (Dodd et al., 2010; Li et al., 2022; Nomura et al., 2008), and subsequently the information is relayed to target proteins through intermediary proteins such as kinases, phosphatases and proteases.

The protein PTMs, particularly the phosphorylation, have been associated with changes in chloroplast calcium levels (Stael et al., 2012). Furthermore, some target proteins involved in the calcium-dependent regulation of photosynthesis have been identified (Hochmal et al., 2015). Nonetheless, the interaction between phosphorylation and the calcium signaling network remains enigmatic. The precise role of calcium in relation to phosphorylation requires in-depth investigation for identification of the intermediate proteins (kinases and phosphatases) (Vainonen et al., 2008) and the target proteins (including proteases) (Stael et al., 2012) linked to the calcium signaling cascade within the chloroplast. Papers I, II, and III explore the regulation of photosynthesis through phosphorylation, emphasizing aspects of thylakoid ultrastructure and thylakoid kinases. In contrast, paper IV specifically delves into the relationship between calcium and phosphorylation-dependent regulatory processes.

The response of chloroplasts to variations in calcium levels was examined in Paper IV. Specifically, the high calcium transient (10µM) occurring exclusively in the absence of light was mimicked using the HCaTC treatment. In contrast, the low calcium condition (0.15 µM) occurring under resting conditions, was simulated using the LCaTC treatment of isolated intact chloroplasts of Arabidopsis. Our study involved a comprehensive analysis (a screening method) of chloroplast proteins using DIGE gel electrophoresis, revealing alterations in the abundance of 25 proteins in response to calcium (Figure 2, table 1 and 2, paper IV). Notably, among these affected proteins, approximately 19 were phosphorylations, as documented in Table S1 of paper IV.

The study of paper IV introduces an innovative method for assessing the impact of calcium on the whole chloroplast proteome. Previous investigations were restricted to in vitro experiments focused on the thylakoid membrane only, as in Stael et al., 2012. Our findings in paper IV highlight the specific protection of two proteases, FTSH2 and FTSH5, against degradation in the presence of high calcium levels (HCaTC treatment). Conversely, a few proteins, namely FNR1, FNR2, AtpA, and Rubisco Small Subunit, were degraded under these conditions. Subsequently, we conducted a thorough examination of the proteins to assess whether their abundance correlated with the phosphorylation level of respective proteins, as outlined in Table 3 of paper IV. Notably, FTSH2, FTSH5, AtpA, FNR1, and FNR2 showed phosphorylation in connection to changing chloroplast calcium.

This study (Paper IV) unveiled previously unexplored phosphorylation targets, which have not been reported in prior comprehensive phosphoproteomic investigations (Baginsky, 2016; Bayer et al., 2012; Mergner et al., 2020; Reiland et al., 2009; Simeunovic et al., 2016). These phosphorylation sites hold significant potential as a basis for future inquiries into calcium-dependent mechanisms. Furthermore, we endeavored to elucidate potential kinases that might be intricately linked with the calcium signaling pathway, thus establishing a potential avenue for crosstalk between calcium signaling and phosphorylation events. This endeavor is based on the phosphorylation sites disclosed in Table S1 (Paper IV). Moreover, the recent bioinformatic tools which integrate omics data to study protein interaction networks (Escandón et al., 2020) can further be used on the current proteomics data to identify the proteins connected with the signaling pathways.

In the context of photoprotective mechanisms, plants engage in both short-term and long-term responses to cope with photoinhibition. Paper IV reveals that proteins crucial for the PSII repair cycle, such as THF1, HCF136, and FTSH protease (Lu, 2016), are phosphorylated and are safeguarded against degradation under high calcium conditions (HCaTC) (Table 2, paper IV). Notably, FTSH5, featuring more than eight phosphorylation sites, exhibited a specific calcium-dependent relationship at phosphosite T666. De-phosphorylation of T666 in FTSH5 under HCaTC

conditions coincided with protection from degradation, suggesting a potential linkage between this phosphorylation, the protein's stability and the chloroplast calcium status.

Involvement of CAS, known to be coregulated with FTSH and THF1 (Albanese et al., 2018), further supports the proposed functional connection between the PSII repair cycle and calcium. Additionally, the formation of protein complexes and the spatial proximity of proteins are pivotal in regulating energy transfer processes within the photosynthetic electron transfer chain and, by extension, play a critical role in modulating photoprotection mechanisms. In this context, we investigated calcium dependency among PSI proteins (PSAE, PSAF, PSAG, PSAD) and LHCA1, which are essential components required for PSI-Fd-FNR interactions (Andersen et al., 1992; Barth et al., 1998; Jin et al., 1999; Marco et al., 2018, 2019; van Thor et al., 1999). These proteins exhibited protection against degradation in the presence of high calcium levels (HCaTC), which contrasts with the behavior of FNR1 and FNR2. Notably, FNR1 displayed exclusive phosphorylation at T167 under HCaTC conditions (Table 3 and S1, paper IV), and this particular site is situated on the outer surface of the protein where FNR is known to interact with Fd. The degradation of FNR in HCaTC and the calcium-dependent phosphorylation of T167 site suggest potential involvement in FNR-Fd-PSI interactions. Furthermore, T167 is a target site of STN7 (Schönberg et al., 2017) and CAS has already been considered as a part of phosphorylation network associated with STN7 (Cutolo et al., 2019) and STN8 (Vainonen et al., 2008).

Collectively, the findings in paper IV not only identify novel phosphorylation targets but also open avenues for investigating the intricate interplay between calcium signaling and phosphorylation events in the chloroplast, based upon the phosphosites uncovered in paper IV.

6 Conclusions and future prospects

My thesis has provided valuable insights into the evolution of thylakoid kinases (STN7 and STN8) and their respective targets, particularly in Physcomitrium, which has a distinct photoprotection strategy involving LHCB6 and LHCBM phosphorylations that is different from that of angiosperms. The formation of both PSI-supercomplexes, PSI-large and PSI-LHCI-LHCII, are shown to depend on LHCBM phosphorylation induced by STN7 kinase. I have also explored the complex interplay of protein phosphorylation in thylakoid architecture, showing how LHCII and CURT1B phosphorylation respond dynamically to changing light intensities, contributing to the fine-tuning of thylakoid membrane structure and function. In addition, my research has explored the potential links between calcium signaling and photoprotective mechanisms, providing a novel screening tool for identifying calcium-dependent proteins. This investigation has revealed the phosphorylation and calcium dependence of critical proteins involved in Photosystem (PS) II repair, and has suggested a possible link between calcium and the PSI-Fd-FNR interaction. These findings not only identify new phosphorylation targets but also provide exciting opportunities to explore the intricate interplay between calcium signaling and phosphorylation.

Improving our understanding of the regulation of photosynthesis is crucial for optimizing biomass production. My PhD research has advanced this understanding, paving the way for resilient organisms that can adapt to changing environments. By introducing innovative tools and approaches to study the intricate mechanisms that influence photosynthesis at the proteome level, my work contributes to improving the production of food, feed, and renewable energy. Ultimately, this research will help to address the global challenges such as food scarcity and climate change, in line with the goals of a sustainable bioeconomy.

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