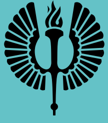
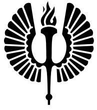


TURUN YLIOPISTO
UNIVERSITY OF TURKU

LIGHT-ACCLIMATION AND REGULATION OF PHOTOSYNTHESIS IN AUTOTROPHIC *CHLAMYDOMONAS REINHARDTII*

Olli Virtanen

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. AI OSA – TOM. 698 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2023



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The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-9394-9 (PRINT)
ISBN 978-951-29-9395-6 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)
Painosalama, Turku, Finland 2023

“Omat valinnat sattuvat eniten.”
-Kansanviisaus

UNIVERSITY OF TURKU
Faculty of Technology
Department of Life Technologies
Molecular Plant Biology
Olli Virtanen: Light-acclimation and regulation of photosynthesis in autotrophic *Chlamydomonas reinhardtii*
Doctoral Dissertation, 139 pp.
Doctoral Programme in Technology
September 2023

ABSTRACT

Photosynthetic light reactions take place in the thylakoid membranes, where light-driven electron transfer occurs from water to carbon dioxide through two photosystems (PSII and PSI), between of which is an electron-transferring agent called plastoquinone (PQ). PQ is present in the thylakoids as a pool (PQ-pool) that consists of its reduced and oxidized forms. The ratio of the two forms of PQ has long been known to control the movement of the Light-harvesting complex II trimers between PSII and PSI, a phenomenon termed as state transitions. Most of our understanding on algal photosynthesis originates from plant studies. However, as our knowledge of the algal system has increased, it is now clear that deeper understanding, e.g. about regulation of algal light reactions, is required.

This thesis set out to examine how the green alga *Chlamydomonas reinhardtii* withstands strong light that damages the photosynthetic machinery. The results demonstrate how mutant strain cells resistant against photoinhibition of PSII perform better under high light and how they divert the resources saved from less active PSII repair to biomass production. The photoinhibition-tolerance of the mutant is hypothesized to be partly due to changes in the redox potentials inside PSII.

However, it is also shown that even a wild-type population of *Chlamydomonas* can survive under extreme light intensities via modifications to the photosynthetic machinery. This variation-dependent acclimation enables the cells to cope with the increased photon flux. The demonstrated acclimation contains numerous changes in the properties of PSII, such as significantly reduced rate of singlet oxygen production. However, the acclimated cells exhibit signs of severe photoinhibition.

Lastly, this work highlights the dynamics of the PQ-pool and its relation to state transitions in *Chlamydomonas*. The PQ-pool of *Chlamydomonas* is shown to be of similar size as in cyanobacteria and plants. It is highly reduced under almost all light conditions applied, possibly due to fairly similar wavelength dependence profiles of algal PSII and PSI. The non-photochemical reduction and oxidation of PQ are very active in the used alga. It is also shown that state transitions do not correlate with the PQ-pool redox state in *Chlamydomonas* as in plants, and that light states respond more to the intensity, rather than the quality of light in autotrophic *Chlamydomonas*.

KEYWORDS: Light-acclimation, Chlamydomonas, high light, photoinhibition, photosynthesis, photosystem II, plastoquinone pool, state transitions

TURUN YLIOPISTO
Teknillinen tiedekunta
Bioteknologian Laitos
Molekulaarinen Kasvibiologia
Olli Virtanen: Omavaraisen *Chlamydomonas reinhardtiin* yhteyttämisen säätely ja sopeutuminen eri valo-oloihin.
Väitöskirja, 139 s.
Teknologian tohtoriohjelma
Syyskuu 2023

TIIVISTELMÄ

Yhteyttämisen valoreaktiot tapahtuvat tylakoidikalvoilla, joihin ankkuroituneet valoreaktiokeskukset (PSII ja PSI) käyttävät kerättyä valoenergiaa elektronien siirtämiseen. PSII:n ja PSI:n välissä elektroneja siirtää plastokinoni (PQ), jonka pelkistyneet ja hapettuneet muodot muodostavat fotokemiallisesti aktiivisen plastokinonivarannon (PQ-pooli). PQ-poolin hapetus-pelkistys-asteen on jo kauan tiedetty säätelevän valoa keräävien antenniproteiinien liikettä PSII:n ja PSI:n välillä. Tätä ilmiötä kutsutaan tilasiirtymiksi. Tietomme viherlevien yhteyttämisestä pohjautuu paljolti kasvitutkimuksiin. On kuitenkin selvää, että esimerkiksi levien valoreaktioiden säätely eroaa kasveista, mikä painottaa levillä tehtävän tutkimuksen tärkeyttä niiden yhteyttämisreaktioiden ymmärtämiseksi.

Tämä työ tutki viherlevä *Chlamydomonas reinhardtiin* kykyä sietää valo-olosuhteita, joiden tiedetään vahingoittavan yhteyttämiskoneistoa. Tuloksissa esitetään, kuinka PSII:n fotoinhibitiolle vastustuskykyinen mutanttikanta kykenee uudelleenohjaamaan resursseja biomassan tuotantoon, jotka villityypissä kuluvat PSII:n korjausreaktioihin. Mutantin fotoinhibition sietokyvyn arvellaan osittain johtuvan PSII:n sisäisten redox-potentiaalien muutoksista.

Myös villityypin *Chlamydomonas* kykenee kasvamaan hyvin korkeassa valon intensiteetissä. Tämä ominaisuus riippuu solupopulaation sisäisen vaihtelun määrästä, joka lisää akklimaatiokykyisten solujen lukumäärää kasvatuksessa. Akklimaation seurauksena useat yhteyttämiskoneiston ominaisuudet muuttuvat, joista huomionarvoisin on singlettihapen muodostumisen merkittävä hidastuminen. Muutoksista riippumatta, akklimoituneet solut ovat silti alttiita fotoinhibitiolle.

Työ tarkasteli myös *Chlamydomonaksen* PQ-poolin hapetus-pelkitys-tilaa ja sen vaikutusta tilasiirtymiin. Tuloksena todettiin *Chlamydomonaksen* PQ-poolin olevan samaa kokoluokkaa kuin syanobakteereilla ja kasveilla. Lisäksi se pysyi korkeasti pelkistyneenä lähes kaikissa valo-olosuhteissa, minkä oletettiin johtuvan levien PSII:n ja PSI:n hyvin samankaltaisesta kapasiteetista kerätä valoa. Ei-fotokemialliset reaktiot vaikuttivat myös voimakkaasti levien PQ-pooliin. Toisin kuin kasveilla, *Chlamydomonaksen* tilasiirtymien todettiin reagoivan pääasiallisesti valon intensiteetin, ei laadun, vaihteluun.

ASIASANAT: Chlamydomonas, fotoinhibitio, plastokinonivaranto, sopeutuminen, tilasiirtymät, valoreaktiokeskus II, voimakas valo, yhteyttäminen

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Abbreviations

$^1O_2$	Singlet oxygen
^3Chl	Excited state chlorophyll
$^3P_{680}$	Excited state of PSII reaction center chlorophyll
ADP	Adenosine diphosphate
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
Car	Carotenoid
Chl	Chlorophyll
CEF	Cyclic electron flow
CP26	26 kDa PSII monomeric antenna protein
CP29	29 kDa PSII monomeric antenna protein
CP43	43 kDa PSII core antenna protein
cyt b_6f	Cytochrome b_6f-complex
D1	D1 (or PsbA) subunit of the PSII core
DBMIB	2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone
DCBQ	2,6-dichloro-1,4-benzoquinone
DMBQ	2,6-dimethylbenzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
Fd	Ferredoxin
FDP	Flavodiiron protein
F_V/F_M	ratio of variable fluorescence and maximal fluorescence (a proxy of the yield of PSII photochemistry)
H_2O_2	Hydrogen peroxide
HSM	High Salt Medium (photoautotrophic mineral growth medium)
LET	Linear electron transfer
LHC	Light harvesting complex
Lhca	Minor PSI light harvesting antenna protein
LhcbM	LHCII apoprotein (1-9)
LHCI	PSI-associated light-harvesting complex
LHCII	PSII-associated light-harvesting complex
LHCSR1	Light-harvesting complex stress-related protein 1

LHCSR3	Light-harvesting complex stress-related protein 3
MDA	Monodehydroascorbate
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDA2	Type II NAD(P)H dehydrogenase
NPQ	Non-photochemical quenching
$O_2^{\bullet -}$	Superoxide anion radical
OD_x	Optical density of sample at designated wavelength x
OEC	Oxygen evolving complex
Pheo	Pheophytin
P_{680}	Reaction center of PSII (consisting of four designated chlorophylls molecules)
P_{700}	Reaction center chlorophylls of PSI
PC	Plastocyanin
(P)PFD	(Photosynthetic) photon flux density ($\mu mol\ m^{-2}\ s^{-1}$)
PQ	Plastoquinone
PQH_2	Plastohydroquinone; doubly reduced plastoquinone
PQ-pool	Photochemically active fraction of total plastoquinone, localized in the thylakoids
PsbS	Photosystem II subunit S
PSI	Photosystem I protein complex
PSII	Photosystem II protein complex
PTOX	Plastid terminal oxidase
qE	Energy-dependent fluorescence quenching
qI	Photoinhibition of photosystem II-dependent fluorescence quenching
qT	State transition-dependent fluorescence quenching
ROS	Reactive oxygen species
SOSG	Singlet oxygen sensor green; sensor reagent for $^1O_2$ detection
SOD	Superoxide dismutase
STT7	State transition kinase (STN7 in plants)
TAP	Tris-acetate-phosphate growth medium
Q_A	Primary quinone electron acceptor site in PSII
Q_B	Secondary quinone electron acceptor site in PSII

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 Virtanen O, Valev D, Kruse O, Wobbe L, Tyystjärvi E. Photoinhibition and continuous growth of the wild-type and a high-light tolerant strain of *Chlamydomonas reinhardtii*. *Photosynthetica*, 2019; 57:617-626. doi:10.32615/ps.2019.056.

II Virtanen O, Khorobrykh S, Tyystjärvi E. Acclimation of *Chlamydomonas reinhardtii* to extremely strong light. *Photosynthesis Research*, 2021; 147:91-106. doi:10.1007/s11120-020-00802-2.

III Virtanen O, Tyystjärvi E. Plastoquinone pool redox state and control of state transitions in *Chlamydomonas reinhardtii* in darkness and under illumination. *Photosynthesis Research*, 2023; 155: 59-76. doi: 10.1007/s11120-022-00970-3.

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1 Introduction

Chlamydomonas reinhardtii (hereafter *Chlamydomonas*) is a unicellular green alga belonging to the order *Chlamydomonales*, estimated to have diverged from the rest of the green algal lineage approximately 250 million years ago (Herron et al. 2009). Species of this order have spread widely across different biomes and can be found in most fresh and saltwater bodies, but also in soils. *Chlamydomonas* is a soil-dwelling organism that can move rapidly in its environment in response to both internal and external stimuli by using its two flagella. In the last twenty years, *Chlamydomonas* has become a commonly used model organism in green algal photosynthesis research for many reasons. Most importantly, it is easy to culture and store for long periods of time and protocols enabling its modification are widely used. Recently, a huge library of *Chlamydomonas* mutants was generated in University of Princeton (Li et al. 2019), giving access to mutants with highly specific deletions. This has enabled faster-paced access to research without having to go through the trouble of mutant line generation. Currently, studies with green algae (and hence also *Chlamydomonas*) gather a great deal of interest as they offer potential solutions in various fields of circular bioeconomy, e.g. wastewater treatment and nutrient reabsorption.

1.1 Photosynthesis captures light energy to chemical form

In a nutshell, photosynthesis captures light energy in chemical form, measured in two types of energetic biomolecules: NADPH and ATP. In photosynthetic eukaryotes, the whole process takes place in double-membrane enclosed cell organelles called chloroplasts. A distinguishable difference between plant cells and *Chlamydomonas* cells is that plant cells contain numerous chloroplasts, whereas a *Chlamydomonas* cell contains only one big chloroplast that takes up approximately 50 % of the cell volume (Engel et al. 2015). Chloroplasts in both plants and algae contain membranes called thylakoids that can either be appressed in tightly packed structures called grana stacks or as non-appressed, more loosely ordered stroma thylakoids.

A schematic view of the light reactions of oxygenic photosynthesis is represented in Fig. 1. However, even though often illustrated side by side, the different parts of the linear electron transfer (LET) chain are separated by lateral heterogeneity in green algae and plants. This means that the majority of photosystem IIs (PSII) are usually found in the grana stacks, while photosystem Is (PSI) and ATP-synthases are localized to the non-appressed thylakoids. Cytochrome b_6f s (cyt b_6f) can be found evenhandedly distributed across the thylakoid membranes (Wietrzynski et al. 2020).

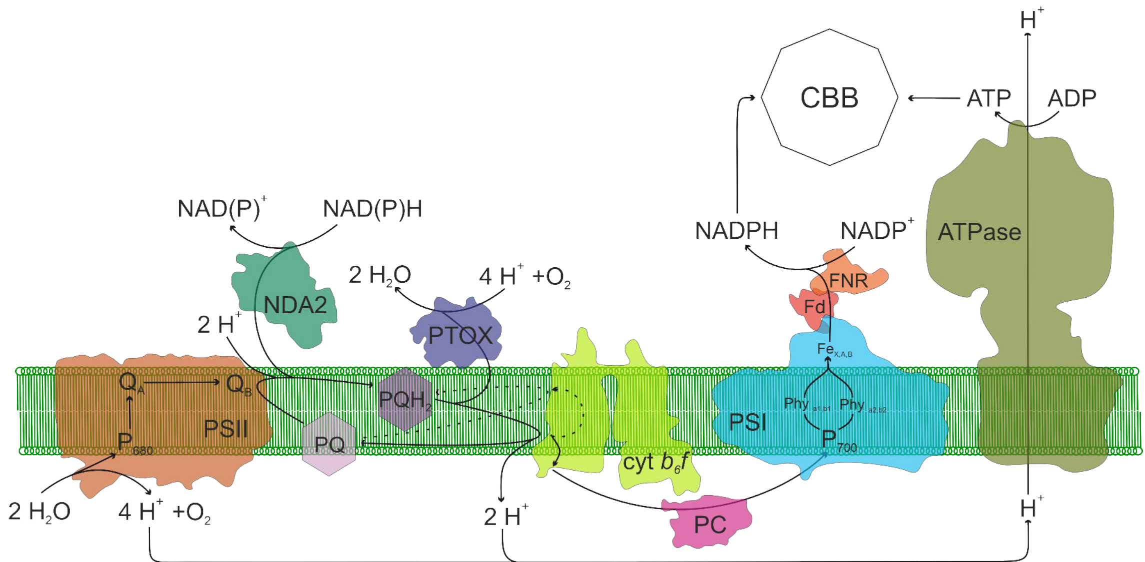

Figure 1. Light reactions in Chlamydomonas thylakoid membranes. A characteristic view of the linear electron transfer chain from water to NADPH. The transfer occurs via PSII, PQ, cyt b_6f, PC and PSI. Absorbed light energy is utilized at the reaction centers (P_{680} and P_{700}) to cause charge separation that drives the electron transfer forward. Water splitting and PQ-oxidation at cyt b_6f pump protons to lumen, which drives ATP-synthesis at ATPase that utilizes the electrochemical gradient across the thylakoid membrane. The generated NADPH and ATP can then be utilized in energy-demanding processes, such the Calvin-Benson-Bassham cycle (CBB). Auxiliary transfer agents NDA2 and PTOX reduce and oxidize the PQ-pool in a light- independent manner. The dashed line shows the recycling of electrons in the Q-cycle that increases the ratio of protons pumped to electrons transferred.

The generation of NADPH occurs when excitation energy of photons is captured by chlorophyll (Chl) molecules and transferred to the cores of PSII and PSI. At PSII, this excitation energy is then used to split water to molecular oxygen and protons, which is a biologically unique reaction. The oxidation of water yields electrons that are then transferred in a series of reactions involving a donor with more negative redox potential and an acceptor with more positive redox potential (see Table 1). In addition, excitation of the reaction center (RC) of PSI produces a highly reducing

molecule, P_{700}*. Eventually, electrons are used to reduce $NADP^+$ to NADPH. This molecule is then used to fuel energy-requiring reactions, such as carbon fixation or lipid synthesis.

The balance between the flow of electrons through the photosystems is maintained by a phenomenon called state transitions (see Chapter 1.2.1) that equilibrate the rate of electron transfer when the excitation favors the turnover of one type of photosystem over the other. This equilibration occurs via movement of the light-harvesting antenna proteins. Simply put, when the electron flow is faster through PSII than PSI, some light-harvesting antennae move to PSI, which increases its light-harvesting capacity and consequent electron flow through it (State 2). Vice versa, the light-harvesting antennae move back to PSII (State 1) when there is less electron flow through PSII than PSI.

Table 1. Estimates of midpoint redox potentials (E_m) of the key electron transferring redox pairs in the linear electron transfer chain of photosynthesis.

Redox pair	E_m (mV)	Reference
½ O_2 + 2 H^+ / H_2O	+820	*Hohmann-Marriott and Blankenship (2011)*
P_{680}^+ / P_{680}	+1170 – +1210	*Kato et al. (2009)*
P_{680}^+ / P_{680}^*	-660 – -620	*Kato et al. (2009)*
Q_A / Q_A^-	-170 – -104	*Shibamoto et al. (2009, 2010)*
Q_B / Q_B^-	-60 – +93	*Shibamoto et al. (2009), Kato et al. (2016)*
P_{700} / P_{700}^+	+400 – +470	*Nakamura et al. (2011)*
P_{700}^+ / P_{700}^*	≈ -1300	*Nakamura et al. (2011)*
$NADP^+$ + H^+ / NADPH	-320	*Hohmann-Marriott and Blankenship (2011)*

The other form of chemical energy, ATP, is generated by the thylakoid-bound ATP-synthase. This enzyme utilizes the proton gradient formed across the membrane by the electron transfer-reactions that pump protons to the lumen in different parts of the chain. The protons in the lumen flow back to stroma through the enzyme in a controlled manner to balance out the electrochemical gradient between the two sides of the thylakoids, powering the ATP-synthase to phosphorylate ADP to ATP.

1.1.1 Light-dependent water-oxidation: photosystem II

PSII operates in apparent cycles. These cycles consist of energy of four absorbed photons that induce charge separation in the RC, a water molecule being oxidized by the high oxidizing potential of the charge-separated P_{680}, and finally two PQ

molecules being doubly reduced to PQH_2 by the electrons transferred from the OEC. This cycle also contributes in generating the proton gradient across the thylakoid by splitting water into oxygen and protons on the lumenal surface of a PSII complex.

The transfer of electrons through PSII is initiated when excitation energy, absorbed by the pigments in the core or the various LHC-proteins of the complex, reaches the RC of the complex. This center consists of four Chl molecules in close proximity of one another, but the majority of charge separations are thought to lead to oxidation of a pigment bound to the D1-protein (Nagao et al. 2017). Anyway, after the excitation has reached the reaction center and consequent charge separation is induced, the charge pair $P_{680}^{+}/Pheo^{-}$ is formed. This radical pair, as the name suggests, is unstable. Hence, the energy is stabilized by transferring the energy forward to Q_A. The differences in redox potentials between subsequent charge pairs are essential, because they enable the electrons to (mainly) flow in unidirectional manner. The Q_A/Q_A^--pair donates the electron to a PQ molecule bound to the Q_B-site and forms a semiquinone Q_B^-. After accepting a second electron and binding two protons, the redox potential of the PQ-molecule shifts by 50 mV, which severely decreases its affinity to bind the Q_B-pocket of PSII (De Causmaecker et al. 2019) and causes it to dissociate from the pocket to the PQ-pool.

The electron transfer between the two quinone sites, Q_A and Q_B, and its fine-tuning is enabled by a non-heme iron binding a bicarbonate molecule (Takahashi et al. 2009). Interestingly, this bicarbonate between the Q_A and Q_B in PSII can be replaced with acetate in *Chlamydomonas* if present in the medium (Shevela et al.2007). This changes the midpoint redox potentials inside PSII, stabilizing the S_2/Q_A^--pair (S_2 refers to the state of the manganese complex of the OEC) instead of the Q_B/Q_B^--pair, discouraging the recombination reactions that produce singlet oxygen ($^1O_2$) and consequently protect the acetate-grown cells from photoinhibition (Roach et al. 2013). However, this does not mean immunity from photoinhibition but yields only an alleviation in physiological conditions.

Once oxidized, the reaction center Chl P_{680}^{+} is re-reduced to its ground state by electrons from a tyrosine residue of D1, which in turn can receive an electron from OEC that finally strips it from water molecules bound to the manganese complex.

The structure of a typical PSII-LHCII supercomplex is represented in Fig. 2. The depicted structure is of a dimeric $C_2S_2M_2L_2$-supercomplex, where the letters refer to the core proteins (C), and LHCII trimers bound strongly (S), mediocrely (M) or loosely (L) to the core units. The structures of the PSII-LHCII supercomplexes vary *in vivo* in response to illumination and other environmental conditions from C_2S_2 to the $C_2S_2M_2L_2$ shown below, from smallest to largest antenna structure, respectively. The dimer also contains monomeric antenna proteins, CP26 and CP29, one of both per reaction center. These monomeric antennae, especially CP29, function in light harvesting, but they also bridge together the peripheric antenna and the PSII core

antennae, CP43 and CP47, and consequently enable energy transfer between the two ends of the chain transferring excitation energy from Chl to Chl and eventually to the PSII core (Barber et al. 1999, Nield et al. 2000, Yakushevska et al. 2003, Minagawa and Takahashi 2004).

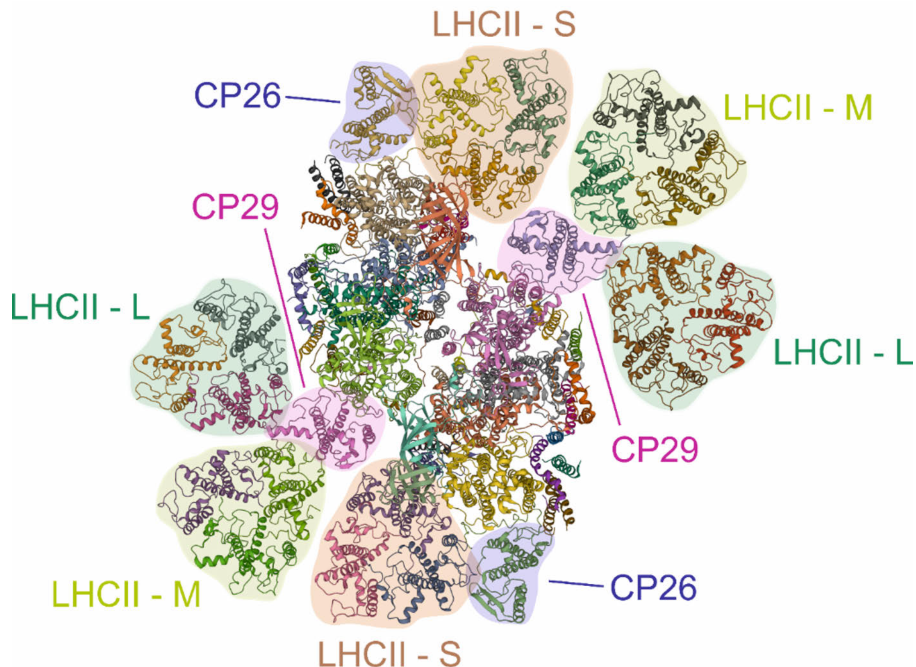

Figure 2. Architecture of the dimeric *Chlamydomonas* PSII-LHCII $C_2S_2M_2L_2$-supercomplex as seen from above. The structure is downloaded from PDB (ID: 6KAD) and the added overlays depict the various antenna complexes as described in the text. The notations L, M and S refer to the affinity of LHCII-trimer to bind the complex as Loose (Green), Medium (Yellow) and Strong (Red). The structure is originally cited in Sheng et al. (2021).

1.1.2 Plastoquinone pool – a crossroad between photosystems

PQ can be localized in three different membrane structures in the chloroplast: thylakoid membranes, chloroplast envelope membrane or membrane structures called plastoglobuli. The thylakoid-bound fraction is here classified as the photoactive fraction as it partakes in the photosynthetic electron transfer. This fraction is henceforth referred to as the PQ-pool. The plastoglobuli-localized fraction of PQ was found already by Lichtenhaler et al. (1981), but its importance has recently become more elucidated. Plastoglobuli of *Chlamydomonas* are localized to areas of flat sheets of the thylakoids, with which they share only scarce, point-like

connections (Engel et al. 2015). Pralon et al. (2019) showed that there is an exchange of PQ molecules between the two pools in *Arabidopsis thaliana*. They suggested this transfer of PQ molecules to act as a protective measure against over-excitation of the PQ-pool under high light. However, whether *Chlamydomonas* possesses similar mechanism, remains uncertain. Interestingly, the eyespot of *Chlamydomonas*, an organelle responsible for light-sensing, contains a lot of proteins that are homologous to proteins found in plant plastoglobuli (Schmidt et al. 2006, Wagner et al. 2008, Eitzinger et al. 2015), suggesting that the eyespots and plastoglobuli share a common ancestry. Even though plastoglobuli have a capacity to store a large fraction of PQ that is non-photoactive, meaning it is not reduced by PSII or other component reducing PQ in the thylakoids, they are also central for various biochemical pathways and storage of biomolecules e.g. α-tocopherol recycling and lipid storage (for review see van Wijk and Kessler 2018).

Here the main interest is in the thylakoid-localized PQ-pool, as it is the first electron-transfer agent between PSII and PSI. It consists of a reserve of both PQ and PQH_2. The PQ-pool redox state is determined as the ratio between the reduced PQH_2 to the total PQ ($PQH_2/(PQH_2+PQ)$). The relative size of the PQ-pool varies between organisms and it has been shown to be 30-35 % and 43-45 % of the total PQ in higher plants (Kruk and Karpinski 2006, Yoshida et al. 2010) and cyanobacteria (Khorobrykh et al. 2020a), respectively. Furthermore, it has been estimated that there are 5-10 PQs per active PSII RC in the thylakoids of *A. thaliana* (Kruk and Karpisnki 2006). The PQ-pool is the first branching point in the LET chain and once in the PQ-pool, instead of a linear funnel for the electrons to flow through, there are optional paths the electrons can go to and come from. These alternative paths are described in Chapter 1.1.4. Nevertheless, the majority of electrons are transferred through the cyt b_6f, a PQH_2-plastocyanin-oxidoreductase, to plastocyanin and eventually to PSI. The oxidation of PQH_2 in the Q_0-site of the cyt b_6f releases the PQ back to the pool to be reduced again and the two protons are released to the lumen. The two electrons are transferred via the high potential and low potential transfer chains of cyt b_6f. The latter recycles electrons to the Q_i-site of the cyt b_6f, which binds a PQ-molecule that is doubly reduced and bound together with two protons from the stroma. This recycling of electrons is continuously active in the thylakoids (Sacksteder et al. 2000), and it causes cyt b_6f to be responsible of 2/3 of the proton pumping the thylakoids by doubling the ratio of protons pumped per electron transferred (Cramer and Zhang 2006).

In the light, the most influential factors contributing to the PQ-pool redox kinetics are the rates of charge separation and consequent electron transfer rates through PSII and PSI (Mattila et al. 2020). The redox state of the PQ-pool is often considered to be a probe for the organisms to read the illumination conditions they are under and acclimate to function in the most optimal way. The cyt b_6f controls the

rates of PQH_2 oxidation and further LET when the thylakoid lumen is acidified (Foyer et al. 2012). This regulation is often referred as photosynthetic control and it was recently shown to function similarly *Chlamydomonas* as in plants (Ozawa et al. 2022). Photosynthetic control provides photoprotection for PSI by limiting electron flow on the donor side of P_{700} to balance the capacity of PSI donor and acceptor sides (Chaux et al. 2015). Other examples of direct PQ-pool-dependent regulation are the STN7-dependent state transitions in plants that function in response to increased/decreased PQ-pool reduction (Bellafiore et al. 2005, Mattila et al. 2020), and the control of the electron-transferring efficiency of PSII that occurs in response to the PQ-availability in the thylakoids (Suslichenko and Tikhonov 2017). All of these regulatory elements act as a feedback mechanism to maintain a balanced reduction state of the LET chain to maintain optimal function in varying light conditions.

In addition to its function in the electron transfer chain, PQH_2 is active also in other reactions. It can scavenge reactive oxygen species (ROS), i.e. $^1O_2$ (Kruk and Szysmanska 2012, Khorobrykh et al. 2015, Ferretti et al. 2018) generated by e.g. photosensitization, but also superoxide radicals (Borisova-Mubarakshina et al. 2018, Khorobrykh and Tyystjärvi 2018) originating either from PSI or auto-oxidation of PQ (Khorobrykh and Ivanov 2002, Vetoshkina et al. 2017, Khorobrykh et al. 2020b). The quenching capacity yields PQH_2 antioxidant characteristics as it can protect the thylakoid lipids from peroxidation by different ROS (Hundal et al. 1995, Ksas et al. 2015, Kruk et al. 2016). These reactions lead to oxidation of PQH_2 to PQ and generation of H_2O_2 termed as membrane-generated hydrogen peroxide. H_2O_2 is a signaling molecule that has been suggested to function as a retrograde signaling agent to the nucleus in plants (Pfannschmidt et al. 2003).

In addition to H_2O_2, plants and algae have a Chloroplast Sensory Kinase (CSK) (Puthiyaveetil et al. 2008) that is homologous to the cyanobacterial sensory kinase enabling sensing and signal-transduction of the PQ-pool redox state (Ibrahim et al. 2016). CSK can interact directly with PQH_2 and mutants lacking this kinase have been shown to have significantly altered expression of several photosynthesis genes (Ibrahim et al. 2020).

In several studies, PQ-pool redox state has been shown to affect gene expression in three major types of photosynthetic organisms: plants (Pfannschmidt et al. 1999, 2001, 2009, Piippo et al. 2006, Frigerio et al. 2007, Bräutigam et al. 2009, Bode et al. 2016), green algae (Escoubas et al. 1995, Giordano et al. 2005) and cyanobacteria (Hihara et al. 2003). These genes were often related to photosynthesis. However, similar studies have reported varying results on what genes are regulated and how the PQ-pool regulates them. Furthermore, the exact signaling mechanisms remain to be elucidated. Hence, the work on PQ-pool redox state as a regulator of gene expression is still a work in progress.

1.1.3 Algal photosystem I is very different from plant photosystem I

The core of a PSI-complex is highly conserved between plants, green algae, cyanobacteria and red algae (Allen et al. 2011). In the PSI core, absorbed light energy is used to induce charge separation between the reaction center chlorophyll-pair (P_{700}) and the primary electron-accepting Chl (A_0) in either of the branching transfer routes found inside the PSI core. This charge separation oxidizes P_{700} to P_{700}^+, which is re-reduced by electrons from plastocyanin that interacts with PSI on its lumenal surface. The two routes within PSI start at their respective A_0-Chls. These routes are highly similar to one another, and they are located in the two major Psa-subunits (PsaA and PsaB) (Santabarbara et al. 2015). They are both utilized, even though they function at significantly different rates from one another (Guergova-Kuras et al. 2001). Interestingly, even when their electron transfer rates are very different, the charge separation still occurs at the ratio of 77:23 (PsaA:PsaB) in physiological temperatures in cyanobacteria (Makita and Hastings 2015). After a transfer of an electron to an intermediate phylloquinone in either of the branches, the paths converge again as the electron is transferred to a shared iron-sulfur cluster. In fact, there are three iron-sulfur clusters in total in the PSI-complex and the last of these clusters donates the electrons to reduce ferredoxin, from which there are various types in *Chlamydomonas* that function in different conditions (for review see Sawyer and Wrinkler 2017). Ferredoxins then enable the ferredoxin-NADP+ reductase to function and form NADPH (see Mulo and Medina 2017), a stable storage of reduction energy that can then be utilized in carbon fixation and/or other metabolic processes requiring an input of energy.

In contrast to the well conserved core structure, a special characteristic of the algal PSI-LCHI complex is that it harbors an additional belt of Lhca-antennae located on the fringes of the complex (Fig. 3) (Pan et al. 2021), that is not present in the plant PSI-LHCI (Caspy and Nelson 2018). The Lhcas in this additional belt of antennae have significantly different pigment composition compared to the inner belt, enabling efficient transfer of excitation energy from the outermost antenna to the PSI core through the two layers of the fan-shaped antenna (Su et al. 2019, Suga et al. 2019). This gradient causes the ratio of Chls *a* and *b* of *Chlamydomonas* PSI-LHCI (4.4, Drop et al. 2014) to be closer to the ratio that of PSII-LHCII-complex (2.1, Shen et al. 2019) than in plants (Caspy and Nelson 2018, Su et al. 2017), leading to more similar absorption profiles between the photosystems in this alga than in plants. In addition, the two monomeric antennae on the side of the core complex, Lhca2 and Lhca9, are unique to the algal system and are not found in plants (Caspy and Nelson 2018).

A recent discovery by Naschberger et al. (2022) illustrated that the PSI-complexes of *Chlamydomonas* can be found in the thylakoids as monomers or state transition complexes, but also as dimers. This dimerization was found to be accomplished via two monomeric PSI-LHCIs becoming tethered via Lhca9. The

three forms of PSI-complex were also suggested to be transformable between one another. Another study, by Younas et al. (2022), argued this dynamic nature of the different supercomplex formations to be achieved via stabilization dependent on the phosphorylation states of PsaG and PsaH. PsaG was shown to be present in all the reported structures and its phosphorylation was speculated to strengthen the stability of associations between PsaG, PsaB and Lhca9. In contrast, PsaH was considered to be involved in excluding the option for PSI-dimerization in algae once the state transition structure PSI-LHCI-LHCII is formed. Moreover, while PsaH has been shown to be integral to the state transition complex formation by providing a docking site for the L-LHCII/LHCII-1 in PSI of both plants (Pan et al. 2018) and algae (Younas et al. 2022), in maize this subunit lacks the three specific residues found to be responsible for the LHCII-2-docking in algae (Younas et al. 2022). Hence, the PsaH (together with Lhca2) is most probably the key in enabling the algal PSI to bind an additional LHCII trimer in State 2. Importantly, the joining of two LHCII-trimers to PSI further evens out the difference in the Chl *a*:*b*-ratio between PSII and PSI mentioned above by decreasing the *a*:*b*-ratio at PSI (to 2.95, Drop et al. 2014).

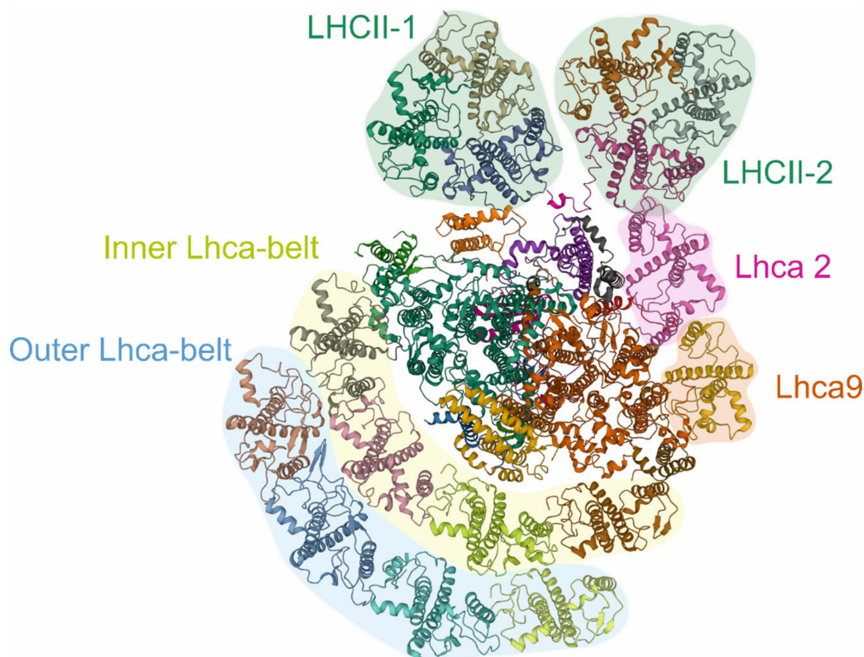

Figure 3. Architecture of the PSI-LHCI-LHCII supercomplex of *Chlamydomonas* viewed from top. The structure was downloaded from PDB (ID: 7DZ7) where it was originally cited by Pan et al. (2021). The masking colors depict the two antenna belts (Yellow: Inner consisting from Lhca1, 8, 7 and 3; Cyan: outer belt consisting of Lhca1, 4, 6 and 5) of the complex, as well as the two monomeric antennae Lhca2 (purple) and 9 (orange). The two LHCII trimers (green) attached to the complex are depicted as LHCII-1 and LHCII-2 (see text for details).

Indeed, the striking feature of the algal PSI-complex are the two LHCII trimers consisting of LhcbM1-M3/4-M2/7 (LHCII-1) and LhcbM5-M3/4-M2/7 (LHCII-2), capable of binding the PSI-LHCI-complex through interactions via Psa-subunits O, H and L (Pan et al. 2021). All the monomeric LhcbM-proteins in these trimers have been characterized to take part in state transitions between PSI and PSII and have been found in the "loose" LHCII-fraction (Takahashi et al. 2006, 2014, Ferrante et al. 2012, Girolomoni et al. 2017, Kim et al. 2020), aside from LhcbM1 that has been associated with heat-dissipation (Elrad et al. 2002, Ferrante et al. 2012) (see Chapter 1.2.2.).

1.1.4 Auxiliary electron transfer

In addition to the linear electron transfer, there are several different ways for electrons to flow in and out from the thylakoids. These pathways are designated as alternative electron transfer routes that have two main functions: 1. Enable production of ATP and NADPH in an optimal ratio to fuel reactions of carbon fixation but also other metabolic processes requiring an input of energy. 2. Provide a mechanism to safely regulate the varying redox poises in different parts of the LET chain and hence reduce the probability for harmful reactions such as lipid peroxidation.

The most commonly cited alternative electron transfer pathway is the cyclic electron flow (CEF) around PSI. This pathway cycles electrons again through PSI by re-introducing the electrons to the donor side of PSI in the LET chain. The pathways for CEF, even when it has been almost 70 years since the first report of cyclic electron transfer (Arnon et al. 1954), are still being studied in different organisms. The difficulties of *in vivo* measurements of CEF hamper final definitions of the accurate pathways from PSI-acceptors back to the thylakoid. However, it is known that both the PQ-pool and the cyt b_6f are involved in the recycling of electrons (see Nawrocki et al. 2019a). Notably, the transition to CEF in *Chlamydomonas* has been shown to involve a transition to State 2 (Finazzi et al. 2002, see Chapter 1.2.1.) and formation of a PSI-LHCI-cyt b_6f-supercomplex (Steinbeck et al. 2018). These changes occur in response to the need to re-balance the intracellular ATP-to-NADPH-ratio, to which CEF contributes by increasing the production of ATP at the expense of $NADP^+$ reduction (Alric et al. 2010).

In plants, there are two identified routes for CEF. First pathway requires Proton Gradient Regulator 5 (PGR5)-protein that (as its name suggests) helps regulate the proton motive force across the thylakoid membrane by reintroducing electrons in the PQ-pool (Munekage et al. 2002). This pathway is often referred as the antimycin A-sensitive pathway (see Labs et al. 2016). As in plants, PGR5 has been shown to be crucial for photosynthesis maintenance also in *Chlamydomonas* (Johnson et al. 2014,

Buchert et al. 2020), but its accurate molecular function is still elusive. The second CEF-route in plants is through type I NADH dehydrogenase (NDH-1). This thylakoid-bound enzyme transfers electrons to the PQ-pool from ferredoxin (Yamamoto et al. 2011) and contributes to the proton gradient formation by pumping electrons across the thylakoid membrane (see Peltier et al. 2016 for review). However, NDH-1 is missing in many green algal lineages, also in *Chlamydomonas* (Robbens et al. 2007, Martín and Sabater 2010). Instead, majority of green algae utilize NDA2, a type II NAD(P)H dehydrogenase to light-independently reduce the PQ-pool. Interestingly, homologues of NDA2 can be found in the mitochondria of plants, fungi and bacteria (Rasmusson et al. 2008). Jans et al. (2008) showed NDA2 to be localized to the chloroplasts of *Chlamydomonas*, where it utilizes the reducing equivalents in the chloroplast stroma to reduce the PQ-pool. A study using a recombinant protein showed NDA2 to oxidize both NADH and NADPH, with the former being the preferred substrate (Desplats et al. 2009). However, unlike NDH-1, NDA2 is non-proton-pumping and hence does not generate the proton motive force to fuel ATP-synthesis (Nawrocki et al. 2015). As NDA2 can utilize stromal reductants generated in both LET and fermentative metabolism, it can be considered as both a CEF pathway (reduction of PQ by PQ-reductase that consumes PSI-acceptors) but also to function in chlororespiration (process involving a PQ reduction by PQ-reductase utilizing stromal reductants and reoxidation of PQH_2 by plastid terminal oxidase (PTOX) (see below) that reduces oxygen to water). Under dark anoxia, NDA2-dependent reduction of PQ is responsible for enabling STT7-activation and transition to State 2 (Jans et al. 2008). NDA2 is upregulated under deficiency from both nitrogen (Wei et al. 2014) and sulphur (De Mia et al. 2019).

There are two genes encoding for PTOX in *Chlamydomonas*, *PTOX1* and *PTOX2*, from which the protein encoded by *PTOX2* has been shown to be the more prominent PQ-oxidizer (Houille-Vernes et al. 2011) and to be crucial for growth under fluctuating light conditions (Nawrocki et al. 2019b). However, since they nonetheless cover similar function and are hard to distinguish without specific mutant strains, the two proteins will hereafter be referred collectively as PTOX. This enzyme was described already by Cournac et al. (2000) to be responsible for PQ-pool oxidation not involving the cyt b_6f by closing the smaller water-water-cycle (Fig. 4) in the thylakoids and reducing oxygen to water with electrons directly from PQH_2. PTOX was originally considered as a safety valve for electrons in case of imbalance of electron transfer through the photosystems (Cardol et al. 2008). However, later it was shown to be substantially (over two orders of magnitude) slower at transferring electrons than the competing route to PSI (Nawrocki et al. 2015), lessening but not refuting the suggested role as a safety valve. Interestingly, similarly to NDA2, also the expression of PTOX is upregulated during nitrogen (Wei et al. 2014) and sulphur starvation (De Mia et al. 2019). The co-function of NDA2

and PTOX link chlororespiration (Jans et al. 2008), but also mitochondrial respiration (Cardol et al. 2003, 2009) with state transitions. Such mitochondrial-chloroplastic coupling is also an efficient strategy for *Chlamydomonas* to cope with high light stress (Kaye et al. 2019).

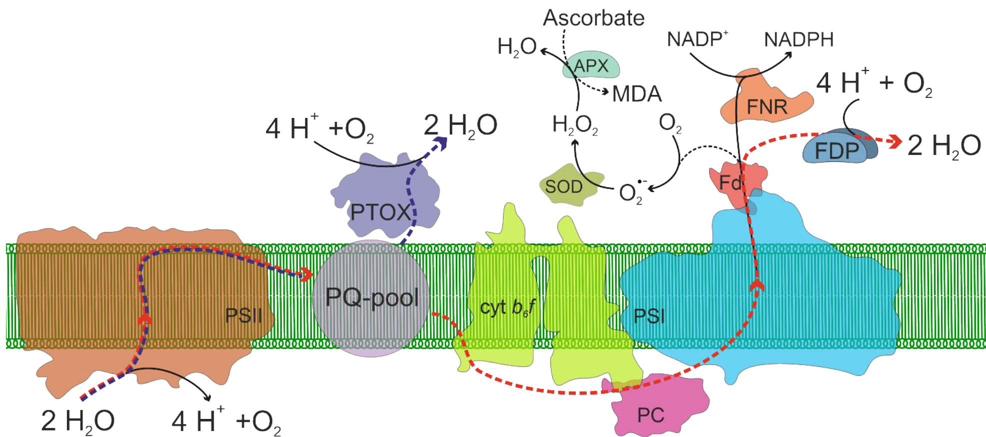

Figure 4. The main water-water cycles in Chlamydomonas thylakoids. The reactions in the linear electron transfer classified to take part in the small (blue, dashed line) and the large (red, dashed line) water-water cycle. The small cycle occurs via plastid terminal oxidase (PTOX), whereas the larger utilizes the dimeric flavodiiron proteins (FDP) downstream of PSI. These two types of proteins catalyze similar reactions, where molecular oxygen is transformed into water with electrons from photosynthetic electron transfer. These two cycles are thought to prevent the overreduction of the electron transfer chain and consequently decrease the probability for production of reactive oxygen species under conditions where other sink activity is not enough to consume enough electrons from the photosynthetic light reactions. The Mehler reaction involving the superoxide dismutase (SOD) and ascorbate peroxidase (APX) is also depicted. However, even though this pathway can alleviate the excitation pressure, it includes production of ROS and is thus not considered to have such protective role as the two other water-water-cycles described.

PTOX has been suggested to be more efficient at oxidizing the PQ-pool than the NDA2 is at reducing it (Houille-Vernes et al. 2011). These data hint towards a similar preference in *Chlamydomonas* to oxidize the PQ-pool in the dark as in higher plants (Mattila et al. 2020). However, the results by Houille-Vernes et al. (2011) are based on fluorescence data, meaning that reduction of PQ is quantified via a proxy, making it difficult to directly compare the rates of PTOX and NDA2 turnover. Furthermore, as the rate of PQ-reduction by NDA2 is dependent on both the concentration of the stromal reductants and the presence of oxidized PQ molecules, whereas PTOX activity is mostly dependent on the presence of reduced PQH_2 (as oxygen can be considered to be abundant in the chloroplasts under standard conditions), the accurate interpretation and comparison of the kinetics is difficult. Nonetheless, the

reduction of PQ by NDA2 in the dark (usually recorded as fluorescence transient or state transitions) is seen only when using anaerobic (total inhibition of PTOX, Jans et al. 2008) or micro-oxic conditions (PTOX activity significantly hindered, Patil et al. 2022), suggesting that the interpretation on kinetics of non-photochemical PQ-pool reduction and oxidation in Houille-Vernes et al. (2011) is correct and that PTOX really has more rapid turnover than NDA2.

The co-function of NDA2 and PTOX in the dark is interesting, since it does not contribute to ATP-synthesis; NAD(P)H oxidation and PQ-pool reduction by NDA2 and a subsequent oxidation of PQH_2 by PTOX does not cause translocation of protons to thylakoid lumen (Johnson and Alric 2013, Nawrocki et al. 2015). Interestingly, this feature and its implications have not been studied in detail to gain insight on how the light-independent non-electrogenic reduction and oxidation of PQ benefits algae. However, this kind of electron shuttling from reducing equivalents to water emphasizes the interplay between organelles of *Chlamydomonas* by acting as a mechanism to regulate the intracellular NADPH concentrations, altering the utilization of lipids and carbohydrates as carbon storages (see Burlacot et al. 2019).

In addition to PQ-pool reduction and oxidation, there are also demonstrated safety-valves for electrons. An excellent example of such safety-valves are the flavodiiron proteins (FDP) that facilitate safe dissipation of energy on the acceptor side of PSI, enable growth in fluctuating light conditions and prevent damage from ROS (Allahverdiyeva et al. 2013, 2015, Jokel et al. 2018). The activity of flavodiiron proteins causes photoreduction of O_2, which contributes to the ATP-to-NADPH-ratio by decreasing the production of NADPH via closing of the larger water-water-cycle (Fig. 4).

1.2 Acclimation to high light

NPQ is a collective term for protective measures that photosynthetic organisms utilize to control and safely dissipate absorbed light energy that cannot be utilized in photochemistry of PSII (Wobbe et al. 2016, Ruban and Wilson 2020). This way the probability for triplet chlorophyll formation and consequent possible production of $^1O_2$ is decreased by minimizing the probability of recombination reactions inside PSII, which in turn decreases the oxidative stress the cells are exposed to (Bassi and Dall'Osto 2022). There are several mechanisms involved in NPQ and they are generally divided into three major categories, based on the time it takes the system to recover: energy dissipation as heat (qE, fastest), state transitions (qT, medium) and fluorescence quenching by photoinhibition of photosystem II (qI, slowest). In addition, also xanthophyll cycles and long-term, sustained NPQ (Malnoë 2018) contribute to photoprotection. Six different cycles of xanthophylls have been identified already some time ago (García-Plazola et al. 2007), but also new ones have

emerged recently, namely the loroxanthin-cycle found operating at longer time scales in *Chlamydomonas* (van den Berg and Croce 2022). In addition to NPQ, e.g. changes in overall pigment and protein contents help cells to avoid the light-induced damage altogether, as there is less light-absorbing material in the cells. The features of algal NPQ are described in the Chapters below. However, due to the overlapping roles of quenching mechanisms in *Chlamydomonas*, instead of differentiating between the two fastest operating mechanisms (qE and qT), the operating agents (STT7, LHCSR3/1 and xanthophylls) will be described separately instead.

1.2.1 STT7-kinase

State transitions are a phenomenon supposed to equilibrate the light-harvesting capacity of the photosystems in fluctuating light (Minagawa 2011, Wientjes et al. 2013a), and under low-light conditions (Muillineaux and Emlyn-Jones 2004, Tikkanen et al. 2006). The adjustment of the antennae occurs via LHCII-trimers that move to PSI upon phosphorylation and increase its functional antenna size when conditions favor excitation of PSII (Allen et al. 1981). In conditions favoring the excitation of PSI, LHCII-movement is reversed via dephosphorylation of the trimers that then disassociate from PSI and reattach to PSII. The kinase responsible for the LHCII phosphorylation in plants, STN7, is characterized to be regulated by imbalance in the electron transfer in the thylakoids via PQH_2-occupancy of the Q_0-site of the cyt b_6f (Vener et al. 1995, 1997, Shapiguzov et al. 2017, Dumas et al. 2017). More recently, the modulation of light-harvesting capacity via state transitions has been shown to be curvilinearly related to the PQ-pool redox state and a portion of the PQ-pool to be maintained at a reduced state even in the dark in *A. thaliana* (Mattila et al. 2020). The algal kinase, STT7, is a close ortholog of the plant STN7 (Fleischmann et al. 1999, Bellafiore et al. 2005) in terms of both function (Fleischmann et al. 1999, Lemeille et al. 2010) and activation mechanism (Finazzi et al. 2001, Depége et al. 2003, Shapiguzov et al. 2017, Dumas et al. 2017).

Originally, the LHCII-phosphorylation-dependent, equilibrating state transitions have been thought to alter the antenna sizes of the photosystems to a much larger degree in algae than in plants. STT7 is capable of phosphorylating many different antenna proteins of PSII (Lemeille et al. 2010), which together with the assumption of algal state transitions being larger than in plants, has originally led to suggestions of the monomeric antenna (CP26 and CP29) units also being shuttled back and forth between photosystems and to act as additional anchoring surfaces for LHCII trimers at PSI (Kargul et al. 2005, Takahashi et al. 2006, Turkina et al. 2006, Takahashi et al. 2014). However, in their recent structural study, Pan et al. (2021) argue that CP29 has no interaction whatsoever with PSI under State 2 conditions.

In their study regarding the structure of *Chlamydomonas* PSI-LHCI-LHCII supercomplex, Pan et al. (2021) demonstrated that the pocket in algal PSI for binding one of the two LHCIIs ("LHCII-2", Fig. 3) is not conserved in plant PSI. This pocket binds the LHCII via interaction between the phosphorylated LHCII apoprotein LhcbM5 and the PsaH-subunit of PSI, which stabilizes the PSI-LHCI-LHCII supercomplex in *Chlamydomonas*. Biophysical and biochemical studies (Takahashi et al. 2006, 2014) have confirmed LhcbM5 to be required for algal PSI-LHCI-LHCII supercomplex formation, suggesting it to be an important agent in qT of *Chlamydomonas*. Intriguingly, this LhcbM5-containing LHCII-trimer has not been found in the PSII-LHCII complex of *Chlamydomonas* (Shen et al. 2019, Sheng et al. 2019). Together these studies suggest that the LHCII trimer apoprotein target for STT7-dependent phosphorylation and consequent PSI antenna modulation, LhcbM5, does not bind PSII even in State 1 conditions, but remains in (and is the main constituent of) the LHCII-fraction in the thylakoids not associated with either of the Photosystems.

The second PSI-interacting LHCII apoprotein, LhcbM1, has also been shown to be a target for STT7-dependent phosphorylation (Lemeille et al. 2010). The LHCII containing LhcbM1 docks to *Chlamydomonas'* PSI LHCII-1-site ("LHCII-1", Fig. 3) via interaction with both PsaH and PsaO subunits (Pan et al. 2021). In contrast to LhcbM5-containing LHCII, this type of LHCII trimer has been detected in both PSI-LHCI-LHCII (Takahashi et al. 2006, 2014) and PSII-LHCII supercomplexes (Sheng et al. 2019). However, LhcbM1 is not associated with state transitions (Elrad et al. 2002), but instead its mutants have been described to have diminished qE capacity (Ferrante et al. 2012, Liu et al. 2022 preprint) and will be discussed in more detail in Chapter 1.2.2.

To truly balance the light absorption between the photosystems, the activity of STT7 needs to be counteracted by LHCII-P dephosphorylation and detachment from PSI. There are two phosphatases functioning in land plants: Protein Phosphatase 1/Thylakoid Associated Phosphatase 38 (PPH1/TAP38) and Photosystem II Core Phosphatase (PBCP). These enzymes have defined areas of function in plants: PPH1/TAP38 interacts specifically with antenna trimers (Shapiguzov et al. 2010) and PBCP dephosphorylates mostly monomeric antenna and core subunits of PSII (Liu et al. 2019). In contrast, the homologs of these proteins in *Chlamydomonas* are both able to dephosphorylate LHCII trimers, but with some substrate specificity. In their work, Cariti et al. (2020) showed that PBCP reacts with type I and II LhcbMs, and PPH1 with type III and IV LhcbMs. In addition, it was shown that both of these phosphatases are able to dephosphorylate the monomeric core units of PSII. Furthermore, PBCP was proposed to be a constitutively active phosphatase with a slightly wider target protein range than the more selectively active PPH1. The different activities of these phosphatases indicate a dynamic balance between the two

light states that depend on the rates of phosphorylation and dephosphorylation of LhcbM5 and other light-harvesting units. Overall, the two phosphatases, even with certain substrate specificity, both function in algal state transitions and in maintaining effective balance in light-harvesting.

For some time, state transitions have been considered to be more protective than equilibrating in algae than in plants. This protection was recently quantified to be responsible of approximately 40 % of the measurable NPQ with noticeable dependency on the presence of Light-harvesting complex stress-related protein 3 (LHCSR3) in the cells (Steen et al. 2022), indicating a connection and a functional overlap of STT7 and LHCSR3. Nonetheless, qT is generally thought to be responsible for longer-term quenching as suggested before by Allorent et al. (2013). Importantly, it has been demonstrated that even though the PSII antenna size can indeed be reduced by 70 % under State 2 (Nagy et al. 2014), only a small fraction of the LHCIIs move to PSI, seen as only 20 % modulation in PSI antenna under these conditions (Nagy et al. 2014, Ünlü et al. 2014). The large difference in PSII antenna modulation has been attributed to be mostly due to rapid quenching and energetic dissociation of LHCII-trimers from PSII by LHCSR3 (Roach and Na 2017), which speaks for the protective nature of state transitions in *Chlamydomonas.* This mechanism will be described in detail in the following Chapter.

1.2.2 LHCSR3 and LHCSR1

When considering NPQ in green algae, it is impossible to neglect the LHCSR proteins, characterized originally by Peers et al. (2009). These proteins are responsible for the efficient NPQ-response in green algae, and *Chlamydomonas* possesses three different genes that encode LHCSRs: *LHCSR3.1 LHCSR3.2* and *LHCSR1*. The first two genes encode an identical protein, LHCSR3, that is crucial for qE induction and growth under fluctuating light conditions (Peers et al. 2009, Cantrell and Peers 2017, Girolomoni et al. 2019, Roach 2020, Liu et al. 2022 prepint). LHCSR3 has been found to be expressed in a light-dependent manner (Peers et al. 2009, Maruyama et al. 2014, Petroutsos et al. 2016), even when the LET is chemically inhibited via addition of DCMU (Redekop et al. 2022). Recently, the ambient CO_2-concentrations were shown to affect the expression of LHCSR3 at least on the mRNA-level (Redekop et al. 2022). Later, a study performed by Ruiz-Sola et al. (2023) demonstrated how both transcript and protein level-accumulation of LHCSR3 was significantly inhibited in conditions with 5 % CO_2, independently of high light. This was an interesting demonstration of the connection between photoprotection regulation and carbon availability. Importantly, LHCSR3 has been identified in PSII but also in the antenna of PSI (Allorent et al. 2013, Bergner et al. 2015).

The thylakoid-bound LHCSRs are activated when their lumenal residues become protonated upon lumen acidification (Bonente et al. 2011, Ballottari et al. 2016, Tian et al. 2019). Interestingly, these proteins also have sites for STT7-dependent phosphorylation that are not, however, required for their activation (Bonente et al. 2011). Instead, these phosphosites have been suggested to play a role in guiding the LHCSR3 to a proper quenching site (Bergner et al. 2015). More recently, zeaxanthin has also been found to activate the LHCSR3 in a pH-independent manner (Troiano et al. 2021). Overall, LHCSR3 appears to have different ways for quenching induction that form a dynamic range of mechanisms for the organisms to protect themselves from high light.

Both LHCSR3 and 1 contain pigments (Liguori et al. 2016, Dinc et al. 2016), which enables the LHCSRs to act as the quenching agent on site upon activation (Bonente et al. 2011, Dinc et al. 2016). The quenching itself is suggested to have two different mechanisms: First, LHCSRs can quench excitation energy via energy transfer between a carotenoid (Car) and a Chl molecule incorporated in the LHCSR structure. Secondly, there is also a possibility for the quenching to occur via protein interactions between the LHCSR3 and LHCII. De la Cruz Valbuena et al. (2019) suggested that the functioning of these mechanisms depends on which LHC is to be quenched and the environmental conditions. To outline, in algae the LHCSRs act both as the sensing and quenching agent in qE, which is in contrast to our understanding of the plant qE where photosystem II subunit S (PsbS) is the key sensor for NPQ-induction, but in which the quenching itself is still under debate (Fan et al. 2015, Ruban and Wilson 2020). Interestingly, in *Chlamydomonas* PsbS is required only transiently during the high light response (Redekop et al. 2020).

When active, LHCSR3 interacts with the PSII-LHCII supercomplex via PsbR, CP26 and a strongly bound LHCII trimer (Xue et al. 2015, Semchonok et al. 2017). The quenching by LHCSR3 offers a competing route for excitation energy transfer from the antennae to the PSII core via CP43, which increases the relative energy flow to the PSII core through CP47 in quenched state (Kim et al. 2017). This way LHCSR3 can quench the PSII complex without direct interaction with the PSII core (Semchonok et al. 2017). The quenching itself significantly decreases light-utilization of PSII (Tian et al. 2019) by energetically detaching LHCII units from the PSII core (Roach and Na 2017). However, even when detached, PSII-LHCII supercomplexes have been suggested to be maintained physically intact under State 2 (Minagawa and Tokutsu 2015), which has led to a hypothesis of more of an energetic rather than physical detachment of LHCII from the PSII. This characteristic is shared with plants, where qE-induction has been shown to not require physical disassembly of the PSII supercomplex (Bielczynski et al. 2022). LHCSR3 most probably specifically interacts with the monomeric antenna protein, LhcbM1, found at PSII mostly in the LHCII-S trimer (Sheng et al. 2019, Fig. 2), as

their co-presence is required for induction of sufficient qE (Elrad et al. 2002, Ferrante et al. 2012, Liu et al. 2022 preprint). However, whether this interaction is an enhancer or a prerequisite for the quenching, remains elusive (Liu et al. 2022 preprint).

Less is known about the second quenching protein, LHCSR1. The clearest characterization of it is that it is overall expressed less than LHCSR3 (Cantrell and Peers 2017). Perozeni et al. (2020) set out to examine the *in vivo* dominance of the LHCSR3 over LHCSR1 and suggested the division in expression to be due to different occupancy in one of the Car-binding sites, which makes the LHCSR1 less efficient quencher than LHCSR3. Nonetheless, LHCSR1 is able to quench PSI (Kosuge et al. 2018) but also the free fraction of LHCII (Dinc et al. 2016). In addition, LHCSR1 can replace the activity of LHCSR3 to some degree upon genetic repression of both genes encoding LHSCR3 (Girolomoni et al. 2019). Specifically, LHCSR1 is expressed during UV-exposure (Tokutsu et al. 2021), which depends on photoreceptor UVR8 activation that increases the expression of LHCSR1 and PsbS (Allorent et al. 2016, Tokutsu et al. 2019). The expression of the latter protein is also transiently required in *Chlamydomonas* for high-light acclimation, probably through thylakoid architecture modification (Redekop et al.2020) but it does not contribute to qE on its own (Girlomoni et al. 2019). Overall, LHSCR1 seems to function similarly as LHSCR3, but at a slower rate and in response to more specific environmental cues.

1.2.3 Xanthophylls

Carotenoids protect light-harvesting machineries from oxidative damage by quenching ^3Chl (Mozzo et al. 2008) but also $^1O_2$ (Ramel et al. 2012). The “loose” fraction of LHCII trimers has been suggested to quench excitation energy through formation of aggregated groups of trimers (Horton et al. 2005), a phenomenon proposed to occur via zeaxanthin-accumulation in the LHCII-pool (Shukla et al. 2020). Kim et al. (2020) showed that the two types of LHCII-trimers found in PSI-LHCI-LHCII (see Chapter 1.1.3.) were specifically shown to possess the capacity for fluorescence quenching *in vitro* in a pH-dependent manner when aggregated. On the basis of their data Kim et al. (2020) suggested that the two types of LHCII trimers capable of quenching have similar photoprotective capacity as their plant counterparts. Moreover, Fujita et al. (2022) were recently able to localize highly quenched LHCs *in vivo* by measuring fluorescence lifetime combined with a cryo-microscopy method. They suggested these highly quenched LHCs to be accumulated in PSI-enriched areas of the thylakoids and the quenching to occur via aggregation.

Interestingly, xanthophylls are shown not to be required for sufficient qE induction in *Chlamydomonas* in high light (Bonente et al. 2011, Quaas et al. 2015), but their absence decreases the total NPQ capacity (Niyogi et al. 1997). In contrast,

Troiano et al. (2021) argued that xanthophylls do indeed play a crucial role in activating NPQ via pH-independent mechanism upon introduction to high light. However, the complex antenna structures, dynamic nature and rapid movement of the LHCIIs at PSI (Pan et al. 2021) and the qE induction mechanisms not dependent on xanthophylls in *Chlamydomonas* (Bonente et al. 2011) severely complicate the interpretation of any model generated for a xanthophyll-dependent quenching mechanism. Hence, the accurate mechanism involving xanthophylls in NPQ in *Chlamydomonas* still remains obscure. Anyway, the LHCII-localized quenching induced by LHCRS3, especially at PSII, does not include any LHCII aggregation (Tian et al. 2019), even though aggregation is still considered to function in quenching as described above.

1.3 Photoinhibition of photosystem II

Even though PSII has ways to enhance its survival via mechanisms described above e.g. by tuning of redox chemistry inside the complex (Vass and Cser 2009, Rutherford et al. 2012, Brinkert et al. 2016) and via LHCSR3-mediated qE (Peers et al. 2009, Takahashi et al. 2009, Roach et al. 2013, Roach et al. 2020), it is still susceptible to damage by light (Tyystjärvi and Aro 1996) via inactivation and slowly recovered PSII photoinhibition (see Tyystjärvi 2013). The exact mechanism of this inactivation is still debated, even though the phenomenon has been known for over half a century. Below are described the probable theories, and how the damaged PSIIs are being repaired.

1.3.1 Light-dependent inactivation of photosystem II

The mechanism of PSII photoinhibition remains elusive. In the literature the debate has landed on possible mechanisms that will be here divided rather crudely into two categories. The first category is photoinhibition caused by the donor side of the P_{680}. In the classical donor side mechanism, the damage is suggested to be caused by long-lived P_{680}^+ or Tyrosine Z radical (TyrZ•) that unintentionally oxidize some PSII structure (Callahan et al. 1986, Jegerschöld et al. 1990, Anderson et al. 1998). This kind of photodamage may be caused by “misses” occurring in the OEC that involve light-absorption but failure to reduce P_{680}^+. These “misses” occur constantly in the light (Pham et al. 2019) and have been suggested to be causing formation of both long-lived P_{680}^+ and $^1O_2$ (Mattila et al. 2022), putting them in prime position to be causing photoinactivation of PSII via both donor side and $^1O_2$-dependent mechanisms. In turn, the Mn-mechanisms of photoinhibition of PSII describes the damage to be caused by P_{680}^+ after a release of a Mn-ion that inactivates the OEC (Hakala et al. 2005). This mechanism has

also been described as two-stepped mechanism (Ohnishi et al. 2005). In the first phase of this mechanism, the Mn-cluster of the OEC becomes damaged e.g. by radiation of different wavelengths of light, rendering it unable to oxidize water (Hakala et al. 2005, Ohnishi et al. 2005, Zavafer et al. 2017, Mattila et al. 2022). The second phase involves unintended oxidation of PSII component by P_{680}^{+} or TyrZ• in a similar manner as in classical donor side photoinhibition. This latter phase would be required to cause additional damage and secure the inactivation of PSII in the cases where OEC manages to recover from its initial damage without inactivation of the PSII complex. However, in this scenario the PSII inactivation is dependent on two subsequent reactions, and hence the yield of overall kinetics cannot follow the first-order reaction kinetics observed in photoinhibition of PSII (e.g. Sarvikas et al. 2010). As deviation from such reaction kinetics has not been recorded, the current understanding points towards the inactivation of the PSII reaction center in the described mechanism to be dependent on the primary reaction that is considered irreversible. Any following reactions are considered to act on an already inhibited PSII. With this assumption, the described mechanism would yield the observed kinetics with both fluorescence (e.g. Havurinne et al. 2022) and oxygen evolution-based measurements (e.g. Hakala et al. 2005).

The second proposed mechanism occurs via $^{1}O_2$. PSII is the key producer of $^{1}O_2$ in the thylakoids (Hideg and Vass 1995, Fufezan 2002, Krieger-Liszkay et al. 2008, Vass and Cser 2009, Cazzaniga et al. 2012, Telfer 2014, Mattila et al. 2022). $^{1}O_2$ is produced when a triplet state Chl molecule donates energy to molecular oxygen. Triplet state Chl in the antenna or RC can be formed either via intersystem crossing or recombination reactions that form ^{3}Chl or $^{3}P_{680}$, depending whether the formation occurs in the antenna Chl or in the RC, respectively. The produced $^{1}O_2$ has been thought then to damage surrounding structures causing inactivation of the RC (Vass 2012, Kale et al. 2017, Nawrocki et al. 2021). The recombination pathway and consequent $^{1}O_2$ production have been shown to be dependent on the rate of misses occurring in the OEC (Mattila et al. 2022) that cause the P_{680}^{+} not to receive electrons from the OEC after charge separation and hence cause rapid recombination of P_{680}^{+}/Q_A^{-}. Indeed, the rates of photoinhibition and singlet oxygen production have been shown to correlate with the light intensity (Rehman et al. 2013) and singlet oxygen has been shown to be the cause behind photoinhibition at very low temperatures (Mattila and Tyystjärvi 2022), supporting the previous claims of its role in photoinhibition (Treves et al. 2016). However, the amount of protection that heat dissipation provides (Sarvikas et al. 2006) suggests that the damage by $^{1}O_2$ is not alone sufficient explanation for the damaging reaction of photoinhibition. Furthermore, photoinhibition occurs also in anaerobic conditions where no singlet oxygen can be assumed to be produced (Hideg et al. 1994, Hakala et al. 2005, Mattila and Tyystjärvi 2022).

Several studies have suggested different mechanisms to function or dominate in different conditions, which leads to hybrid-type mechanisms (Zavafer 2021, Nawrocki et al. 2021, Mattila et al. 2022), but the overall story is still blurry. Regardless of mechanism, the damage is always associated with both inactivation of the PSII RC and to damage that requires degradation and resynthesis of the D1-protein. There is also evidence of actual protein damage (Kale et al. 2017). This will be described in the Chapter below.

1.3.2 Repair – resources-consuming but necessary cycle

The damage caused by photoinhibition requires replacement of the D1-subunit of the damaged PSII-complex. This D1-replacement is crucial as the PSII-complex is incapable of performing any electron transfer without having gone through the repair cycle taking place throughout the chloroplasts (see e.g. Järvi et al. 2015, Theis and Schroda 2016). However, in the chloroplasts of *Chlamydomonas*, the newly synthesized PSIIs are mainly assembled near the pyrenoid organelle (Uniacke and Zerges 2007). The repair cycle consists of three key steps: First, the PSII-complex is partially disassembled, which exposes the damaged D1-protein to both FtsH and Deg-proteases (Kato et al. 2012). These two proteases are both required for the repair cycle, but they have different energetic requirements (Kato and Sakamoto 2009, Kley et al. 2011). They also require specific phosphorylation of target proteins to function efficiently (Tikkanen et al. 2008). The second step is the *de novo* synthesis and insertion of the chloroplast encoded D1-protein to the PSII complex. Once the new D1-protein has been inserted to the complex, the last step is to reassemble the PSII complex and move it back to the grana thylakoids, where it resumes its function.

Overall, the PSII repair cycle is a complex process and demands resources (Murata and Nishiyama 2018), but the tradeoff for minor protection of PSI at the cost of PSII photodamage (Huang et al. 2016) has been suggested to be favorable over damage to PSI as PSI does not have efficient repair mechanisms (Lima-Melo et al. 2018). However, the PSI protection via this mechanism is limited and e.g. in *Chlorella ohadii* PSI structure changes in response to high light (Caspy et al. 2021) in the absence of the suggested protective role of PSII photodamage. PSII repair is significantly hindered via various environmental stresses (Allakhverdiev and Murata 2004). Specifically, different ROS inhibit protein synthesis and hence also PSII repair cycle (Nishiyama et al. 2001, Kato et al. 2018), suggesting that ROS quenchers, such as zeaxanthin, protect photosynthetic machinery not only from the damaging reaction, but also by enhancing the effectiveness of the repair reactions. The two opposing reactions of photoinhibition, damage and repair, can be measured individually by measuring the rates of oxygen evolved in conditions at which the

rate-limiting step is the PSII photochemistry and by using the antibiotic lincomycin that blocks chloroplastic protein translation and consequently PSII repair.

1.4 Source of carbon matters

Photosynthesis and metabolic fluxes in *Chlamydomonas* are adjusted in response to the source of carbon. Mostly this means a switch or balancing of resource fluxes in response to the amounts of carbon being acquired either from inorganic (CO_2) or organic (acetate) sources. With the former being the sole source, the cells are fully autotrophic. In the latter case, cultures can be mixotrophic or heterotrophic, depending on the presence of photosynthetically active radiation, as CO_2 can be assumed to be available at all times in ambient air. To enhance the availability of CO_2, *Chlamydomonas* has effective carbon-concentrating mechanisms that act to increase the intracellular CO_2-concentations (see Mackinder 2018). The maintenance of the CO_2-concentration in the pyrenoid organelle is crucial, as majority of the Rubisco is also localized there and sufficient concentration of inorganic carbon enables its carboxylase activity (Wang et al. 2015).

When in the presence of acetate (also some other two-carbon molecules can be used), the cultures of *Chlamydomonas* proceed to grow in the dark without an input from the photosynthetic light reactions (Thompson et al. 1985). *Chlamydomonas* cells metabolize acetate to acetyl-coenzyme A to be utilized in the mitochondrial respiration and consequent production of NADH and ATP. These energy-containing molecules can be transported between organelles to support chloroplastic metabolism via transport through malate-shuttles (Scheibe 2004) and yet unknown transporters that transport NADH and ATP, respectively. This is also probably why *Chlamydomonas* cells manage to maintain their photosynthetic machineries during dark periods lasting for several days (Hemschemeier 2013). Acetate-originating carbon can also be stored as lipids or starch, just like CO_2 (for review on carbon metabolism see Johnson and Alric 2013 or Burlacot et al. 2019).

Heifetz et al. (1997, 2000) studied the effects of acetate in the medium on photosynthetic efficiency and showed it to have a concentration-dependent effect on *Chlamydomonas* cells when they switched to mixotrophic metabolism. This effect is seen as rapidly decreasing O_2-evolution and inorganic carbon assimilation rates as the amounts of organic carbon in the medium increases. Their data also show how heterotrophic metabolism substituted over 50 % of the photoautotrophic biomass accumulation under optimal illumination and CO_2-concentrations when the cells were supplied with acetate. These results indicate a preference for the acetate to be used as a carbon source over CO_2 under ambient air and limited illumination. In general, heterotrophy may be more energy-efficient type of metabolism than autotrophy. This may hold true especially in aquatic environment where the key

substrates for heterotrophy (chemical substrates such as sucrose or acetate) and autotrophy (light and CO_2) have very different spatial dispersion. Heterotrophy is in this case more suitable option as light distribution in large volumetric space can prove to be very uneven. In the case of *Chlamydomonas* and acetate, the preference for heterotrophy probably arises also from acetate being a two-carbon molecule with less carbon losses than photosynthesis-originating three-carbon glyceraldehyde 3-phosphate when incorporated e.g. in fatty acid biosynthesis.

One of the most attention-receiving research in food technologies recently was published by Hann et al. (2022). In their paper, they hypothesized that they could improve photon-to-feed conversion efficiency by growing various organisms (different fungi, algae and plants) in the dark with acetate generated via solar-powered photovoltaic system they claimed to function at higher efficiencies than biological photosynthesis. They did indeed manage to grow *Chlamydomonas* with their photosynthesis-bypassing system as well as with standard Tris-acetate-phosphate (TAP) medium. Arguably, the presence of light is indeed redundant, since *Chlamydomonas* can grow in the dark if the cells are fed enough organic carbon and have oxygen to respire. However, light is a major driving force for cell division and biomass accumulation even in mixotrophy: Heifetz et al. (2000) showed that when cells were given acetate in concentration exceeding the amounts of standard TAP medium, photosynthesis still contributed 23 % of total carbon accumulated in the biomass. In addition, the usage of inorganic carbon further increased to 54 % of total carbon biomass when supplied with excess CO_2. Furthermore, photosynthesis has more functions than just to fix carbon. Light gives the cells telltale signs about their environment and help in developmental processes (Szechynska-Hebda et al. 2017) i.e. guide algal cells in their surroundings through phototaxis.

In conclusion, the relationship between autotrophic and heterotrophic metabolism is very dynamic in *Chlamydomonas*. Hence, if we consider *Chlamydomonas* as a candidate for future biotechnological applications, we need to see it from the aspect of whole metabolism in addition to the engineering of specific production pathways.

2 Aims of the Study

The present study was set to examine photoinhibition of photosystem II and tolerance against light stress in *Chlamydomonas*. In addition, it was meant to examine how light intensity together with redox poise in the thylakoid membranes guides and regulates the phosphorylation and concurrent movement of the light-harvesting antennae, a well-known phenomenon in plants but still rather elusive in algal systems. More specifically, the aims were:

1. Examine the characteristics of a *Chlamydomonas* mutant line lacking a gene responsible for UV-B-signaling in regard of tolerance against high-light induced photoinhibition of photosystem II.
2. Attempt to map what changes need to occur in the photosystem II of the commonly used control strain of *Chlamydomonas* for it to be able to grow under strong light.
3. Explore how the plastoquinone pool in the thylakoid membranes of *Chlamydomonas* behaves under various visible wavelengths and in aerobic and anaerobic dark-incubation.
4. Explore the mechanisms regulating the state transitions and consequent light states of the photosynthetic machinery in *Chlamydomonas*.

3 Materials and Methods

3.1 Strains and growth conditions

All studies were conducted with the commonly used cell wall-containing laboratory wild type of *Chlamydomonas reinhardtii*, *CC124*. This strain is characterized to be incapable of using nitrate as its primary nitrogen source (Pröschold et al. 2005) and to have negative phototaxis (Smyth and Ebersold 1985). Two mutants were also used. *Hit2*, generated via selection by exposing the control strain *CC124* to subsequent UV and high light stress (Schierenbeck et al. 2015) was used to examine the relationship between high-light tolerance and biomass productivity (Paper I). The second mutant was s*tt7-9* (Cardol et al. 2009) lacking the STT7-kinase. Even though this mutant strain was originally generated from an arginine-deficient line with no cell wall (Fleischmann et al. 1999), *CC124* was considered to be a suitable control for this slightly leaky (Bergner et al. 2015) mutant. The strains were maintained on TAP-plates (Gorman and Levine 1965). Prior to any experiments however, cells were always cultured photoautotrophically in high salt mineral medium (HSM) (Sueoka 1960) under continuous light with PPFD 100 µmol of photons m^{-2} s^{-1}, and supplied with ambient air with 1 % CO_2. All pigment quantifications were performed according to Wellburn (1994) by using overnight methanol extraction.

3.1.1 Biomass production

Volumetric productivities of both *CC124* and *hit2* were measured with a flat panel photobioreactor (Photobioreactor FMT-150, PS Instruments, Brno, Czech Republic), which for this purpose was run as a turbidostat; fresh medium was pumped in as old medium was pumped out as the cell density had reached a set threshold. This way the cell density inside the chamber was maintained in the exponential growth phase throughout the experiment, determined via OD_{735}. The light intensity was increased in a stepwise manner as: 200, 500, 750, 1000, 1250 and 1500 µmol m^{-2} s^{-1}, each step lasting for 5-6 days. Cultures were bubbled with air supplied with 3 % CO_2 and temperature was maintained at 20 °C with an external water bath. Productivity was calculated from the mass of the culture pumped out, as

the relationship of between the OD_{735} and the average dry biomass concentration of the culture in the photobioreactor was known.

3.1.2 Extreme light experiments

Survival of two types of cultures was examined in extreme light conditions (PPFD 3000 µmol m^{-2} s^{-1}, RT and ambient air) (hereafter EL-conditions). The first type of cultures originated from single, individual cells of the control strain *CC124*. Diluted inocula of *CC124* were spread on TAP-plates and upon identification of individual colonies on plates, the colonies were transferred to liquid HSM and placed in growth conditions described above. The second type of cultures were inocula from isolated subpopulations of *CC124* that were initiated from a very dilute cell concentration in the growth conditions. This way the difference between the tested cultures was the amount of genetic variance in the initiated cultures. The cultures were defined to be EL-tolerating if they grew in HSM from the initial OD_{730} of 0.01 to 0.05 by 96 h from the introduction to EL-conditions.

3.2 Measurements of oxygen evolution and photoinhibition of photosystem II

PSII activity was estimated as the rate of light-saturated oxygen evolution by using a Clark-type oxygen electrode (Hansatech Instruments Ltd., Norfolk, United Kingdom) from samples with known chlorophyll concentrations (Papers I and II) or cell densities (Paper II). Measurements with isolated thylakoids were always done on chlorophyll basis. 1 ml samples were supplemented with DCBQ and ferricyanide, or with DMBQ, all in 0.5 mM concentration. Where applicable, DBMIB was used in 0.5 µM concentration. Rates of photoinhibitory reactions, both damaging and recovering, were estimated via changes in the light-saturated oxygen evolution during 40-minute high-light treatments (PPFD 950 µmol m^{-2} s^{-1}, Xenon lamp (201-1 k, 1000 W, Science Tech Inc., London, ON, Canada)) with and without 0.5 mg ml^{-1} lincomycin. To obtain the rate constant of damaging reaction, the values obtained with lincomycin-treated samples were fitted to values obtained from a first-order reaction kinetics equation

$$\frac{dA(t)}{dt} = -k_{PI} \times A(t), \quad (1)$$

where k_{PI} is the rate constant of photoinhibion, *t* time and *A(t)* the oxygen evolution rate at time *t*. The rate of recovering reactions was calculated by estimating a best fit for the solution for equation

$$\frac{dA(t)}{dt} = -k_{PI} \times A(t) + k_{REC} \times (A(0) - A(t)), \quad (2)$$

where k_{REC} is the rate constant of the recovery reaction. The k_{PI}-value was kept fixed after fitting to equation (1).

3.3 Chlorophyll fluorescence and P700$^+$ signal

Fluorescence decay and fluorescence induction curves in Paper II were measured from 2 ml samples with 5 and 1 µg Chl ml^{-1}, respectively. The former was measured with an FL 200 fluorometer equipped with a Superhead high-sensitivity detector (Photon Systems Instruments, Drásov, Czech Republic). The fluorescence signal was measured for 120 s after giving the sample a 30 µs single-turnover flash (PPFD 10^5 m^{-2} s^{-1}, according to the manufacturer). The fluorescence induction, in turn, was measured with a standard handheld PAM-fluorometer, AquaPen (AquaPen AP100, Photon Systems Instruments, Drásov, Czech Republic). In both protocols, the samples were dark-incubated for 15 min prior to the measurements and DCMU was used in 10 µM concentration when applied.

Simultaneous measurements of Chl *a* fluorescence and P700$^+$ absorbance in Paper III were done with a Dual-PAM-100 (Heinz Walz GmbH, Effeltrich, Germany) from 1.5 ml samples with 40 µg Chl ml^{-1}. After incubating the samples in the dark for 1 h and measuring the values for F_V/F_M and P_M, illumination with monochromatic LEDs (See 3.6.1.) was turned on for 5 minutes, during which a saturating pulse (PPFD 4000 µmol m^{-2} s^{-1}) was given every 30 s to yield an estimate of the rETR in both PSII and PSI.

3.4 Thermoluminescence

Thermoluminescence was recorded *in vivo* from samples with known chlorophyll concentrations with custom equipment (Tyystjärvi et al. 2009). In Paper I, the samples were collected from cells grown under moderate light and during a similar growth phase as in the turbidostatic photobioreactor to probe the possible differences between the strains without applying the stress caused by high light. In comparison, the samples in Paper II were collected from cells grown either under moderate or the extremely strong light intensity used in experimental setup to specifically probe the changes caused by the high-light stress. For the measurement of thermoluminescence, cells were applied on a filter, on which they were shortly incubated in the dark. For Q-band measurements, DCMU was added in 20 µM concentration prior to the application on a filter. After the dark-incubation, the samples were cooled down to either -10°C (B-bands) or -20°C (Q-bands). The frozen samples were then given a single-turnover flash and warmed to 60°C at a heating rate of 0.66 °C s^{-1}, during which light emission was recorded.

3.5 Singlet oxygen production

Singlet oxygen production was examined by using SOSG (Invitrogen by ThermoFischer Scientific). This reagent was used at the final concentration of 50 µM in samples with 50 µg Chl ml^{-1}. Singlet oxygen production was induced via illumination at >650 nm red light with PPFD 2000 µmol m^{-2} s^{-1}. SOSG fluorescence (λ_{ex}: 500 nm) was recorded every 10 min with a QEPro spectrometer (Ocean Insight, Ostfildern, Germany) and the rate of increase in emission between 535 and 540 nm was interpreted as the relative rate of $^1O_2$ production.

3.6 Low-temperature fluorescence emission

Low-temperature fluorescence emission was recorded with a QEPro Spectrometer (Ocean Insight, Ostfildern, Germany) in liquid nitrogen temperature from samples with 1.5 – 6 µg Chl ml^{-1} chlorophyll concentrations, as indicated. The Chl concentrations were maintained as low as possible to prevent the artifact of self-absorption, which was examined to be negligible with these chlorophyll concentrations. The fluorescence emission was probed by exciting the samples with 442 nm (Paper I), 440 nm (Paper II) or 470 nm (Paper III) blue light. In Paper III, sodium fluorescein with an emission peak at 525 nm was added to the samples to act as an external control, to which all spectra were first normalized. The final spectra were normalized to either the signal at 686 nm, originating from PSII core, or at 714 nm, originating from PSI core.

3.7 Plastoquinone measurements

3.7.1 HPLC measurements with green algal material

PQH_2 can be easily detected and quantified with HPLC, as already described by Kruk and Karpinski (2006). This method is based on the attribute of PQH_2 to absorb light at 290 nm, which causes it to emit fluorescence at 330 nm. However, PQ can also be detected due its ability to absorb light at 255 nm. The original method was later modified to be used with cyanobacterial material by Khorobrykh et al. (2020a). Here we applied this modified method to *Chlamydomonas*. The protocol consisted of three phases: First, the material was filtered on a glass microfiber filter (VWR, USA, Cat. No. VWRI516-0862), on which any treatments were performed. Second, PQ in the sample on the filter was extracted rapidly via grinding the cells and the filter in dry, ice-cold ethyl acetate, which was then evaporated under a nitrogen flow. Third, the samples were resuspended in methanol and divided into two halves that were then subsequently run through HPLC measuring fluorescence (λ_{ex}: 290 nm, λ_{em}: 330 nm)

and absorption (255 nm). The PQ in the latter half of the sample was chemically fully reduced to PQH_2 with 5 µM $NaBH_4$. The peak at 9 – 10 min in the fluorescence signal, directly corresponding to the amount of PQH_2 in the samples, was then used to estimate the fraction of PQ reduced via given treatments (F_{Sample}) as:

$$F_{Sample} = \frac{A_{Treated}}{A_{NaBH_4}}, \tag{3}$$

where the notations *Treated* and *$NaBH_4$* refer to the two aliquots of the extracted sample described and A to the area of fluorescence peak at 9-10 min. PQ gave signal after 27-31 min, and it was checked to ensure total reduction of PQ by $NaBH_4$. The relative size of the photochemically active PQ-pool was estimated with light treatments with either far red (10 min, PFD 50 µmol m^{-2} s^{-1}) or high light (30 s, PPFD 2000 µmol m^{-2} s^{-1}) (Fig. 5a). These threshold values were then used as limits for determining the redox state of the PQ-pool after various treatments.

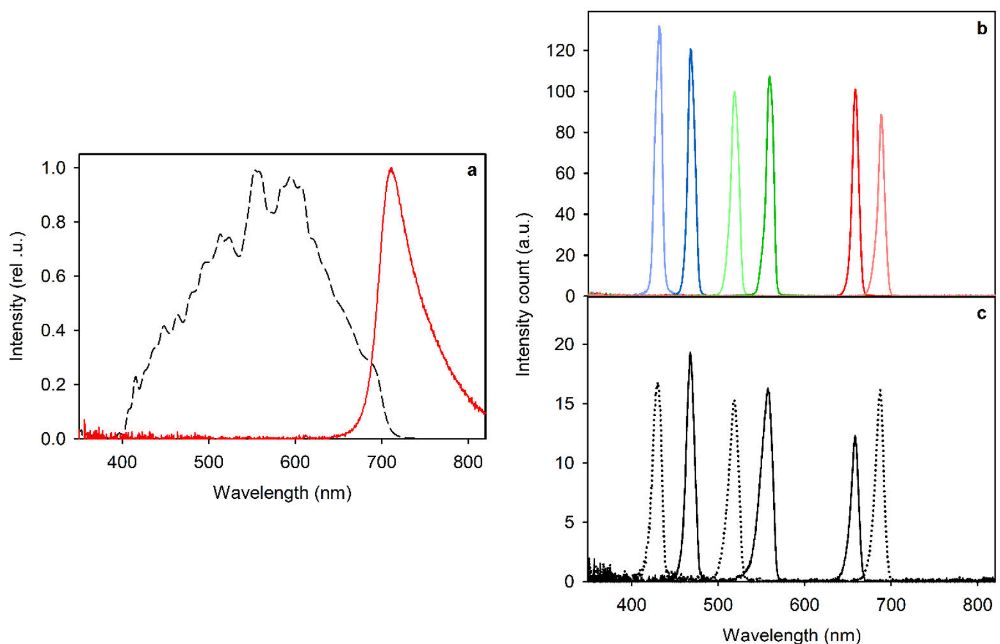

Figure 5. The spectra of the various light sources used in the treatments to manipulate the redox state of the PQ-pool in *Chlamydomonas*. a. The far-red (solid, red line) and high light (dashed, black line) used in measurements to estimate the threshold levels of the PQ-pool. b. Spectra of monochromatic light sources favoring PSII (solid line) or PSI (dashed line) each as their respective color, and the spectra of these wavelengths combined to generate the two polychromatic light types (c, PSII-WL solid line, PSI-WL dashed line). All spectra were measured with an STS-VIS spectrometer. The values in a are normalized to the maximal value to better visualize the continuous spectra. Values in b and c are raw arbitrary values of the spectrometer corresponding to PFD of 50 (b) or 30 (c) µmol m^{-2} s^{-1}.

3.7.2 Light treatments with LEDs

A custom-built LED illumination system was used to provide monochromatic light (Fig. 5b) for the light treatments. These wavelengths were previously determined to favor PSII (470, 560 and 660 nm) or PSI (430, 520 and 690 nm) (Mattila er al. 2020), and were combined together to generate the two white light types thought to favor the excitation of one of the photosystems (PSII-WL, PSI-WL) (Fig. 5c). These light types were used for 5 min to induce changes in the PQ-pool redox state, after which the PQ was extracted and run through HPLC in two aliquots as described above. The redox state was also examined under the growth light at different intensities by transferring the cells back in the growth chamber for 5 min after filtering, after which the PQ was extracted as described. The redox state of the PQ-pool was then estimated as

$$F_{Sample} = 100\ \% \times \frac{(F_{Sample} - F_{Oxidized})}{(F_{Reduced} - F_{Oxidized})}, \quad (3)$$

where *F* is the percentage of PQ-pool that was reduced by the applied treatment, and notations *Sample*, *Oxidized* and *Reduced* refer to the total PQ reduction level of the sample in question, and PQ reduction values in samples with fully oxidized (FR-treated) and reduced (high light-treated) PQ-pools, respectively.

3.7.3 Incubations in darkness

For estimations for the PQ-pool redox state after dark incubations in the presence of oxygen, 10 ml cell suspensions were placed on a horizontal shaker and bubbled with air for 2 h prior to the PQ-extraction. For the anaerobic incubations, special equipment was built. Cell suspensions were placed inside an air-tight chamber, which was then sealed and flushed with nitrogen. After the oxygen concentration inside the chamber had decreased to sufficient degree, the gas line inside the chamber was submerged in the cell suspension for 2 h. The PQ extraction (filtering and grinding in ethyl acetate) was then performed inside the chamber. Oxygen concentrations were, in both cases, observed with a FireSting Fibreoptic Oxygen Meter (PyroScience GmbH, Aachen, Germany) directly in the suspension for aerobic treatments and from the gas phase of the sealed chamber. DBMIB was used at 5 µM concentration.

3.8 Western blotting

Proteins were extracted after breaking the cells with three subsequent freeze-thaw cycles in an extraction buffer, as described in Paper II, either directly from growth conditions, or after specific light treatments. In paper III, phosphatase inhibitor

(PhosSTOP, Roche Diagnostics, Mannheim, Germany, Cat. No. 04 906 845 001) was added to the isolation buffer to prevent changes in the phosphorylation pattern of LHCII proteins. Isolated protein samples were then loaded to SDS-PAGE on protein basis, depending on the optimization of the antibody in question. Antibody binding was detected via alkaline phosphatase. Equal loading of proteins was examined by Coomassie-staining the developed membranes.

4 Overview of the results

4.1 Connection between photoinhibition of photosystem II and biomass production

4.1.1 *Hit2* had increased resistance towards the damaging reaction of photoinhibition of photosystem II

Photoinhibition affects photosynthetic organisms regardless of light intensity (Tyystjärvi and Aro 1996). The damage and repair of photoinhibition of PSII occur in tandem and their rates determine an equilibrium, which can vary from complete inhibition of PSII activity to retainment of full PSII turnover rate during high light exposure. Paper I demonstrated *hit2*, mutant strain that has a mutation in the UV-B-sensing gene *Cr-COP1*, to have significantly slower damaging reaction of photoinhibition than its respective control strain, *CC124*. The rate constant for the damaging reaction in *hit2* was less than 50 % of that of *CC124*. After 40 min of high-light treatment in the presence of lincomycin, *hit2* was still producing oxygen at a rate corresponding to 29 % of the oxygen production rate of the control value at t = 0 min. In comparison, at this point samples of *CC124* no longer produced oxygen (Paper I, Fig. 2a). However, the rate of the repair-reaction successfully compensated for the faster damaging reaction in *CC124*, as the oxygen evolution rates at 40 min of high-light treatment without lincomycin equilibrated to similar levels in both strains: 52 % of control in *CC124* and 50 % of control in *hit2* (Paper I, Fig. 2b).

The rate of photoinhibition was measured also *in vitro* from isolated thylakoids. It was shown that when isolated thylakoids were subjected to the similar 40-minute high-light treatment, the high-light tolerant phenotype of *hit2* was less pronounced. This was observed as *hit2*-thylakoids producing oxygen only marginally more than *CC124*-thylakoids throughout the high-light treatment (Paper I, Fig. 3).

As there was no clear indication why a mutation in gene responsible for UV-B signaling would provide protection from high light, Paper I also explored possible reasons behind the photoinhibition tolerant phenotype of *hit2*. Two main discoveries were made. First, the pigment composition of *hit2* showed both lower ratio of Chls *a* and *b* (Paper I, Fig. 5), but also smaller Car-to-Chl ratio than *CC124* (Paper I, Fig. 4c). There was also a similar decrease in PSI-to-PSII fluorescence emission ratio

between the strains in response to increasing light intensity (Paper I, Fig. 6). However, as the simultaneous changes in pigments were more or less similar between the strains, the observed decrease in PSI-to-PSII fluorescence emission ratios were most likely due to identical decrease in the number of photosystems, rather than any modification of the antennae. Second, the examination of the redox potentials of the charge pairs inside PSII via thermoluminescence showed that the while the potential of the Q_A/Q_A^- -pair (Q-band) appeared at the same temperature between the strains, the Q_B/Q_B^--pair (B-band) appeared at a 3.3 °C lower temperature in *hit2* cells than in the *CC124* (Paper I, Fig. 7). As in these experiments thermoluminescence emission is a result of charge recombination of either Q_A^- or Q_B^- backwards to the OEC in S_2-state, the results suggested a shift in the redox potential of the Q_B/Q_B^--pair of *hit2* PSIIs towards more negative from that of Q_B/Q_B^- -pair of *CC124*.

4.1.2 Slower photodamage to photosystem II increased biomass productivity in a turbidostatic photobioreactor

When studying the biomass production rates, the benefits of turbidostatic system are undeniable when compared to a batch system, as the cultures can be maintained in exponential growth phase for prolonged periods of time and nutrients in form of fresh medium can be injected to the system to replace the pumped-out culture. Interestingly, the most optimal PPFD to photoautotrophically accumulate *Chlamydomonas* biomass was found to be relatively high, 750-1000 µmol m^{-2} s^{-1} (Paper I, Fig. 1), even though *Chlamydomonas* is commonly cultivated in laboratories at PPFD matching to 10 % or less of this intensity (Roach and Na 2017, Xing et al. 2017, Lin et al. 2018, Yang et al. 2018, Pan et al. 2021, Naschberger et al. 2022, Paper III).

As stated, PSII is susceptible to be inactivated in light-dependent manner throughout all light intensities and the rate of damage is directly proportional to the intensity of incident light (Tyystjärvi and Aro 1996). Since repairing the damaged PSII-complexes by synthesizing new D1-proteins consumes resources (Aro et al. 1993, Murata and Nishiyama 2018), the repair can be considered as a hindrance to growth as these resources could be diverted to more beneficial metabolism. Indeed, when the strain with slower damaging reaction of photoinhibition of PSII, *hit2*, and its respective control strain, *CC124*, were grown under increasing intensities of light, *hit2* produced biomass at a higher rate than *CC124* throughout the experiments. This gap kept increasing as the light intensity was increased, but the only statistically significant difference was recorded at 1250 µmol m^{-2} s^{-1} (Paper I, Fig. 1), where *hit2* had 19.1 % higher volumetric productivity than *CC124*.

In conclusion, the data suggest that under high light *hit2* was able to direct resources to growth that would had been otherwise been spent on the synthesis of D1-protein and PSII re-assembly, which was seen as faster biomass production rates than with *CC124*.

4.2 Growth of photoautotrophic cells at extreme light intensities

4.2.1 Survival in high light was enabled by reversible acclimation in the laboratory strain *CC124*

It is long known that high light intensities are detrimental for plants. Hence, they have developed several ways of either protecting themselves or completely avoiding the damaging effects of high light. Against previous knowledge (Förster et al. 1999, Schierenbeck et al. 2015), an unexpected observation was made during previous studies that the commonly used laboratory strain of *Chlamydomonas*, *CC124*, was suddenly able to grow under EL-conditions (RT, ambient air, PPFD 3000 µmol m^{-2} s^{-1} and in photoautotrophic medium). In paper II, this observation was further studied and confirmed to be a frequently occurring phenomenon.

The growth of the cultures was consistent, as after 96 h under EL-conditions, 80 % of isolated subpopulations and 56.7 % of the single-cell-originating cultures managed to grow past the set threshold cell density of OD_{730}: 0.05 (Paper II, Table 1). These surviving cultures from isolated subpopulations had an average OD_{730} of 0.18 ± 0.02, whereas the single-cell-originating cultures had grown slower, to an average of 0.09 ± 0.02 (Paper II, Figure 1a). In addition, if left in EL-conditions for a longer period of time, practically all cultures from both types of inocula would have had eventually grown. However, the ability to exceed the set threshold density in 96 h was not permanent and some cultures determined to be EL-tolerating could not perform in a similar manner after 1 week in standard growth conditions after the initial exposure to EL-conditions (Paper II, Fig. 1b).

4.2.2 Acclimation to extreme light conditions was accompanied by a wide range of modifications on photosystem II

Several acclimatory changes were observed to occur in PSII during the acclimation to withstand the extreme light intensity of 3000 µmol m^{-2} s^{-1}. Importantly, these changes were similar in both types of inocula used to generate EL-tolerating cultures (hereafter EL-cells). During the experiments, four key changes were observed in the EL-tolerating PSIIs.

First, it was observed that cells grown in control conditions (hereafter control cells) would not produce oxygen in light-saturated conditions (H_2O to CO_2) at similar rates as the EL-cells. In these experiments, when normalized to the average chlorophyll concentration in the two types of cultures (11.08 ± 0.40 and 3.89 ± 0.33 μg Chl ml^{-1} in control and EL-acclimated cells, respectively), the control cells produced oxygen at a significantly slower rate than the EL-cells (Paper II, Fig. 4a). At first, this appeared to be due to the decreased number of PSII (and PSI) units an EL-acclimated cell (Bonente et al. 2012, Paper II, Fig. 3), but when the oxygen evolution rate was normalized to the average cell density in the samples instead of chlorophyll, the EL-acclimated cells performed poorly in comparison to the control cells (Paper II, Fig. 4b).

The EL-cells were very inefficient at producing oxygen with any kind of artificial electron acceptor used. The best rate in EL-cells was obtained with electron transfer from H_2O to DMBQ, but it was still significantly slower than in measurement without artificial electron acceptors. Addition of DBMIB, an inhibitor of PQH_2-oxidation at cyt b_6f, to the measurements with DCBQ improved the rate of oxygen evolution in control cells, but no difference was observed in EL-cells. When measured from isolated thylakoids, the thylakoids from EL-cells produced oxygen at approximately half of the rate of the thylakoids of the control cells, and the values were similar with both DCBQ and DMBQ (Paper II, Fig. 4c).

Second, measurements of decay of Chl *a* fluorescence yield revealed that the first, most rapid phase of decay after single turnover flash was faster in the control cells than in EL-cells (Paper II, Fig. 5a). Furthermore, the rate of fluorescence decay in the control cells was three times that of the EL-cells when supplemented with DCMU that blocks the electron transfer $Q_A \rightarrow Q_B$ (Paper II, Fig. 5b). Chl *a* induction, in turn, revealed that the F_0 level of EL-cells is at all times higher than in the control cells (Paper II, Fig. 5c) and subsequently the F_V/F_M of EL-cells is less than half of that of control cells. Interestingly, in EL-cells there was a consistent decrease in F after F_M, which was less pronounced in the control cells. However, the time that the F_M-level was reached remained similar between control and EL-cells both with and without DCMU (Paper II, Fig. 5c and d).

Third, as in the case of *hit2*, small changes in the peak temperatures of the thermoluminescence curves were observed between control and EL-cells, indicating changes in the redox potentials of Q_A/Q_A^- and Q_B/Q_B^-. The Q-band peaked at 13.6 °C in EL-cells and at 15.5 °C in the control cells (Paper II, Fig. 6). The temperatures for the B-band were 21.6 °C in EL-cells and 20.0 °C in control cells. Hence, the temperature difference between the Q and B bands was 3.5 °C wider in the EL-cells than in the control cells.

Fourth, as the results so far suggested that the recombination reactions inside PSII were affected by the EL-acclimation, we wanted to see their consequent effect.

Since the production rate of the highly harmful $^1O_2$ depends on the formation of $^3P_{680}$ via PSII recombination reactions (Krieger-Liszkay et al. 2008), we decided to measure the rate of $^1O_2$ production with SOSG. During 30 min illuminations with red light, EL-cells produced 90 % less $^1O_2$ than the control cells (Paper II, Fig. 7). However, even with the low $^1O_2$-production rate the EL-cells showed signs of being severely photoinhibited (Paper II, Fig. 8).

4.3 Plastoquinone pool redox state in *Chlamydomonas*

4.3.1 Photoactive plastoquinone-pool in *Chlamydomonas*

The interphotosystem electron carrier molecule, PQ, is in plants mainly reduced by PSII-activity and oxidized by PSI-activity. The ratio between the activities of the two photosystems determines the redox state of the pool of PQ-molecules in the thylakoids under moderate light intensity (Mattila et al. 2020). The relative size of the photoactive PQ-pool was estimated similarly as previously with cyanobacteria (Khorobrykh et al. 2020a). After illumination with far-red light, which was considered to fully oxidize the PQ-pool, 17.6 ± 3.9 % of the total PQ in the cells was reduced. On the other hand, after a short high-light treatment to fully reduce the PQ-pool, 47.5. ± 5.2 % of the total PQ was reduced (Paper III, Fig. 1a). After confirming these threshold values of fully oxidized and reduced PQ-pool by repeating the similar light treatments with additions of DCMU (far-red) and DBMIB (high light) to control electron flow through PSII and PSI, respectively, 29.9 ± 9.2 % of the total *Chlamydomonas* PQ was estimated to belong in the photoactive PQ-pool.

4.3.2 Light quality-dependent modulation of the *Chlamydomonas* plastoquinone pool redox state

Once the threshold values for maximal oxidation and reduction of the PQ-pool by light treatments have been established, the PQ-pool redox state under various light conditions is available for measuring. We wanted to know how the PQ-pool is reduced under our growth light and saw that under PPFD 50 µmol m^{-2} s^{-1} the PQ-pool was more or less completely reduced, seen as 100.9 ± 14.4 % reduction of PQ-pool (Paper III, Fig. 1b). When PPFD was increased to 100 µmol m^{-2} s^{-1}, the apparent reduction of the PQ-pool increased to 165.5 ± 12.8 %, regardless of the cells having also been grown under this light.

By using our custom-built LED equipment, samples of *Chlamydomonas* were treated with low-intensity light using the wavelengths described to see whether the action spectrum of the PQ-pool redox state appeared similar to the one observed

earlier in *A. thaliana* (Mattila et al. 2020). First, the efficiency of the two described white monochromatic wavelength combinations (PSII-WL and PSI-WL) in PQ-pool reduction was examined. The PSI-WL reduced the PQ-pool close to the value obtained with high-light treatment, to 85.5 ± 6.1 % (Paper III, Fig. 1b). In contrast, the PSII-WL reduced the PQ-pool well above the apparent maximal redox state, to 165.5 ± 1.7 %. According to these results, these white wavelength combinations caused PQ-pool redox state to vary only between highly reduced and extremely reduced.

When the monochromatic components of the PSII-WL and PSI-WL were used, the lowest PQ-pool reduction was achieved with 520 nm green light that was still able to reduce 50.5 ± 6.7 % of the photoactive PQ (Paper III, Fig. 1c). The two other PSI-wavelengths (430 and 690 nm) caused higher reduction, 81.1 ± 13.4 % and 89.7 ± 8.3 %, respectively, but not as high as the high-light treatment. In comparison, the PSII-wavelengths, 470, 560 and 660 nm, exceeded the 100 % threshold level and caused the apparent PQ-pool to become 128.3 ± 10.8 %, 117.0 ± 13.7 % and 129.8 ± 13.4 %, respectively. Overall there was much less variation in the PQ-pool redox state throughout the action spectrum than in *A. thaliana* (Mattila et al. 2020).

To explain the highly reduced PQ-pool, rETR values were measured during illumination with the monochromatic light wavelengths used in PQ-measurements. Unsurprisingly, the blue wavelengths were shown to be the most and green wavelengths the least efficient at inducing electron transfer. More interestingly, the ratio of rETRs through photosystems suggested the electron flow to be faster through PSII than PSI (Paper III, Fig. 2), which was considered to explain the high reduction of PQ-pool observed after treatments with these light wavelengths. However, due to the fickle nature of algal rETR-measurements (Havurinne et al. 2019), their dependency on the wavelength-dependent absorption coefficients of PSII and PSI, and the recent reports about the nature of measurements involving variable fluorescence (Sipka et al. 2021), these values were and should be considered as descriptive.

4.3.3 Plastoquinone pool reduction by NDA2 and oxidation by PTOX in the dark, and consequent state transitions

Algal STT7-dependent LHCII-movement has been studied for a long time by dark-incubating the samples either in the presence (Finazzi et al. 2001, 2002, Ünlü et al. 2014, Cariti et al. 2020) or absence (Delphin et al. 1995, Lemeille et al. 2009, Ünlü et al. 2014, Cariti et al. 2020) of oxygen. In these conditions, the PQ-pool is either efficiently oxidized by PTOX (Houille-Vernes et al. 2011) or reduced by NDA2 (Jans et al. 2008), respectively. The changes in the PQ-pool redox state via these two pathways have been suggested to successfully induce STT7-dependent state

transitions, however the redox state has not been previously measured directly. Here it was demonstrated that 2 h in anaerobic darkness reduced 42.4 ± 10.0 % of the total PQ (Paper III, Fig. 3c), closely matching the redox state obtained with high light. In contrast, the redox state after aerobic darkness nicely corresponded to the value after the illumination with far red light, and 14.6 ± 4.0 % of PQ was reduced. These conditions also induced clear STT7-dependent state transitions (Paper III, Fig. 3a) that were absent in the *stt7*-mutant (Paper III, Fig. 3b). Hence, the assumption in the literature regarding PQ-pool reduction and oxidation in the dark via reducing equivalents from fermentation and chlororespiration was confirmed via direct measurements of both light state and PQ-pool redox state.

4.3.4 STT7-dependent state transitions induced with two types of weak white light

Interestingly, upon inspection, the changes in low-temperature fluorescence emission were similar in dark-incubated samples and samples treated with the two types of low-intensity white light. The treatments with PSII-WL and PSI-WL (following subsequently a pretreatment with PSI-WL or PSII-WL, respectively) caused significant transition to either State 2 (PSI-WL) or State 1 (PSII-WL) (Paper III, Fig. 4a and b). 20 min of illumination with PSII-WL caused a 26.5 ± 3.7 % decrease, whereas the PSI-WL induced a 18.9 ± 3.8 % increase in the ratio of fluorescence originating from PSII to fluorescence from PSI (F_{686}/F_{714}). In *stt7*, the former treatment caused 16.4 ± 6.1 % decrease in the F_{686}/F_{714} ratio, in which the PSI-WL caused no observable change (Paper III, Fig. 4c and d).

4.3.5 Algal state transitions were affected more by intensity than quality of light

As the role of state transitions in green algae have been considered to have a photoprotective role, Paper III set out to examine the yet dubious link between the regulation of the algal light state as a function of the PQ-pool redox state. In Figure 5 of Paper III, the F_{686}/F_{714} ratio decreased from 1.51 ± 0.11 to 1.32 ± 0.08 as the light intensity decreased by 50 %. Hence, cells grown under 50 µmol m^{-2} s^{-1} had equilibrated more towards State 2 than their 100 µmol m^{-2} s^{-1}-grown counterparts.

Upon transfer to the monochromatic light wavelengths with PPFD of 50 µmol m^{-2} s^{-1} from the growth conditions, almost universal response was recorded from the algal samples. When the transfer included also a decrease in light intensity, the F_{686}/F_{714} decreased under all wavelengths (Paper III, Fig. 5b). Further illumination increased the transition towards State 2 under all wavelengths (apart from 560 nm).

In contrast, when only the light quality was changed while quantity remained, the monochromatic wavelengths did not induce any significant changes in light state during the first 5 minutes (Paper III, Fig. 5d). However, the further illumination did again cause the transition towards State 2, but this change was, in all cases but with 470 nm light, not statistically significant.

To disclose the possible contribution of the phosphorylation of LHCII, relative amounts of LHCII-P were measured. The samples from different time points showed that the amount of LHCII-P decreased during the first 5 min of illumination after transferring the cells from the growth conditions with PPFD 100 µmol m^{-2} s^{-1} to under monochromatic LEDs with PPFD of 50 µmol m^{-2} s^{-1} (Paper III, Fig. 6). The levels of LHCII-P were re-equilibrated after 20 min of illumination in a similar manner throughout the used light wavelengths to the similar level as in growth conditions.

5 Discussion

5.1 Survival in varying light conditions

5.1.1 Mechanisms to survive in high light

From the literature regarding high-light tolerance and the work done in this thesis, it is impossible to pinpoint a single factor that would be universal in granting a resistance against strong illumination. However, the acclimation generally gravitates towards two common trends: avoiding light absorbance altogether (Durnford et al. 2003, Kouril et al. 2013) or alleviating the harmful effects of already absorbed light energy (Ruban et al. 2012, Ruban 2015, Ruban and Wilson 2020). To complicate the matter even further, these mechanisms can work in tandem, i.e. the damage caused by initial increase in light intensity acts as the signaling tool to induce changes to minimize the future damage in the same conditions. One example of signaling tools is the PQ-pool that affects the antenna movements by activating the STT7-kinase. Another example of regulation dependent on the PQ-pool is the expression of chloroplastic genes (Pfannschmidt et al. 1999, 2009) e.g. via chloroplast sensory kinase (Ibrahim et al. 2020), but also nuclear genes through retrograde signaling mechanisms (Pfannschmidt et al. 2001, 2003).

Even if the cell suspensions in Paper II grew under the used PPFD of 3000 µmol m^{-2} s^{-1}, it is clear that they were not thriving (Paper II, Fig. 1a). The most probable cause for the populations of cells to survive was that upon transferring the diluted cell cultures under the extreme light intensity, there were enough cells so that at least some of them could withstand the extreme light conditions. This feature was not universal, however, as demonstrated by the variation in the growth curves and the reversible nature of the acclimation (Paper II, Fig. 1b). However, the acclimation mechanism itself remains a mystery. The topic is not common because most studies regarding high-light conditions use moderate or high (e.g. Bonente et al. 2012, Kouril et al. 2013), but not such extreme light intensities. However, the observations gathered here support either acclimation via modification of the photosynthetic machinery as described (Paper I), or by epigenetic regulation of certain essential genes or genomic areas (Umen and Goodenough 2001, Paper II). The latter types of acclimation mechanisms have been previously shown be involved in acclimation to

withstand other kinds of environmental stress (Kronholm et al. 2017, Duarte-Aké et al. 2018). It is also possible that the cells can utilize different acclimatory mechanisms between Papers I and II, since the exposure to high light is very different: stepwise increase in Paper I vs. instant blast with extreme light in Paper II.

5.1.2 Photosystem II in the center of acclimation

The photoinhibition-tolerating phenotype of *hit2* is largely explained by a high capacity for NPQ (Schierenbeck et al. 2015). NPQ most probably protects the cells efficiently from photoinhibition, as the resistant phenotype is best seen in the native environment (Paper I, Figs. 2 and 3). However, NPQ is a wasteful process that converts absorbed energy to heat, and as such it cannot be considered beneficial if the outcomes are not more beneficial than just repairing the damage caused by high light that would have had occurred without the NPQ-increasing mutation. *Hit2* showed not to have any advantage or disadvantage in comparison to *CC124* in terms of productivity under moderate light (Paper I, Fig. 1). This is not surprising, as susceptibility to photoinhibition was shown not to necessarily thwart algal growth under moderate light (Mattila et al. 2021). However, under light-saturated conditions the *hit2*-mutant produced more biomass than *CC124*, undoubtedly demonstrating the more effective utilization of resources that were saved over from less active PSII repair cycle.

Interestingly, even if Paper I shows how photoinhibition-resistant organisms can provide tools for isolating potential producers in high light, the ability to grow under high light is not only the luxury feature of these organisms, as shown in Paper II. Interestingly, something occurred to PSII in EL-acclimation that highlighted the characteristics of the PSII-binding electron acceptors, as additions of artificial quinones, especially those that bind to the Q_B-pocket of PSII (Graan and Ort 1986), significantly affected the PSII activity, measured as oxygen evolution (Paper II, Fig. 4). However, even if cells can grow under high light, they may not work optimally under such harsh conditions. Such performance cutoff is here seen as the really slow initial growth of the EL-cultures (Paper II, Fig. 1a) and the highly photoinhibited state of these cells (Paper II, Fig. 8). Moreover, our data demonstrate how the incredibly rapid photodamage may end up overpowering the repair cycle, which in the case of EL-cells leads to the permanent degradation and decrease in PSII units observed (Paper II, Fig. 3).

During acclimation processes that last longer times, more permanent modulation of the whole antenna structure of photosystems provides relief from excitation pressure (Wientjes et al. 2013b, Bielczynski et al. 2016). This modification has been suggested to be the key in unlocking photosynthesis' potential, as algal species and strains with small antenna structures tend to perform better under high light (Kirst et

al. 2012, 2017, Treves et al. 2016, Jeong et al. 2017) and have hence been suggested to have an advantage as production platforms (Kosourov et al. 2011). Smaller antennae have also been proposed to function better in crops due to better within-canopy light distribution (Ort et al. 2015). Long-term antenna modulation is also a probable response in extreme light conditions and could explain the apparent equalization of the low-temperature fluorescence emission between the peaks originating from PSII (686 nm) and PSI (714 nm) during EL-acclimation (Paper II, Fig. 1c). The fluorescence emission most probably decreases at both PSII and PSI wavelengths, as both CP43 and PsaA-content of the cells decrease similarly (CP43 slightly more than PsaA) during the acclimation (Paper II, Fig. 3). Moreover, the photoacclimation of *Chlamydomonas* includes a decrease in PSII antenna already at PPFD 400 µmol m^{-2} s^{-1}, which does not yet induce changes in PSI antenna (Bonente et al. 2012). This may explain the observed stronger decrease of PSII fluorescence emission than that of PSI. This kind of antenna reduction of PSII could be an expected response to the increase in light intensity, a phenomenon observed in Paper I (Paper I, Fig. 6). However, the pigment data suggest that the antenna size of either of the strains was not changing significantly during the stepwise acclimation (Paper I, Fig. 5). Our data demonstrate cooperation of different kinds of survival strategies against high light. These strategies enable the photosynthetic machinery to be highly flexible in finding a balance to enable its optimal function (Sakowska et al. 2018, Bielczynski et al. 2020, Maroudas-Sklare et al. 2022).

5.1.3 High-light tolerance and ROS

Maintaining electron transfer through PSII is crucial for efficient photosynthesis. The differences between the redox pair potentials inside PSII can vary to favor more the forward transfer of electrons, as suggested by the data in Paper II. In these data the redox potentials between Q_A/Q_A^- and Q_B/Q_B^- have changed between the control and EL-cells (Paper II, Fig. 6), and the EL-cells show signs of more efficient forward electron transfer over recombination reaction (Paper II, Fig. 5). This reduced probability of recombination reactions would consequently reduce the production of ROS, major damaging agents in the thylakoids (see Khorobrykh et al. 2020b). Hence, it would naturally be beneficial for the system not to produce such harmful oxidizing agents in the first place. Here, the changes in acclimation of *CC124* during exposure to extreme light intensity indeed resulted the acclimated cells to have lower $^1O_2$-production rate than the control cells: EL-cells produced $^1O_2$ at a rate of only 10 % of the control cells (Paper II, Fig. 7), which was hypothesized to be due to the apparent slower recombination reaction in the EL-cells. The slower recombination reaction, in turn, was partly caused by a change in the pre-exponential factor of the rate constant of recombination (Paper II, Fig. 6).

In comparison, the *hit2* strain had better resistance towards chemically produced ROS than the used wild-type (Schierenbeck et al. 2015) but showed no similar change in the redox potentials that would decrease the recombination and subsequent $^1O_2$-production probability (Paper I, Fig. 7). Hence, the features of the EL-cells and *hit2* are different. EL-cells have the ability to avoid ROS production and *hit2* most probably has the capacity to withstand their presence, which showcases how both features would be beneficial in high light. It would be interesting to examine what kind of an advantage *hit2* would have over *CC124* in TAP medium, where the swap of the bicarbonate between Q_A and Q_B of PSII to acetate increases the probability of non-radiative recombination and consequently decreases $^1O_2$ production from the rate observed in autotrophic cells (Roach et al. 2013).

The pigment changes that occurred during the EL-acclimation in Paper II were caused mainly by reduction in overall Chl content, a common high-light response also in cyanobacteria (Muramatsu and Hihara 2012) and plants (Schumann et al. 2017). However, the Car contents of the cells remained at similar levels during the acclimation (Paper II, Fig. 2). Furthermore, in terms of pigment composition, the similar acclimation of *hit2* and *CC124* (Paper I, Fig. 4) to increasing light intensity in terms of pigment composition probably reflects a similar capacity for quenching or scavenging ROS. In addition, as the cells in both Paper I and II were photoautotrophic, they most probably contained LHCSR3 that was shown to lower $^1O_2$ production *in vivo* (Barera et al. 2021). Hence, LHCSR3 probably provided both EL-cells and *hit2* similar amount of $^1O_2$ tolerance. As the activities of water-water cycles were not examined, their role remains to be uncertain in the high-light acclimation of *hit2* and *CC124*.

In Chapter 1.3.1., it was mentioned that several reports speak for the PSII photoinhibition to be $^1O_2$-dependent. Interestingly, here the results in Paper I speak for this, whereas in Paper II the results point towards $^1O_2$-independent mechanisms. In Paper I, *hit2* had both slower damaging reaction of photoinhibition (Paper I), and increased tolerance against artificially generated ROS (Schierenbeck et al. 2015), suggesting that higher resistance against ROS equals a decrease in susceptibility to photoinhibition of PSII. However, the high-light tolerance of *hit2* was nonetheless mostly attributed to its NPQ-capacity that was twice of that of the wild-type (Schierenbeck et al. 2015). In contrast, the EL-cells in Paper II produced negligible amounts of $^1O_2$ when compared to the control cells, but the rates of the damaging reaction of photoinhibition were similar in both control and EL-cells. Interestingly, the green alga *Chlorella ohadii* has the best of both worlds: the alga is rather immune to photoinhibition of PSII and it produces no (or at most negligible amounts of) $^1O_2$ (Treves et al. 2016). However, as Treves et al. (2016) disclose, *C. ohadii*'s ability to withstand photoinhibition relies on PSII modifications, but also on the sink capacity after PSII. Hence, our data are in line with literature that support the assumption that

$^1O_2$ does not solely explain photoinhibition, but more multiplex mechanisms are probably involved (Mattila et al. 2022).

5.2 Regulation of light harvesting in *Chlamydomonas*

5.2.1 Differences between algal and plant system

Algae and plants have very different light harvesting capacity. PSII antenna structures are larger in green algae than in plants (Minagawa and Takahashi 2004, Sheng et al. 2019). Furthermore, PSI antenna is hugely larger in *Chlamydomonas* due to an extra Lhca-belt (Kubota-Kawai et al. 2019, Su et al. 2019) with different Chl-composition from the inner antenna belt shared with plants (Galka et al. 2012, Drop et al. 2014). These characteristics of the algal antenna structures were suggested to cause similar rates of electron flow through PSII and PSI under the monochromatic light wavelengths used (Paper III, Fig. 2). Consequently, this was thought to be one of the reasons behind the less pronounced effect of the monochromatic light wavelengths on the PQ-pool redox state in *Chlamydomonas* (Paper III, Fig. 1) than in plants (Mattila et al. 2020). Furthermore, it appears that the algal PQ-pool is affected more by light-independent factors than in plants. Indeed, several non-photochemical reducers and oxidizers of PQ have been identified in the thylakoids (Jans et al. 2008, Houille-Vernes et al. 2011), plastoglobuli (van Wijk and Kessler 2018, Pralon et al. 2019) and mitochondria (Kaye et al. 2019) of *Chlamydomonas*.

Overall, the relative size of the PQ-pool from the total PQ in *Chlamydomonas* appears to be slightly smaller than in other photosynthetic organisms (Kruk and Karpinski 2006, Khorobrykh et al. 2020, Mattila et al. 2020). This probably reflects the general differences in the architecture of cells between the plants and *Chlamydomonas*. In addition, *Chlamydomonas* retains its PQ-pool at more reduced state than higher plants under all studied light-conditions (Mattila et al. 2020, Paper III Fig. 1). A highly reduced algal PQ-pool could serve as a more sensitive sensor requiring only small amount of stimulus to initiate a signal than a more oxidized pool would, reflecting a possible adaptation to aquatic light conditions with lower light intensities and more gradual variation in light intensity throughout the day than in many terrestrial environments where sun, shade and spectrum may change rapidly. Importantly, another green alga *Acetabularia acetabulum* was also shown to maintain a highly reduced PQ-pool (Havurinne and Tyystjärvi 2020), suggesting highly reduced to be a shared feature of green algae. The PQ-pool may also remain highly reduced because of the wavelength-wise more balanced absorbance of light

in algal antennae described above, especially under the light wavelengths that were shown to favor PSI in plants (Mattila et al. 2020).

As mentioned in Chapter 1.1.4, interorganellar regulation has been found to protect the cells when photosynthetic electron carriers are highly reduced via safe dissipation of reducing equivalents by the mitochondrial alternative oxidase that was suggested to offer photoprotection via decreased $^1O_2$ production (Kaye et la. 2019). Hence there could be an effect of mitochondrial metabolism in the observed state transitions that should not be overlooked. However, it should be noted that cells in our experiments were grown photoautotrophically, while Kaye et al. (2019) cultured their cells mixotrophically, which may increase the potential for mitochondrial metabolism to affect the regulation of photosynthesis, further complicating the comparison as these two types of cells have different metabolic profiles.

To summarize, algal light-harvesting antenna structures appear as a rather blunt tool that can be modulated to a large extent, mostly in the terms of overall efficiency. However, this modulation comes with a need for other levels of regulation, provided probably by at least partly by connections between chloroplasts and other cellular organelles, especially mitochondria.

5.2.2 Take on algal state transitions

The presence of LHCII-phosphorylation and subsequent movement of the antennae in *Chlamydomonas* is unarguable (e.g. Lemeille et al. 2010, Takahashi et al. 2006, 2014). The contribution of the STT7-protein to the qT-component of NPQ has been estimated to depend on both the intensity (and also presence) of light, but also on the sequence of fluctuating light (Steen et al. 2022). These results, together with earlier work by Allorent et al. (2013), suggested the protective role of qT to be crucial in enabling proper induction of qE-type NPQ: qT protects the system until sufficient amount of LHCSR-dependent NPQ is ramped up after a dark period and, in turn, qT is accumulated during the dark period. Interestingly, Steen et al. (2022) also showed that STT7-dependent NPQ was highly dependent on the presence of LHCSR3, further supporting the idea for the joint function of these proteins. STT7 was also here more dominant in the absence of light. Light affected more the PSI-originating fluorescence peak as LHCII trimers were phosphorylated and shuttled to PSI (Paper III, Fig. 3). This mechanism was shown to function rather similarly as in plants and to depend on the PQ-pool redox state (Paper III, Figs. 1 and 3). In contrast, almost any light treatment most probably caused also LHCSR3 to function, distorting the apparent effects of STT7. The only light treatment suggesting involvement of (almost) exclusively STT7, were the treatments with low-intensity white light (Paper III, Fig. 4). However, even the PSII-WL still probably induced a small amount of LHCSR-activation (Paper III, Fig. 4a), as shown via parallel treatment with the *stt7*-

mutant that also showed a small increase in relative fluorescence at 714 nm during the PSII-WL treatment (Paper III, Fig. 4c).

It is intriguing to hypothesize that the state transitions seen here (as PSII-to-PSI emission ratio) under darkness (Paper III, Fig. 3) are smaller than under PSII-WL and PSI-WL (Paper III, Fig. 4a and b) because of LHCSR3. The results in Paper III show that even if STT7-function may not be observed in the data similarly as the function of STN7 of plants in Mattila et al. (2020), both kinases appear to modulate the antennae via similar type of phosphorylation. The PQ-pool redox state seems to have no clear correlation with the light state in *Chlamydomonas*. However, when we compare data in Paper III from treatments with white light or between dark-incubations, we can see that it is the change in the PQ-pool redox state during the swap between treatments that is the governing factor that regulates STT7 activity, rather than any absolute quantification of the redox state of the PQ-pool.

5.2.3 On the dual role of state transitions in green algae

State transitions have been generally thought as an equilibrating mechanism to balance out the light utilization between photosystems. Paper III demonstrates how the green algal state transitions clearly differ from the ones observed in *A. thaliana* (Mattila et al. 2020). Most straightforward explanation for this comes from the above-mentioned co-function of STT7 and LHCSR-proteins. Moreover, just as the number of LHCIIs capable of binding algal PSI, their role in PSI-photochemistry is also doubly difficult to interpret when compared to plants. The case of the trimer containing the LhcbM5 subunit appears simpler, as LhcbM5 is mainly found in the LHCII-fraction not associated with either of the photosystems (Shen et al. 2019, Sheng et al. 2019). However, LhcbM5 is required for the PSI-LHCI-LHCII-supercomplex formation (Takahashi et al. 2006, 2014) that occurs under State 2-promoting conditions that reduce the PQ-pool, consequently causing STT7 to phosphorylate the LhcbM5 (Lemeille et al. 2010). Hence, the decoupling and recoupling of the LHCII trimers containing LhcbM5 to PSI can most probably be seen purely as modulation of PSI light absorption. This assumption does not, however, consider the possible role of the PSII monomeric antennae that have been proposed to partake in algal state transitions (Kargul et al. 2005, Takahashi et al. 2006, Turkina et al. 2006, Takahashi et al. 2014). However, as mentioned in Chapter 1.2.1., structural studies do not support such an interaction taking place under any circumstance (Pan et al. 2021).

As mentioned in Chapter 1.2.2., mutants lacking the LhcbM1 apoprotein have diminished qE capacity, suggesting that an interaction between LhcbM1 and LHCSR3 is a prerequisite for LHCSR3-dependent qE. As LhcbM1-containing LHCII-trimers can be found in both PSII (Sheng et al. 2019) and PSI-LHCI-LHCII-

supercomplexes in phosphorylated form under State 2 (Takahashi et al. 2006, 2014), they can be assumed to be shuttled between photosystems in STT7-dependent manner. Hence, the STT7-dependent movement of LhcbM1-containing LHCII-trimers is probably not only for the equilibration of light energy distribution, but also a requirement for the protection of PSI by LHCSRs. As Girolomoni et al. (2019) and Roach et al. (2020) have demonstrated, LHCSR3 can quench also PSI in addition to PSII. It is tempting to hypothesize that the PSI-bound LhcbM1 is required for LHCSR3-dependent PSI-quenching. However, the capacity for parallel quenching of both photosystems makes interpretation of any fluorescence emission spectrum increasingly complicated. In regard to measuring low-temperature fluorescence emission, the simultaneous quenching of photosystems also highlights the importance of normalization of fluorescence peaks with either biological signals known not to vary, or by using external normalization additives, such as fluorescein, in sample preparation.

The traditional definition of state transitions does not appear to hold in *Chlamydomonas*. Indeed, the LHCII-movement does probably modulate the light utilization of both PSII and PSI via shuttling of LHCII-trimers. However, LHCII-shuttling appears to enable LHCSR-dependent qE of both PSII and PSI. The additive effects of both STT7 and LHCSRs seem to protect the algal antenna system against short term changes in light quantity (Paper III, Fig. 5), whereas the longer-term acclimation includes adjustments in the number of photosystems (Bonente et al. 2012, Paper II, Fig. 3). The double-layered mechanism of qE and qT depend on both light and PQ-pool redox state, respectively. This partly explains why PQ-pool redox state and light state have a different relationship in *A. thaliana* (Mattila et al. 2020) and *Chlamydomonas* here. Furthermore, the layered protection mechanisms also explain why the algal state transitions appear to be more dependent on the quantity, rather than quality of light. This kind of preference for state transitions to be regulated primarily by the intensity of light could be favorable in aquatic environments that tend to have lower ambient light irradiance than the terrestrial habitat of plants.

6 Conclusions

This thesis has provided new information regarding the robustness and flexibility of the photosynthetic machinery of the green alga *Chlamydomonas reinhardtii* by providing details on how its photosynthetic light reactions are responding to various light conditions.

The results in Papers I and II demonstrated different mechanisms on how autotrophic *Chlamydomonas* can tolerate light intensities greatly exceeding photosynthetic requirements. In Paper I, it was shown that a mutation in the *Chlamydomonas* gene involved in UV-signaling has potential to permanently decrease the susceptibility of the system towards photoinhibition of PSII. This decreased rate of photodamage and consequently less active PSII repair cycle saved the cells resources that could be reallocated to fuel cell division and biomass production. These results provide clues about using photoinhibition-tolerant photoautotrophs as production platforms. Such organisms are a potential option in environments where strong light is persistent e.g. in Southern parts of Europe where microalgae are already used in industrial-scale applications due to the sufficient natural irradiance levels and suitable climate conditions.

Paper II showed that growth in strong light is a characteristic reserved not only to the mutant lines, but control cell lines can possess this ability too. Importantly, this acclimation was shown to be only temporary and reversible, ruling out the possibility for spontaneous mutations. However, the acclimation to high light was associated with a number of changes in the biophysical and biochemical properties of the photosynthetic light reactions. Most striking feature caused by these changes was the significantly reduced $^1O_2$-production, suggesting that oxidative stress is a key factor in inducing such high-light tolerance. However, as the study focused on changes occurring in PSII, we cannot rule out that the acclimation could have had cellular side-effects that we did not discover. Hence, more should be done to find out the possible additional drawbacks to this kind of acclimation.

Paper III focused on the regulation of the light-harvesting apparatus of *Chlamydomonas* in response to changes in the PQ-pool redox state, summarized in four key characteristics. First, the PQ-pool was found to be highly reduced in any light condition applied. This was suggested to be due to the algal light-harvesting

antenna having highly similar Chl compositions between PSII and PSI, which would lead to both photosystems to be able to utilize light at a very broad range. Second, the *Chlamydomonas* PQ-pool redox state is strongly modulated by non-photochemical processes. This feature has long been used to study STT7-dependent state transitions in algae. Third, due to the complex redox kinetics of the *Chlamydomonas* PQ-pool, no clear correlation between PQ-pool redox state and state transitions could be disclosed. This feature showcases the overlapping function of STT7 and LHCSR-proteins that both affect the low-temperature fluorescence emission experiments. Finally, results in Paper III support the idea of the algal state transitions being photoprotective by demonstrating them to work primarily as a function of light quantity rather than quality, a characteristic not shared with plants.

Acknowledgemets

The work was conducted at the Molecular Plant Biology unit of the University of Turku. The work was financially supported by the University of Turku Graduate School, which has motivated me to finish the book within the four years given.

I want to thank Professor Eevi Rintamäki for guidance with the administrative tasks that have enabled me to actually graduate. The contributions of both Assistant Professor Mikko Tikkanen and Docent Taina Tyystjärvi, who acted in my advisory committee and gave me feedback on the PhD project every year, are greatly appreciated. I wish to express my gratitude also for Associate Professor Thomas Roach and Professor Markku Keinänen for critically examining my thesis. Additionally, I thank Professor Roberta Croce for accepting the invitation to act as my opponent and dedicating the time to thoroughly assess this thesis.

There are several people I want to mention specifically, but I think the first one has to be Esa. You have always had your door open for questions and have given me more or less a free choice in what to pursue in the lab (with some suggestions steering me in a more realistic direction, of course). It is difficult to express how much respect I have for your genuine excitement and interest towards new ideas and results. Thank you for giving this first-year student the opportunity to prove his skills and for having patience when I've made mistakes.

There are three people who started some time before me in the unit and have had a big influence on me. Hence, these three need to be mentioned. First, Heta, you taught me the basics of working in the unit and always showed me how things really work. In addition, your passion for just science has been impressive. Second, Vesa, you demonstrated how much one's own ambition and interest can carry. For setting this example, I am grateful and hope I can someday reach the same level of professional competence as you have. Third, Juha, you have tolerated my outbursts in the last few years and have always answered my (let's face it, dumb) questions about molecular biology, which enabled me progress through the lab work. Moreover, you have shown that one can balance work, sports and personal life while working in the academia. It's been awesome to share an office with you and someday it is going to be weird to have an office without your company. With this, I wish to thank all three of you for acting as my peers and colleagues, and showing that

researchers can also have a good sense of humor (this last bit may have some generosity to it).

Of course, the everyday life in the workplace mostly consists of socializing with others, and hence all the people in the Molecular Plant Biology unit are thanked for their support during the last eight (hngg…that long?) years. Special mentions are in place for Tapio L, Laura L, Hari, Sergey and Dimitar for insightful discussions about both personal and/or professional stuff on the corridors and at the coffee table, or just for distracting my mind from work-related problems. I also wish to mention the technical staff in our unit that has always provided top-tier assistance whenever needed.

Lastly, I want to thank Tuulia, who has had the patience to endure my quirks every day, both in good and in bad, and cheering me on even when all the spoons have been spent. Thank you for listening my rants, excited explanations and every other word in between. I love you.

8.8.2023

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TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama Oy, Turku, Finland 2023

ISBN 978-951-29-9394-9 (PRINT)
ISBN 978-951-29-9395-6 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)