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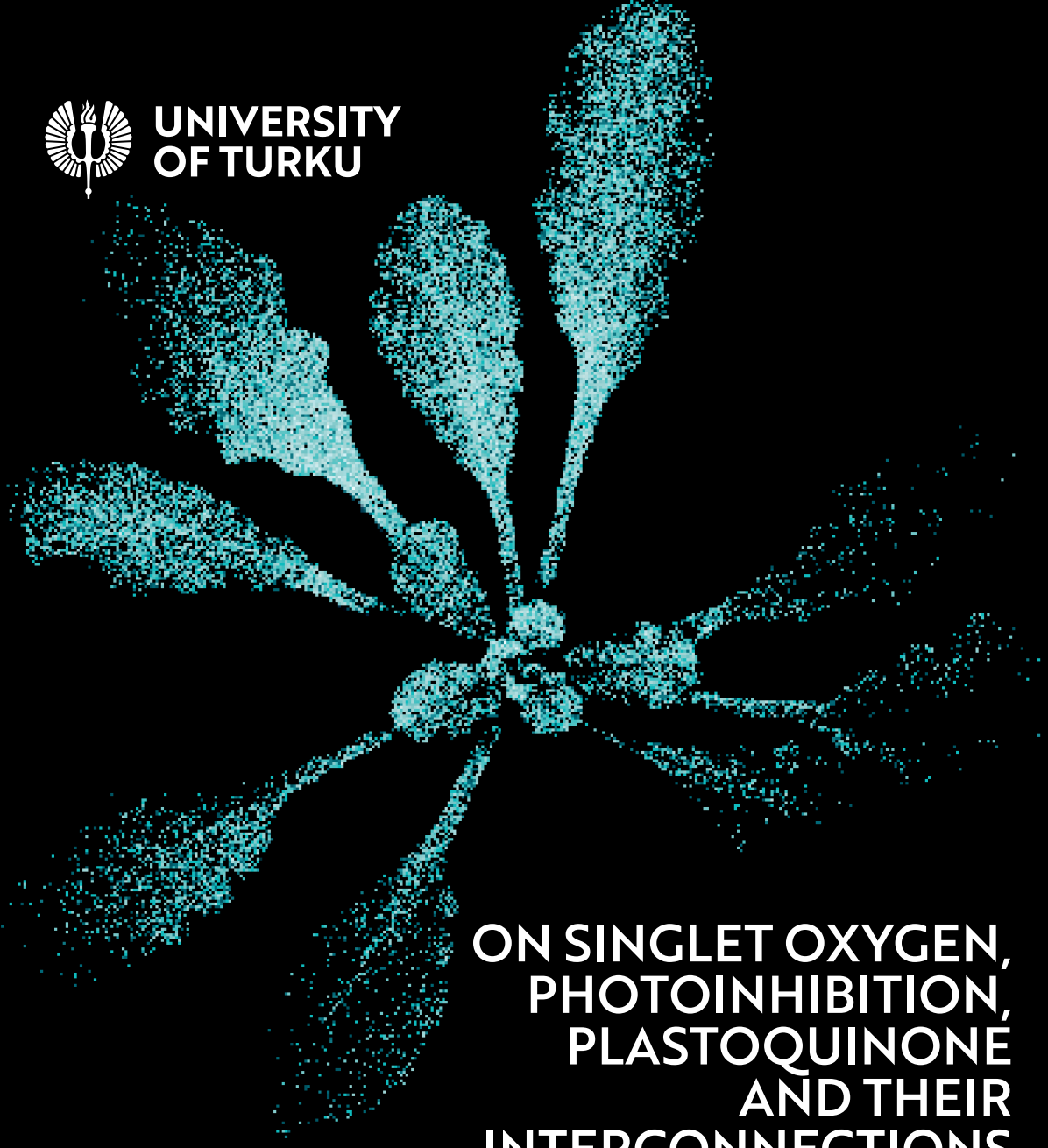

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Heta Mattila

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University of Turku

Faculty of Science and Engineering
Department of Biochemistry
Molecular Plant Biology unit
Doctoral Programme of Molecular Life Sciences

Supervised by

Adjunct Professor, Esa Tyystjärvi
University of Turku
Faculty of Science and Engineering
Department of Biochemistry
Molecular Plant Biology unit

Reviewed by

Assistant Professor, Alizée Malnoë
University of Umeå
Sweden

Professor, Shunichi Takahashi
National Institute of Basic Biology
Okazaki, Japan

Opponent

Associate Professor, Pavel Pospíšil
Palacký University Olomouc
Czech Republic

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

Cover Image: Heta Mattila

ISBN 978-951-29-8269-1 (PRINT)
ISBN 978-951-29-8270-7 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)
Painosalama, Turku, Finland 2020

– –Tämä heleä, kostea multa,
tämä kirkkaus loppumaton– –

Saima Harmaja

UNIVERSITY OF TURKU
Faculty of Science and Engineering
Department of Biochemistry
Molecular Plant Biology
HETA MATTILA: On singlet oxygen, photoinhibition, plastoquinone, and their interconnections
Doctoral Dissertation, 151 pp.
Doctoral Programme of Molecular Life Sciences
November 2020

ABSTRACT

In oxygenic photosynthesis light is captured in series by two protein complexes: photosystem II and photosystem I (PSI and PSII). Electron transfer from PSII to PSI is mediated by the plastoquinone (PQ) pool. Despite being the energy source, light also damages the photosynthetic machinery. Singlet oxygen ($^1O_2$), an excited state of O_2, may be generated when a charge recombination reaction in PSII re-excites the reaction center chlorophylls (P680), producing a triplet state. In this thesis, mass-spectroscopy-based detection methods for $^1O_2$ were developed further, to understand the role of this reactive oxygen species in photosynthesis. It was shown that even though both O_2 and $^1O_2$ are produced in pumpkin thylakoid membranes, most (if not all) of the $^1O_2$ derives from the ambient dissolved O_2, not from the nascent O_2 produced due to the water splitting activity of PSII. The result shows that the O_2 evolving ability of PSII as such does not render PSII vulnerable to oxidative damage.

Nevertheless, light inactivates PSII, and no consensus about the mechanism(s) of the photoinhibitory damage exists. Therefore, the temperature dependence of the rate constant of PSII photoinhibition was measured under various conditions and compared with temperature dependencies of $^1O_2$ production and recombinations. The results show that in plants and cyanobacteria the rate constant of photoinhibition and production of $^1O_2$ increase similarly with temperature as the miss probability of the oxygen evolving complex. Photoinhibition proceeded under anaerobicity, where no $^1O_2$ is produced, and was unaffected by quenchers of $^1O_2$. We suggest that when a miss occurs, but a recombination does not re-reduce the PSII reaction center, $P680^+$ lives long enough to oxidize a vital component of PSII, causing the photodamage.

Plants have also ways to adjust to different light conditions. An initial fluorescence screen and subsequent high-performance liquid chromatography measurements revealed that 470 nm, 560 nm and 660 nm light favors PSII over PSI and reduces 80–90 % of the PQ pool, whereas 440 nm, 520 nm and 690 nm favors PSI and oxidizes 90–100 % of the pool in *Arabidopsis thaliana*, when moderate light was used. Light state curvilinearly followed the redox state of the PQ pool; state 2 was reached with 50 % reduction. All tested white lights, including light from the Sun, reduced less than 50 % of the PQ pool. This PSI light character of white light enables plants to respond to the intensity of light via the redox state of the PQ pool.

KEYWORDS: MIMS, photoinactivation, ROS, signaling, state transitions, TEMP

TURUN YLIOPISTO
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Biokemian laitos
Molekulaarinen kasvibiologia
Heta Mattila: On singlet oxygen, photoinhibition, plastoquinone, and their interconnections
Väitöskirja, 151 s.
Molekulaaristen biotieteiden tohtoriohjelma
marraskuu 2020

TIIVISTELMÄ

Vaikka valo onkin yhteytyksen eli fotosynteesin energianlähde, se myös vahingoittaa yhteyttäviä eliöitä. Happea vapauttavassa yhteytyksessä kaksi proteiinikompleksia, valoreaktiot II ja I (PSII ja PSI), vastaanottavat valoa. Plastokinonivaranto välittää elektroninsiirtoa näiden välillä. Singlettihappi on reaktiivinen happiyhdiste, jota syntyy, kun PSII:n reaktiokeskusklorofylli (P680) siirtyy triplettitilaan elektronin palatessa rekombinaatioreaktioissa takaisin reaktiokeskukselle. Tässä työssä kehitettiin edelleen massaspektrometria-menetelmiä ja havaittiin, että vaikka eristetyt kasvin yhteytyskalvostot tuottavat sekä happea että singlettihappea, suurin osa singlettihapesta on peräisin ympäristöön liuenneesta hapesta eikä PSII:n veden hajotuksessa vastasyntyneestä hapesta. PSII:n kyky vapauttaa happea ei siis itsessään tee PSII:sta herkkää hapettavalle vahingolle.

Valo kuitenkin vaurioittaa erityisesti PSII:ta, eikä valovahingon mekanismista ole yksimielisyyttä. Tästä syystä PSII:n fotoinhibition, singlettihapen tuoton ja rekombinaatioreaktioiden lämpötilavasteet mitattiin kasveista ja syanobakteereista. Sekä fotoinhibitio että singlettihappi lisääntyivät lämpötilan noustessa. Molemmat lämpötilavasteet selvästi muistuttavat PSII:n happea vapauttavan kompleksin “hutien” lämpötilariippuvuutta. Fotoinhibitio ei kuitenkaan vähentynyt, kun yhteytyskalvostoja valotettiin singlettihapen vaimentajien kanssa tai hapettomissa olosuhteissa, jolloin singlettihappea ei synny. Tulosten perusteella muotoiltiin hypoteesi: mikäli huti tapahtuu eli happea vapauttava kompleksi on tilapäisesti kykenemätön luovuttamaan elektronin, eikä rekombinaatiokaan uudelleen pelkistä reaktiokeskusta, $P680^+$:lla on tarpeeksi aikaa hapettaa jokin PSII:n tärkeä osa.

Kasvit kykenevät myös sopeutumaan valo-olosuhteiden muutoksiin. Plastokinonivarannon hapetus-pelkistystila mitattiin nestekromatokrafialla; PSII vastaanotti PSI:tä tehokkaammin 470, 560 ja 660 nm:n aallonpituuksien valoa ja nämä valot pelkistivät 80–90 % plastokinonivarannosta. 440, 520 ja 690 nm:n aallonpituudet sen sijaan suosivat PSI:tä ja hapettivat 90–100 % plastokinonivarannosta, kun kohtalaista valovoimakkuutta käytettiin. Kaikki testatut valkoiset valot, mukaan lukien auringonvalo, suosivat PSI:tä, joten kasvit voivat aistia valon voimakkuutta plastokinonivarannon pelkistyneisyyden avulla.

ASIASANAT: HPLC, MIMS, plastokinoni, reaktiiviset happilajit, signalointi, $^1O_2$, TEMP, tilasiirtymät, yhteyttäminen

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Abbreviations

^{1}Chl	Singlet (ground state) chlorophyll
$^1O_2$	Singlet oxygen; $^1\Delta_gO_2$
$^1\Delta_gO_2$	Singlet oxygen
$^1\Sigma^+{}_gO_2$	Singlet oxygen
^{3}Chl	Triplet chlorophyll
$^3O_2$	Triplet (ground state) oxygen; $^3\Sigma^-{}_gO_2$
^{3}P680	PSII reaction center triplet ($^3Chl_{D1}$ or $^3P_{D1}$)
^{3}P700	PSI reaction center triplet
$^3\Sigma^-{}_gO_2$	Triplet (ground state) oxygen; $^3O_2$
^{16}O	A stable isotope of oxygen (common in the nature)
^{18}O	A stable isotope of oxygen (rare in the nature)
A_0	The primary electron acceptor of PSI (a chlorophyll)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	ATP synthase
Car	Carotenoid
Car_{D2}	A β-carotene close to PSII reaction center
Chl	Chlorophyll
Chl*	Excited singlet chlorophyll
Chl_{D1}	A PSII reaction center chlorophyll
Chl_{D2}	A PSII reaction center chlorophyll
$ChlZ_{D2}$	A chlorophyll close to PSII reaction center
CP24	An antenna protein of PSII
CP26	An antenna protein of PSII
CP29	An antenna protein of PSII
CP43	A core protein of PSII (intrinsic antenna)
CP47	A core protein of PSII (intrinsic antenna)
Cyt b6f	Cytochrome b_6/f complex
D1	A core protein of PSII
D2	A core protein of PSII
D_2O	Deuterium oxide (heavy water); ^2H_2O

DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea (a PSII herbicide)
Deg1	A protease important in PSII repair
EF-G	A cyanobacterial translation elongation factor
EF-Tu	A cyanobacterial translation elongation factor
EPR	Electron paramagnetic resonance
F_0	Minimal fluorescence yield (Q_A oxidized), dark-acclimated sample
F_0'	Minimal fluorescence yield (Q_A oxidized), light-acclimated sample
F_A	A 4Fe-4S cluster of PSI
F_B	A 4Fe-4S cluster of PSI
Fd	Ferredoxin
F_M	Maximal fluorescence yield (Q_A reduced), dark-acclimated sample
F_M'	Maximal fluorescence yield (Q_A reduced), light-acclimated sample
FNR	Ferredoxin $NADP^+$ reductase
FR	Far-red ($\lambda \geq 680$ nm)
FtsH	A protease important in PSII repair
F_V	Variable fluorescence (F_M-F_0)
F_V/F_M	Variable to maximal fluorescence (yield of PSII photochemistry)
F_X	A 4Fe-4S cluster of PSI
FWHM	Full width at half maximum
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
k_{PI}	Rate constant of photoinhibition of PSII
LED	Light emitting diode
LHC	Light harvesting complex
LHCI	Light harvesting complex of PSI
LHCII	Light harvesting complex of PSII
LHCSR	A protein important in NPQ in algae and moss
MIMS	Membrane inlet mass spectroscopy
MS	Mass spectrometer/spectroscopy
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NIR	Near-infrared (~750–2500 nm)
NO•	Nitric oxide (a radical)
NPQ	Non-photochemical quenching (of chlorophyll fluorescence)
O_2•$^-$	Superoxide anion (a radical)
OCP	Orange carotenoid protein (important in cyanobacterial NPQ)
OEC	Oxygen evolving complex of PSII
OJIP	Fluorescence rise from initial (O) to maximal (P) level, via J and I steps
P680	Reaction center chlorophylls of PSII (P_{D1}, P_{D2}, Chl_{D1} and Chl_{D2})
$P680^+$	Oxidized reaction center of PSII (P_{D1}^+)

P700	Reaction center chlorophylls of PSI
PAM	Pulse amplitude modulated
PBCP	PSII core phosphatase
PC	Plastocyanin
P_{D1}	A PSII reaction center chlorophyll
P_{D2}	A PSII reaction center chlorophyll
PGR5	A protein important for PSI cyclic electron transfer
PGRL1	A protein important for PSI cyclic electron transfer
Pheo	Pheophytin
$Pheo_{D1}$	Redox active pheophytin of PSII
$Pheo_{D2}$	A pheophytin close to PSII reaction center
PhQ	Phylloquinone
Pi	Inorganic phosphate
PPFD	Photosynthetic photon flux density
PQ	Oxidized plastoquinone
PQH_2	Reduced plastoquinone; plastoquinol
PsbS	A protein important in NPQ in higher plants
PsaA	A core protein of PSI
PsaB	A core protein of PSI
PSI	Photosystem I
PSII	Photosystem II
Q_A	Primary quinone acceptor of PSII
Q_B	Secondary quinone acceptor of PSII
qL	Photochemical quenching of fluorescence (lake model)
qE	Energy-dependent quenching of excitation energy
qP	Photochemical quenching of fluorescence (puddle model)
ROS	Reactive oxygen species
SD	Standard deviation
SOSG	Singlet oxygen sensor green (a $^1O_2$ sensor)
STN7	State transition kinase (STT7 in *C. reinhardtii*)
STN8	A kinase involved in phosphorylating PSII core
TAP38	A phosphatase involved in state transitions (PPH1 in *C. reinhardtii*)
TEMP	2,2,6,6-tetramethylpiperidine (a $^1O_2$ sensor)
TEMPO	1-oxyl of TEMP (reaction product of TEMP and $^1O_2$)
TyrZ	Redox active tyrosine residue of PSII; Y_Z
(U)HPLC	(Ultra) high-performance liquid chromatography
UV-A	Ultraviolet radiation (315–400 nm)
UV-B	Ultraviolet radiation (280–315 nm)
UV-C	Ultraviolet radiation (200–280 nm)
VIS	Visible light (~400–700 nm)

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 Maarit Karonen*, Heta Mattila*, Ping Huang, Fikret Mamedov, Stenbjörn Styring, Esa Tyystjärvi. A tandem mass spectrometric method for singlet oxygen measurement. *Photochemistry and Photobiology*, 2014; 90: 965–971.

II Heta Mattila, Sergey Khorobrykh, Esa Tyystjärvi. Singlet oxygen derives from ambient dissolved gas, not from nascent oxygen produced by Photosystem II. Manuscript.

III Heta Mattila, Sujata Mishra, Taina Tyystjärvi, Esa Tyystjärvi. Misses of oxygen evolving complex play a crucial role in photoinhibition of Photosystem II. Manuscript.

IV Heta Mattila*, Sergey Khorobrykh*, Marja Hakala-Yatkin, Vesa Havurinne, Iiris Kuusisto, Taras Antal, Taina Tyystjärvi, Esa Tyystjärvi. Action spectrum of the redox state of the plastoquinone pool defines its function in plant acclimation. *The Plant Journal*, 2020; doi: 10.1111/tpj.14983.

*Equal contribution.

Other recent publications related to the topic:

Mattila H*, Mishra KB*, Kuusisto I, Mishra A, Novotná K, Šebela D, Tyystjärvi E (2020) Effects of low temperature on photoinhibition and singlet oxygen production in four natural accessions of Arabidopsis. *Planta* 252: 19

Khorobrykh S, Havurinne V, Mattila H, Tyystjärvi E (2020) Oxygen and ROS in photosynthesis. *Plants* 9: 91

Havurinne V, Mattila H, Antinluoma M, Tyystjärvi E (2019) Unresolved quenching mechanisms of chlorophyll fluorescence may invalidate multiple turnover saturating pulse analyses of photosynthetic electron transfer in microalgae. *Physiol Plant* 166: 365–379

Mattila H, Khorobrykh S, Havurinne V, Tyystjärvi E (2015) Reactive oxygen species: Reactions and detection from photosynthetic tissues. *J Photochem Photobiol B* 152: 176–214

1 Introduction

Some 3.7–2.4 billion years ago, a (pre)cyanobacterium, now extinct, achieved the ability to extract electrons from water with the energy of light (Fischer et al. 2016a, Martin et al. 2018; oxygenic photosynthesis might have evolved also much before the phylum Cyanobacteria, see e.g. Cardona et al. 2019). Light and water are plentiful on Earth, and the (eventual) rise in atmospheric oxygen, due to water splitting, enabled efficient respiration. Consequently, the emergency of oxygenic photosynthesis which, to the present knowledge, has occurred only once in the evolution of life, increased the primary production of Earth by a factor of ~30, as estimated by Raven (2009). Today, (oxygenic) photosynthesis supports almost all life on Earth (Mccollom and Shock 1997, Raven 2009). According to the widely accepted endosymbiosis hypothesis (Mereschkowsky 1905), chloroplasts of algae and plants were once free-living cyanobacteria but became engulfed into an ancient eukaryote and transformed into cell organelles (for a review and a recent phylogenetic analysis, respectively, see Gavelis and Gile 2018, Moore et al. 2019). Chloroplasts of all but one (*Paulinella chromatophora*; Marin et al. 2005, Delaye et al. 2016) photosynthetic eukaryotes known to science originate from the same primary endosymbiosis event.

1.1 Photosynthetic electron transport chain

Membrane embedded protein complexes and lipid- and water-soluble electron carriers of photosynthetic electron transport chain convert light energy to chemical form (Fig. 1); to the universal carriers of energy (adenosine triphosphate; ATP) and reducing power (nicotinamide adenine dinucleotide phosphate; NADPH). The thylakoid membrane system is, in the case of plants and algae, separated from the rest of the cell by the double membrane (called envelope) of chloroplasts. In land plants (and in some green macroalgae), thylakoids are organized to appressed, or stacked, (grana thylakoids) and non-appressed (stroma thylakoids) domains (see Fig. 1B). In the stroma (or, in the case of cyanobacteria, in the cytosol) ATP and NADPH are used by the Calvin-Benson cycle to fix atmospheric CO_2 into sugars.

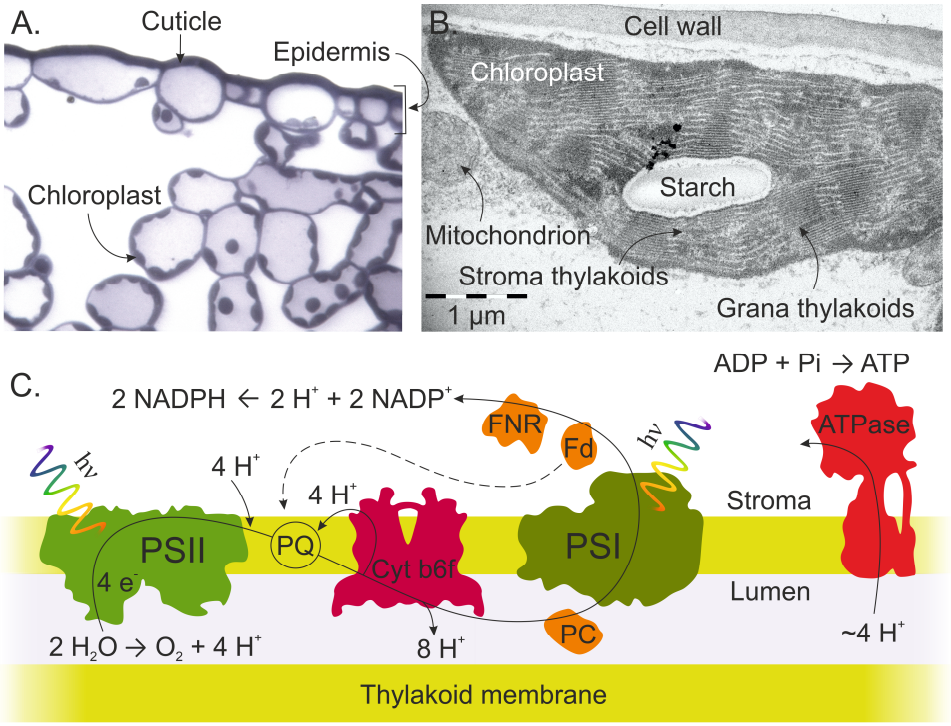

Figure 1. **Photosynthesis in plant chloroplasts.** A. Light microscopy images showing cells and, B. electron microscopy images showing a chloroplast and few mitochondria, of an outdoors-grown dandelion (*Taraxacum officinale*). C. A schematic representation of photosynthetic electron transfer chain and ATP synthesis. Linear electron (e^-) transfer from water to $NADP^+$, via photosystem II (PSII), plastoquinone (PQ) pool, cytochrome b_6f complex (Cyt b6f), plastocyanin (PC), photosystem I (PSI), ferredoxin (Fd) and Fd $NADP^+$ reductase (FNR), is indicated with a continuous arrow. Cyclic electron transfer from Fd to PQ pool is indicated with a dashed arrow. hv (energy of a photon; h is the Planck's constant and v is frequency) indicates absorption of light energy at PSII and PSI. ATP production from adenosine diphosphate (ADP) and inorganic phosphate (Pi) by ATP synthase (ATPase), powered by an electrochemical gradient over the thylakoid membrane created by water splitting and proton (H^+) translocation from stroma to lumen, is indicated by another continuous arrow. Here, a rate of 4 protons/an ATP produced is assumed, based on measurements by van Walraven et al. (1996). D. A general equation of oxygenic photosynthesis, assuming 100 % quantum efficiency. Microscopy images: Olli Virtanen, Molecular Plant Biology, and the Laboratory of electron microscopy, Faculty of medicine, University of Turku.

1.1.1 Energy transfer: (not simply) hop-hop-hop-hop-hop

In oxygenic photosynthesis, light energy is harvested by chlorophylls (Chl; all photosynthetic organisms) and phycobilins (cyanobacteria and some algae) and to a lesser degree by carotenoids (Hofmann et al. 1996, Caffarri et al. 2001, Balevičius

et al. 2017). To increase light absorption (cross-section), photosynthetic organisms have light harvesting complexes (LHC), or antennae, which contain a high number of tightly packed pigment molecules. After absorption of visible light (a photon with wavelength of ~400–700 nm) by a pigment, fast non-radiative excitation energy transfer to the respective reaction center (of either PSII or PSI) occurs (see e.g. Wientjes et al. 2011, Kreisbeck and Aspuru-Guzik 2016). It is not clear whether the rate limiting step is charge separation in the reaction center (trap limited model) or energy transfer to the reaction center, possibly after initial fast equilibrium in the antenna (transfer-to-trap limited model), in the membrane embedded chlorophyll binding antennae of plants and some algae (for discussion, see Renger and Schlodder 2011, Croce and van Amerongen 2013). Excitation transfer from the large, membrane attached protein-pigment complex, phycobilisome, in cyanobacteria and some algae (for a review, see Watanabe and Ikeuchi 2013), to a PSII reaction center is rather slow (~100 ps; Suter et al. 1984). Often, excitation does not reside on a single pigment molecule, but electronic interactions of pigments close to each other lead to delocalized excitations (i.e. several pigment molecules coherently share the excitation), called excitons. Whether excitation transfer is sufficiently described by semi-classical theories (e.g. generalized Förster; Sumi 1999) or whether quantum effects significantly affect excitation transfer *in vivo*, is debated (Engel et al. 2007, Fassioli et al. 2014, Jumper et al. 2018).

1.1.2 Structure and function of photosystem II

PSII core consists of the reaction center proteins D1 and D2, which bind the redox cofactors needed for charge separation, of the inner (or intrinsic) antenna proteins CP47 and CP43, which bind 16 and 13 chlorophylls, respectively, and 3 carotenoids each, and of multiple smaller subunits (for a 1.9 Å X-ray structure, see Umena et al. 2011). In plants, PSII mostly resides in grana and grana margins (Andersson and Anderson 1980), functions as a dimer and is usually surrounded by two strongly and two moderately bound trimeric LHCs (consisting of Lhcb1–3 proteins in different combinations) and by monomeric antenna proteins (CP24, CP26 and CP29), two of each (Caffarri et al. 2009). Bigger complexes, involving also PSI, may exist *in vivo* (e.g. Järvi et al. 2011; for a review, see Rantala et al. 2020a).

P680, the reaction center of PSII, may be considered to consist of four chlorophyll *a* molecules (P_{D1}, P_{D2}, Chl_{D1} and Chl_{D2}; where P stands for pigment and subscripts indicate whether the chlorophylls are bound to D1 or D2). Light energy transfer from LHCII (light harvesting antenna of PSII) or direct light absorption by P680 leads to its excitation, delocalized over at least two of the chlorophylls (Durrant et al. 1992) and maybe also at the primary electron acceptor of PSII (a pheophytin *a* molecule called $Pheo_{D1}$; Durrant et al. 1995, Holzwarth et al. 2006). Fast charge

separation follows (Fig. 2A–B). At least two pathways function; the primary electron donor is either Chl_{D1} or P_{D1} (Diner et al. 2001, Novoderezhkin et al. 2007, Romero et al. 2010, Renger and Schlodder 2011). Irrespective of the primary donor, the hole ($P680^+$) is localized mainly at P_{D1} (Diner et al. 2001, Nagao et al. 2017), though Pavlou et al. (2018) suggested that in far-red light it might reside at Chl_{D1}. Romero et al. (2017) reviewed the proposed charge separation pathways as follows (where δ denotes charge transfer character of excitations or excitons, indicated by *):

1. $(Chl_{D1}^{\delta+}Pheo_{D1}^{\delta-})^* \rightarrow Chl_{D1}^{+}Pheo_{D1}^{-} \rightarrow P_{D1}^{+}Pheo_{D1}^{-}$
2. $(P_{D1}P_{D2}Chl_{D1})^* \rightarrow (P_{D2}^{\delta+}P_{D1}^{\delta-})^* \rightarrow P_{D1}^{+}Chl_{D1}^{-} \rightarrow P_{D1}^{+}Pheo_{D1}^{-}$

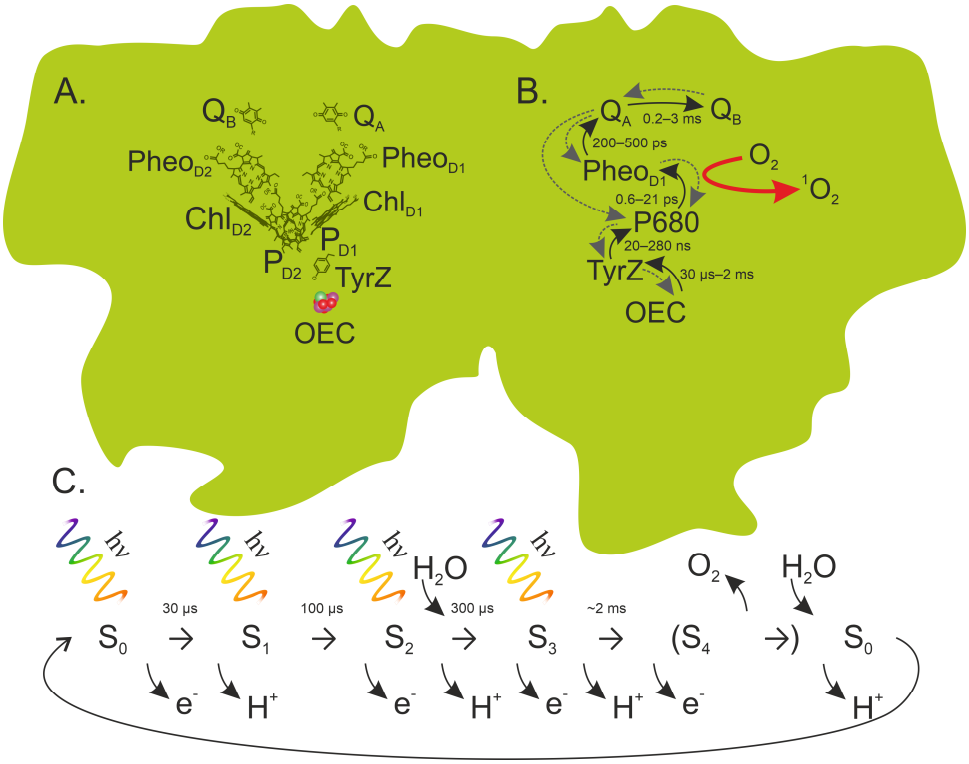

Figure 2. **Electron transfer in photosystem II.** A. Redox cofactors of PSII. The atoms in the oxygen evolving complex (OEC) are colored as follows: calcium with green, oxygen red and manganese purple. B. A schematic representation of electron transfer routes in PSII. Forward electron transfer is indicated with black continuous arrows and recombination (back-reactions) with gray dashed arrows. The red continuous arrow highlights the recombination reaction that may lead to singlet oxygen ($^1O_2$) production. C. The Kok cycle of OEC (according to Klauss et al. 2012, Siegbahn 2013, Kern et al. 2018). S-states ($S_{0–4}$) indicate oxidation of the Mn ions. Time constants are from Meyer et al. (1989), Schatz et al. (1987), Takahashi et al. (1987), Vasil'ev et al. (1996), Hastings et al. (1992), de Wijn and van Gorkom (2001) and Groot et al. (2005).

After the primary charge separation, the charge is stabilized at Q_A (a PQ molecule tightly bound to the D2 protein). Q_A^- donates the electron to a second PQ molecule, Q_B (residing in the D1 protein). After subsequent light absorption and charge separation, another electron is donated to Q_B^-. Q_B^{2-} is reduced sequentially by two protons from the stroma (most probably before and after the second electron donation from Q_A^-) and then released into thylakoid membrane (described in detail in Guskov et al. 2009, Saito et al. 2013, Zobnina et al. 2017, Yao et al. 2018).

$P680^+$ extracts an electron from a tyrosine residue of PSII (TyrZ; also called Y_Z), producing the neutral radical TyrZ• (a proton is transferred to a nearby hydrogen-bonded histidine residue; Barry and Babcock 1987, Hays et al. 1998, Junge et al. 2002). TyrZ• oxidizes the water splitting and oxygen evolving Mn_4CaO_5 complex (OEC). In the Kok cycle (Kok et al. 1970; Fig. 2C), four electrons and four protons are extracted from two water molecules with the energy of four photons (i.e. four charge separations), leading to evolution of a molecule of dioxygen (O_2). In its most reduced state (S_0), three of the Mn ions of OEC are (most probably) Mn^{+3} and one is Mn^{+4}. Each flash of light further oxidizes a Mn ion ($Mn^{+3}\rightarrow Mn^{+4}$), advancing the S-state ($S_0\rightarrow S_1\rightarrow S_2\rightarrow S_3$). After the fourth flash, a transient S_4 (possibly involving a Mn^{5+}) is produced, O_2 is spontaneously released and OEC returns to S_0. Alternating electron donation to TyrZ• and proton release into lumen occur simultaneously with oxidation of the Mn ions (Fig. 2C; Klauss et al. 2012). However, the exact mechanism, specifically formation of the O-O bond, is still under discussion (see e.g. Siegbahn 2013, Kupitz et al. 2014, Kern et al. 2018, Suga et al. 2019).

1.1.3 The mediators: plastoquinone pool, cytochrome b_6/f complex and plastocyanin

The lipid-soluble plastohydroquinone (or plastoquinol) Q_BH_2 (hereafter PQH_2) is oxidized back to PQ in the bifurcated Q-cycle of the Cyt b6f complex (Mitchell 1976). One electron from PQH_2 is donated to the lumenal soluble electron carrier protein PC, via a 2Fe-2S cluster and cytochrome f subunit of the Cyt b6f complex, and the two protons are released into the lumen. The second electron is donated to another PQ molecule (protonation of which doubles the number of protons pumped through the thylakoid membrane) via two hemes (or possibly via three; Lavergne 1983, de Lavalette et al. 2009) of the cytochrome b_6 subunit of the Cyt b6f (Mulkidjanian 2010, Laisk et al. 2016).

Those PQ molecules that mediate electron transfer from PSII to Cyt b6f comprise the photochemically active PQ pool. In spinach, the size of the pool has been estimated to range from four to 12 PQ molecules/PSII (Graan and Ort 1984, Joliot et al. 1992, Kruk and Strzałka 1999). Plants contain PQ also in plastoglobuli

(chloroplasts localized globules surrounded by a lipid monolayer) and in the chloroplast membrane (Kruk and Karpinski 2006).

Translocation of protons over the thylakoid membrane by the photosynthetic electron transport chain creates an electrochemical gradient, which ATPase uses to catalyze ATP formation from ADP and inorganic phosphate (Fig. 1C). Lumenal pH has been measured to range from 7.5 (low light) to 5.7 (high light) in plants *in vivo* (Takizawa et al. 2007). Besides the pH gradient, a significant part of the proton motive force derives from the electric component (Cruz et al. 2001). In addition to light intensity, the proton motive force is modulated by changes in the conductivity of the ATPase (Kanazawa and Kramer 2002) and via anion and cation transport through the thylakoid membrane (for reviews, see Armbruster et al. 2017, Spetea et al. 2017).

1.1.4 Structure and function of photosystem I

It is generally accepted that PSII and PSI have a common ancestry (possibly more closely related to the present-day PSI; see e.g. Orf et al. 2018). Consequently, PSII and PSI resemble each other. However, the PsaA and PsaB proteins of PSI core comprise both "reaction center" and "inner antenna". In plants, PSI is surrounded by four antenna proteins (Lhca1–4; collectively called LHCI), organized like a half-moon (for a recent X-ray structure of plant PSI with 2.6 Å resolution, see Mazor et al. 2017). The PSI complex binds over 150 chlorophylls and 30 carotenoids and consists of more than 15 subunits. Also part of the LHCII may serve PSI (see section 1.4.1). In plants, PSI resides in stroma thylakoids (Andersson and Anderson 1980).

Contrary to PSII, where primary charge separation occurs only between the cofactors bound to the D1 protein, electron transfer in PSI may proceed either in A (via cofactors bound to the PsaA protein) or B (via cofactors bound to the PsaB protein) branch (Guergova-Kuras et al. 2001, Santabarbara et al. 2015). However, if one or another of the branches is favored, is unclear (for a review, see Santabarbara et al. 2010). Both branches bind three chlorophyll *a* molecules and a phylloquinone (PhQ). The reaction center of PSI (P700) is formed by a pair of chlorophylls (one in each branch), oriented chlorin rings parallel to each other. After light absorption, primary charge separation may occur between P700 and a chlorophyll called A_0 (either A_0 in A branch or A_0 in B branch). However, it has also been suggested that charge separation can occur in both the branches: between a so-called accessory chlorophyll (which would therefore not be accessory) and the A_0 chlorophyll of each branch (Müller et al. 2003, Ptushenko et al. 2008, Giera et al. 2010). After the primary charge separation, the electron is donated to one of the PhQs. The two branches converge at a 4Fe-4S cluster (named F_X) after which the electron is donated via two additional 4Fe-4S clusters (F_A and F_B) to Fd, a soluble protein containing a

2Fe-2S cluster. After two subsequent electron donations from two reduced Fds, the flavoenzyme FNR catalyzes two electron reduction of $NADP^+$ ($NADP^+ + 2e^- + H^+ \rightarrow$ NADPH; for a review, see Mulo and Medina 2017). $P700^+$ is reduced by PC.

1.2 Singlet oxygen

In an atmosphere with 21 % oxygen, energy and electron transfer reactions inevitably lead to production of reactive oxygen species (ROS). ROS can be defined, for example, as oxygen-containing compounds that are more reactive towards biomolecules than the ground state O_2. Some relevant ROS include hydroxyl radical (HO•), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), different peroxides (e.g. lipid peroxides), nitric oxide (NO•), ozone (O_3) and singlet oxygen.

1.2.1 Very shortly about the chemistry of singlet oxygen

The two outermost electrons of ground state molecular dioxygen have equal spins and therefore O_2 is a triplet ($^3O_2$ or formally $^3\Sigma^-_gO_2$). The outermost electrons of $^3O_2$ (hereafter O_2) occupy their own orbitals (Fig. 3), as two electrons can reside on the same orbital only if their spins are opposite (Pauli exclusion principle). Because biomolecules are commonly singlets, this restricts the reactivity of O_2; reactions of O_2 can occur via one-electron reduction or after spin inversion (with input of energy) in either of the reactants. Spin inversion of one of the electrons of O_2 produces a singlet form of O_2. In the case of $^1\Sigma^+_gO_2$, the electrons reside on two different orbitals, but this form rapidly decays to $^1\Delta_gO_2$, where the two electrons reside on the same orbital (Fig. 3). The latter form is commonly abbreviated as $^1O_2$.

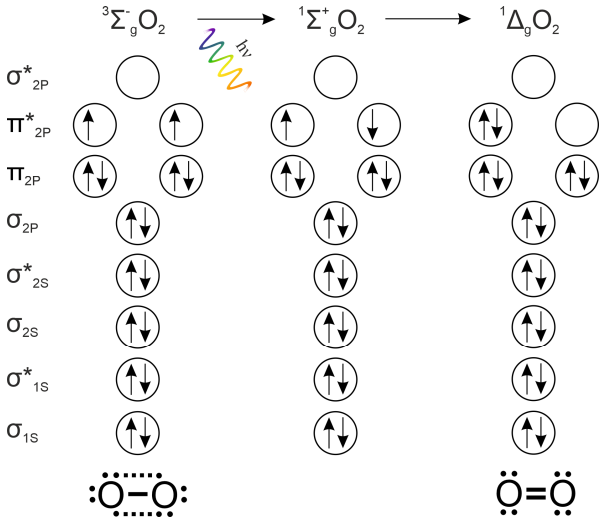

Figure 3. Different spin configurations of molecular dioxygen (O_2). Ground state triplet O_2 ($^3\Sigma^-_gO_2$) can, with addition of energy (hv), be converted to a singlet form ($^1\Sigma^+_gO_2$ or $^1\Delta_gO_2$). The two outermost electrons of $^3\Sigma^-_gO_2$ and $^1\Sigma^+_gO_2$ reside on two different molecular orbitals whereas the electrons of $^1\Delta_gO_2$ reside on a single orbital. Orbitals are represented by circles (names of the orbitals are written on the left). The arrows represent electrons (with spins "up" or "down"). Here, the "double" bond of $^3\Sigma^-_gO_2$ is approximated as a single bond and two "half" bonds, whereas $^1\Delta_gO_2$ forms a true double bond.

Due to the decreased spin demands, compared to O_2, $^1O_2$ rapidly reacts with many compounds, including proteins (e.g. histidine, cysteine, methionine, tryptophan and tyrosine), lipids (specifically unsaturated fatty acids) and nucleic acids (for reviews, see Mattila et al. 2015, di Mascio et al. 2019). Lifetime of $^1O_2$ is a few µs in water (Merkel and Kearns 1972) and 20–30 µs in lipid micelles (Lee and Rodgers 1983) but has not been measured in photosynthetic organisms *in vivo*. Consequently, the diffusion distance is not known (Mattila et al. 2015, Arellano and Naqvi 2016). Measurements from mammalian and plant systems suggest that little $^1O_2$ could diffuse out of the cells (Moan 1990, Krasnovsky 1994, Prasad et al. 2017).

1.2.2 Singlet oxygen production by chlorophylls

Intersystem crossing (spin inversion of one of the outermost electrons) converts excited singlet chlorophyll (^{1}Chl*) to triplet form (^{3}Chl), which may donate the energy to ground state O_2 producing $^1O_2$:

1. $^1Chl + \text{light energy} \rightarrow {}^1Chl^* \rightarrow {}^3Chl$
2. $^3Chl + O_2 \rightarrow {}^1Chl + {}^1O_2$

Intersystem crossing occurs with over 60 % yield in dilute chlorophyll solutions (Bowers and Porter 1967) making free chlorophylls efficient producers of $^1O_2$ (e.g. Krasnovsky et al. 1993). Intersystem crossing may produce triplet chlorophylls (and subsequently $^1O_2$) also in light harvesting antennae.

In the reaction centers, on the other hand, triplet chlorophyll is produced by charge recombination reactions. In a recombination, instead of forward electron transfer, the electron returns to P680 or P700 (see Fig. 2B for PSII). Recombination of the primary radical pair of PSII ($P680^+Pheo_{D1}^-$) can re-excite P680 or produce singlet or triplet reaction center chlorophyll (^{3}P680). If electron transfer to Q_A is not possible, recombination of the primary pair occurs in a few nanoseconds (Schlodder and Brettel 1988). In PSII centers that lack Q_A ^{3}P680 yield is high, 20–30 % per excited reaction center (Takahashi et al. 1987, Durrant et al. 1990). Recombination of electrons at Q_A^- or Q_B^- is slow (1–1.5 and 30–35 s, respectively) as both reactions are very endergonic (Vermaas et al. 1985, Vass et al. 1990). Usually, TyrZ has had enough time to reduce $P680^+$ and to advance the S-state of OEC, and therefore the S-state is reverted during the recombination: $S_{2/3}Q_{A/B}^- \rightarrow S_{1/2}Q_{A/B}$ (S_1 is stable in darkness). In addition to the recombination route re-exciting P680 (excitonic route) and the route leading to either ground state or triplet P680 via $Pheo_{D1}^-$ (indirect route), $S_{2/3}Q_A^-$ can recombine via direct tunneling (without reproducing the primary radical pair; Rappaport and Lavergne 2009). In PSII centers with an inactive OEC, Q_A^- recombines with $P680^+$ with millisecond kinetics (e.g. de Wijn and van Gorkom

2002a). At -269 °C, ^{3}P680 is located at Chl_{D1}, but at room temperature ~30 % of the triplet was suggested to reside at P_{D1} (Diner et al. 2001, Noguchi et al. 2001).

Formation of the triplet reaction center chlorophyll of PSI (^{3}P700), via recombination of the radical pair $P700^+PhQ^-$, occurs in illuminated PSI complexes and chloroplasts (Frank et al. 1979, Rutherford and Mullet 1981). Both ^{3}P680 and ^{3}P700 can be detected based on their electron paramagnetic resonance (EPR) signals (e.g. Rutherford and Mullet 1981).

1.2.3 Antenna or reaction center?

Intersystem crossing takes ~5 ns (Shipman 1979), and transfer of excitation energy from antennae to a reaction center competes with it. In addition, antennae contain carotenoids that efficiently quench both singlet excited and triplet chlorophylls (Peterman et al 1995; see also section 1.2.5), dissipating the energy safely as heat. Spectroscopic studies (Mozzo et al. 2008) showed that certain lutein (a xanthophyll carotenoid) pigments in LHCII quenched 95 % of triplet chlorophylls. Carotenoids can detoxify also $^1O_2$, produced by the remaining triplets, via physical quenching (energy transfer) or chemical scavenging (oxidation). These considerations have usually led to the assumption that not the antennae but the reaction centers (specifically P680) are the major sites of $^1O_2$ production in plants, algae and cyanobacteria (for a contradicting view, see Santabarbara et al. 2007). Calculations by Rehman et al. (2013) show that ^{3}P680 accumulation closely resembles measured $^1O_2$ production. Accordingly, Ramel et al. (2012a) reported that β-carotene (present in PSII and PSI core), but not xanthophylls (in antennae), is oxidized during illumination. Similarly, during a 10 h illumination, no turnover of chlorophyll *b* was observed (Beisel et al. 2010). Chlorophyll *a* turns over in PSII reaction centers but not in LHCs or in PSI (Feierabend and Dehne 1996, Beisel et al. 2010). On the contrary, chlorophylls in LHCII have high turnover in etiolated seedlings upon light exposure (Feierabend and Dehne 1996), suggesting that antennae may be more significant sources of $^1O_2$ (only) during their biosynthesis.

Direct quenching of triplet chlorophylls by carotenoids diminishes $^1O_2$ production also in the reaction centers of non-oxygenic bacteria (deWinter and Boxer 1999, Liu et al. 2005) and possibly in the reaction center of PSI (Cazzaniga et al. 2012). The quenching requires that the emission and absorption spectra of the donor and acceptor overlap as well as an overlap between their electron orbitals (Dexter 1953) and therefore cannot occur in PSII reaction center where the closest two β-carotenes are too far, 20–30 Å from ^{3}P680 (Umena et al. 2011). A carotenoid situated nearer would be irreversibly oxidised by $P680^+$ (formed prior to the triplet), rendering the carotenoid useless (Telfer and Barber 1995). Therefore, if a recombination reaction produces ^{3}P680, it has little other options than to react with

ground state O_2, producing $^1O_2$. β-carotenes in PSII core might still be important quenchers of $^1O_2$, as mutants with fewer β-carotene molecules per reaction center suffer from damage to PSII (Cazzaniga et al. 2012; see also section 1.3). It has also been suggested that when Q_A is reduced, ^{3}P680 would localize over Chl_{D2} and could be quenched by the carotenoid Car_{D2} (Martínez-Junza et al. 2008); ^{3}P680 decays faster when Q_A^- is present (van Mieghem et al. 1995).

Previously it was thought that PSI does not produce $^1O_2$ (Hideg and Vass 1995). Later, maybe due to more sensitive detection methods, $^1O_2$ production by isolated PSI complexes was observed (Cazzaniga et al. 2012, Takagi et al. 2016), at a rate of about 1/10th of the rate of $^1O_2$ production by PSII preparations. It has been speculated that donor and/or acceptor side limitation of PSI may enhance ^{3}P700 formation *in vivo* (Rutherford et al. 2012). Based on the above-mentioned measurements, it cannot be judged, however, if the origin of $^1O_2$ is the antenna of PSI or ^{3}P700. Based on the presence of oxidized amino acid residues in PSI core and antenna, detected by Kale et al. (2020), both sites may generate $^1O_2$. The lifetime of ^{3}P700 is not shortened by the presence of O_2 (Sétif et al. 1981), contrary to what happens to ^{3}P680 (Durrant et al. 1990). Therefore, P700 may be shielded from O_2, or a carotenoid close by very efficiently quenches ^{3}P700. PSI specific illumination could also not induce oxidation of carotenoids (Ramel et al. 2012b). In addition, it has been reasoned that recombination reactions in PSI occur via the A branch, where a recombination route that does not produce $^1O_2$ is favored (Rutherford et al. 2012). Taken together, PSI does produce some $^1O_2$ but little of it may derive from ^{3}P700.

Cyt b6f complex contains a chlorophyll molecule (for a recent cryogenic electron microscopy structure with 3.6 Å resolution, see Malone et al. 2019). Some evidence has been presented that the chlorophyll produces $^1O_2$ in the light (Sang et al. 2010), damaging the complex itself (Taylor et al. 2018). It has been suggested, however, that intact Cyt b6f produces little $^1O_2$ (Ma et al. 2009).

1.2.4 Additional sources of singlet oxygen in plant cells

Apart from photosensitization by chlorophylls, lipid peroxidation is, perhaps, the most relevant route leading to $^1O_2$ production in (photosynthetic) organisms. Oxidation of lipids leads to formation of lipid radicals, tetroxides and triplet species. Decomposition of the tetroxide may directly produce $^1O_2$ (Russel mechanism) or the triplet may donate energy to O_2 producing $^1O_2$. Once initiated, lipid peroxidation and $^1O_2$ production can continue for a long time (for reviews, see Miyamoto et al. 2007, Pospíšil et al. 2019). Oxidation of lipids (and, to a smaller degree, of proteins), possibly by TyrZ•, $P680^+$, $^1O_2$ or HO•, in PSII preparations with nonfunctional OEC has been shown to coincide with $^1O_2$ production (Hideg and Vass 1993, Pospíšil et al. 2007, Khorobrykh et al. 2011, Yadav and Pospíšil 2012). In addition,

lipoxygenase enzymes, present in plants, algae and cyanobacteria, catalyze insertion of O_2 into lipids, producing lipid peroxides (for a review, see Andreou and Feussner 2009). Some plant species also synthetize defense compounds that produce $^1O_2$ in the light (for reviews, see Tyystjärvi 2004, Flors and Nonell 2006).

1.2.5 Non-photochemical quenching and other protective measures against singlet oxygen

Non-photochemical quenching (NPQ) of chlorophyll fluorescence reflects dissipation of light energy as heat. The so-called energy-dependent quenching (qE) comprises most of regulated NPQ; in plants, acidification of lumen induces protonation of the PsbS protein (Li et al. 2000) and conversion of violaxanthin to zeaxanthin (Bilger and Björkman 1994). The exact mechanism of qE is unclear; reorganization of LHCII and interactions within chlorophylls or between chlorophylls and xanthophylls have been proposed (e.g. Holt et al. 2005, Bode et al. 2009, Müller et al. 2010, Johnson et al. 2011). In any case, NPQ decreases the amount of singlet excited chlorophylls, and consequently triplet chlorophylls, in antennae (Carbonera et al. 2012), leading to a decrease in $^1O_2$ production (Dall'Osto et al. 2012, Roach and Krieger-Liszkay 2012, Girolomoni et al. 2017). Whether NPQ affects PSI (Ballottari et al. 2014) or not (Tian et al. 2017) is debated, but also PSI complexes produce less $^1O_2$ when isolated from light-acclimated plants compared to those isolated from dark-acclimated plants (Dall'Osto et al. 2012). Instead of PsbS, in the alga (e.g. *Chlamydomonas reinhardtii*) NPQ depends on the LHCSR protein (Peers et al. 2009) and in cyanobacteria on the OCP. In cyanobacteria, the site of the quenching is the phycobilisome core rather than a chlorophyll containing antenna (Wilson et al. 2006).

Accumulation of zeaxanthin can induce NPQ also independently of lumen acidification or the PsbS protein (Dall'Osto et al. 2005, Verhoeven 2014). A newly indentified lipocalin regulated NPQ mechanism does not require zeaxanthin, lumen acidification or PsbS (Brooks 2012, Malnoë et al. 2018). In addition, damage to PSII units (section 1.3) causes NPQ. State transitions (section 1.4.1) and chloroplast movements (Cazzaniga et al. 2013, Wilson and Ruban 2020) may affect NPQ measurements but do not regulate thermal dissipation in PSII.

In addition to NPQ, the rate of $^1O_2$ production can be affected by modulation of recombination reactions. Some cyanobacteria have high and low light forms of the D1 protein (Kós et al. 2008). Redox properties of the Q_A/Q_A^- or $Pheo/Pheo^-$ pairs in the high light form of D1 favor direct charge recombinations, thereby decreasing $^1O_2$ production (Tichý et al. 2003, Vass and Cser 2009, Vinyard et al. 2013). In plants, exposure to coldness or high light can decrease the redox gap between Q_A and Q_B,

again favoring the direct recombination (Janda et al. 2000, Ivanov et al. 2006, 2008), possibly leading to a decrease in $^1O_2$ production (Ramel et al. 2012a).

Plants and other photosynthetic organisms contain multiple compounds that can quench or scavenge $^1O_2$ (for a review, see Pinnola and Bassi 2018). Lipid-soluble carotenoids (discussed above) and tocopherols (see e.g. Kruk et al. 2005, Rastogi et al. 2014) are good quenchers of $^1O_2$. In addition, lipid-soluble isoprene (Affek and Yakir 2002) and flavonoids, and water-soluble flavonoid glycosides (Majer et al. 2014), ascorbate and glutathione have been reported to offer protection against $^1O_2$.

PQH_2 molecules react with $^1O_2$ both *in vitro* and *in vivo* (Gruszka et al. 2008, Kruk and Trebst 2008, Khorobrykh et al. 2015, Ferretti et al. 2018). In plants, the photochemically active PQ pool was shown to become oxidized during high light, presumably due to a reaction with $^1O_2$ (Kruk and Szymańska 2012). Longer illumination decreased also the total amount of PQ molecules (Ksas et al. 2015, Ferretti et al. 2018). Continued high light stress increased the size of the non-photochemically active PQ pool, supporting the view that *in vivo* PQ serves also as an antioxidant (Ksas et al. 2015).

1.2.6 Where, when and why to bother?

$^1O_2$ is thought to be a major agent of damage to photosynthetic organisms. During illumination, lipid peroxidation in leaves was shown to be mainly due to $^1O_2$ (Triantaphylidès et al 2008) and the majority of the genes induced in *Arabidopsis thaliana* cell cultures by high light were identified as responsive to $^1O_2$ (González-Pérez et al. 2011). $^1O_2$ has been reported to have capacity to damage several photosynthetic protein complexes, including PSII and PSI (e.g. Hideg et al. 2007, Takagi et al. 2016; see also sections 1.3.2 and 1.3.3). Conditions producing $^1O_2$ induce the protective mechanisms discussed in the previous section. $^1O_2$ also directly initiates signaling pathways leading either to high light acclimation or programmed cell death (for a review, see e.g. Crawford et al. 2018).

Under which conditions is $^1O_2$ produced? Even though discussion about $^1O_2$ commonly relates to high light, $^1O_2$-oxidized lipids and carotenoids have been observed even in low light (Triantaphylides et al. 2008, Ramel et al. 2012a). $^1O_2$ production increases linearly with increasing intensity of light, both *in vitro* (Fufezan et al. 2002) and *in vivo* (Rehman et al. 2013). It is also often stated that reduction of the PQ pool increases $^1O_2$ production (by decreasing forward electron transfer and promoting recombination reactions). However, caution should be used, as the rate of $^1O_2$ production is not only determined by the recombination rate but also by the type of the recombination (e.g. Fufezan et al. 2002). Furthermore, only rarely measurements of $^1O_2$ are presented. Chemical inhibition of CO_2 fixation leads to a ~30 % increase in $^1O_2$ production in a dinoflagellate alga *Symbiodinium* (Rehman et

al. 2016a). In an *A. thaliana* mutant (*chlorina1*), $^1O_2$ production increases two to three fold when CO_2 concentration decreases to 50 ppm (Ramel et al. 2013); unfortunately, $^1O_2$ was measured with singlet oxygen sensor green (SOSG) in white light, when the sensor itself may produce $^1O_2$ (Ragás et al. 2009).

Accumulation of free chlorophylls inside cells would pose a risk for photosynthetic organisms (as well as for herbivorous protists; Kashiyama et al. 2012), as demonstrated in mutants with impaired control of chlorophyll synthesis (Przybyla et al. 2008). $^1O_2$ production by chlorophyll catabolites may be relevant also *in vivo*, e.g. in pathogen attack (Mur et al. 2010, Hörtensteiner and Kräutler 2011, Tarahi Tabrizi et al. 2016), but these processes are not well understood. In addition, it has been suggested that $^1O_2$ is produced during repair of damaged PSII units (Feierabend and Dehne 1996, Wang et al. 2016a; section 1.3.3). Removal of OEC favors recombination reactions that do not produce $^1O_2$ (Spetea et al. 1997, Brinkert et al. 2016; see also section 5.2.4) and therefore PSII core may not produce $^1O_2$ during repair. On the other hand, detachment of some of the chlorophylls bound to PSII core or antennae could promote $^1O_2$ production. However, to my knowledge, no direct evidence exists.

How significant are sources other than chlorophyll in $^1O_2$ production? In general, $^1O_2$ production is much higher in the light than in darkness (Hideg et al. 1994) and in photosynthetic tissues than in non-photosynthetic ones (Fig. 4A). Experiments with added electron donors or metal chelates suggest that lipid peroxidation is a significant source of $^1O_2$ in PSII preparations with inactive OEC (Pathak et al. 2017). *In vivo*, this may become relevant during stresses that inactivate PSII, such as high temperature (Yamashita et al. 2008, Rehman et al. 2016a). However, Spetea et al. (1997) measured no $^1O_2$ production in Tris-washed (OEC removed) PSII. Also enzymatic lipid peroxidation may lead to significant $^1O_2$ production under specific stresses. Prasad et al. (2017) showed that a chloroplast localized lipoxygenase induces $^1O_2$ production upon wounding. $^1O_2$ production, probably due to lipoxygenases (Chen and Fluhr 2018), was observed also inside roots after wounding (Mor et al. 2014). In addition, HO• induces lipid peroxidation upon pathogen attack (Zoeller et al. 2012).

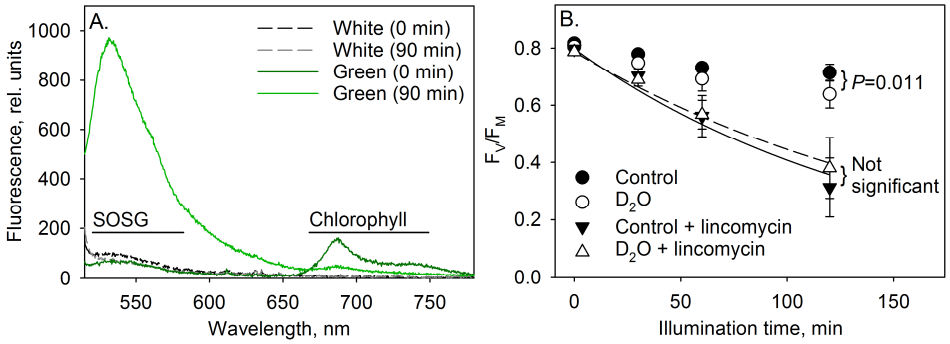

Figure 4. **Singlet oxygen and photoinhibition.** A. $^1O_2$ production in white (containing only undifferentiated plastids) or green (containing functional chloroplasts) leaf sections of *Hordeum vulgare* (cv. Variegata). Leaf discs were vacuum-infiltrated, incubated overnight in 200 µM SOSG and fluorescence (excitation wavelength 500 nm) was measured before and after a 90 min red (λ > 650 nm) light illumination (photosynthetic photon flux density, PPFD, 2000 µmol m^{-2} s^{-1}). Curves are averaged from at least three replications. Wavelength regions corresponding to SOSG and chlorophyll fluorescence are indicated. An increase in SOSG fluorescence corresponds to $^1O_2$ production and a decrease in chlorophyll fluorescence to NPQ formation and photoinhibition. B. Photoinhibition in heavy water (deuterium oxide; D_2O). Excised leaves of *A. thaliana* were incubated overnight with petioles in water (control) or in D_2O, in the absence or presence of 0.4 mg/ml lincomycin and illuminated in white light (PPFD 600 µmol m^{-2} s^{-1}). After 0–120 min of illumination, PSII yield was quantified by measuring the fluorescence parameter F_V/F_M after 30 min in dark. Error bars show standard deviations (SD) from five biological replications. Lines show a fit to the first-order reaction equation (see Materials and methods). Significances of differences between the last time points were calculated with Student's t-test. Measurements by Pooneh Sotoudehnia (A) and own work (B), Molecular Plant Biology, University of Turku.

1.3 Photoinhibition

Here, the term photoinhibition of PSII is used to describe light-induced irreversible damage to PSII that leads to loss of charge separation and O_2 evolution activities of PSII and to proteolytic degradation of the reaction center protein D1. In order to recover from photoinhibition, the D1 protein is replaced in the so-called repair cycle (section 1.3.3). PSII photoinhibition occurs in all oxygenic photosynthesis (with a possible exception of a desert alga *Chlorella ohadii*; Treves et al. 2016), from very low light to intensities several times higher than required to saturate photosynthesis. The rate of the damage is directly proportional to the intensity of light (Tyystjärvi and Aro 1996). Inactive PSII units accumulate (net photoinhibition), if repair reactions cannot keep up with the rate of the damage; this may occur e.g. in high light.

1.3.1 Photodamage to photosystem II in ultraviolet radiation

Ultraviolet (UV) radiation (200–400 nm) causes photoinhibition of PSII with much higher quantum yield than visible light (Jones and Kok 1966a), damaging mostly OEC, while the quinones and TyrZ are also affected (Renger et al. 1989, Vass et al. 1996). Loss of the reaction center activity (i.e. charge separation ability) of PSII coincides with the loss of the D1 protein (Hakala et al. 2005). The reason for the damage is probably absorption of UV radiation by Mn^{+3} or Mn^{+4} ions of OEC (Ruettinger et al. 2000, Szilárd et al. 2007), possibly leading to release of a Mn ion (Hakala et al. 2005). The exact mechanism is not clear. UV-B (280–315 nm) does not induce $^1O_2$ production, but formation of HO• was observed (Hideg and Vass 1996). The HO• scavenger propyl gallate also partly protected PSII from UV-B induced photoinhibition *in vitro* (Lazarova et al. 2014).

UV-A (315–400 nm) damages PSII with a lower yield than UV-B or UV-C (200–280 nm), but sunlight on the surface of Earth contains much more UV-A than UV-B or UV-C. Therefore, UV-A has a high capacity to cause damage under natural conditions (Sarvikas et al. 2006); 35 % of sunlight-induced photoinhibition in indoors-grown pumpkin leaves was caused by UV-A (Hakala-Yatkin et al. 2010). Many organisms have evolved protective mechanisms, such as compounds screening UV radiation (see Rozema et al. 2002, Pescheck et al. 2014, Tilbrook et al. 2016). Indeed, removal of UV-A from sunlight did not affect photoinhibition in outdoors-grown pumpkin leaves as, due to the presence of protective compounds, the UV-A radiation did not reach thylakoid membranes (Hakala-Yatkin et al. 2010).

1.3.2 How does visible light damage photosystem II?

The mechanism of photoinhibition of PSII under visible light is debated (for reviews, see Vass 2012, Tyystjärvi 2013, Nishiyama and Murata 2014). The main mechanisms discussed in recent literature may be roughly divided as $^1O_2$ or donor side hypotheses. According to the latter, the damage to PSII is caused by light absorption of the Mn ions of OEC, similarly as under UV radiation (Hakala et al. 2005, Ohnishi et al. 2005). Alternatively, long-lived $P680^+$ or TyrZ•, observed in OEC-less PSII, may extract electrons from their surroundings (Jegerschöld et al. 1990, Anderson et al. 1998). In the $^1O_2$ (or recombination) hypotheses, PSII is inactivated by oxidation of the D1 protein by $^1O_2$ produced via recombination reactions (Keren et al. 1997, Vass 2012). In addition, $^1O_2$ produced in antennae (Santabarbara et al. 2002) or other ROS (e.g. Song et al. 2006) have been proposed to cause photoinhibition of PSII.

Several lines of evidence support the involvement of recombination reactions and $^1O_2$ in photoinhibition of PSII. $^1O_2$ can damage PSII and induce degradation of the D1 protein *in vitro* (Durrant et al. 1990, Hideg et al. 2007). $^1O_2$ production and

photoinhibition have similar responses to intensity (Rehman et al. 2013) and quality (Jung and Kim 1990) of light, however, Jung and Kim (1990) used a $^1O_2$ detection method (imidazole and p-nitrosodimethylaniline) that inactivates PSII (Stemler and Jursinic 1983). Recombination reactions modulated to decrease or increase $^1O_2$ production have been shown to decrease or increase, respectively, the rate of the damage to PSII (Fufezan et al. 2002, Cser and Vass 2007, Rehman et al. 2013, Davis et al. 2016, Treves et al. 2016). In addition, quenchers and scavengers of $^1O_2$, such as carotenoids, protect PSII (e.g. Barényi and Krause 1985, Cazzaniga et al. 2012, Hakkila et al. 2014), even though the protection is not always observed (Tschiersch and Ohmann 1993, Nishiyama et al. 2001, Hakala-Yatkin et al. 2011). Dissipation of excess light energy decreases photoinhibition of PSII (e.g. Sarvikas et al. 2006), however, to a degree that may be too small to fully support the recombination hypothesis (Tyystjärvi et al. 2005). On the other hand, it was argued that conventional NPQ parameters do not correctly estimate the protective effect (Lambrev et al. 2012). Measurements by Dall'Osto et al. (2012) suggest that an NPQ deficient mutant (*npq1*) produces about two times as much $^1O_2$ as the wild type. Photoinhibition proceeded, according to Sarvikas et al. (2006), 5–20% faster in the mutant than in the wild type.

Experimental results support also $^1O_2$-independent mechanisms. Photoinhibition proceeds under anaerobic conditions (Satoh 1970, Chaturvedi et al. 1992, Hakala et al. 2005, Fan et al. 2016), where no $^1O_2$ is produced (Hideg et al. 1994). The lifetime of $^1O_2$ is lengthened by ~10 times in D_2O, but photoinhibition *in vivo* does not proceed faster in D_2O than in water (Fig. 4B). The action spectrum of photoinhibition of PSII (reviewed by Zavafer et al. 2015a) resembles the absorption spectra of Mn-clusters (Hakala et al. 2005; for an artificial Mn-cluster closely resembling that of PSII, see Zhang et al. 2015) and is dramatically different in OEC-less PSII (Hakala et al. 2005). However, peaks at orange and red lights, observed in some of the measured action spectra, support the idea that photoinhibition is at least partially driven by light absorbed by PSII antenna (e.g. Santabarbara et al. 2002, Tyystjärvi et al. 2002, Havurinne and Tyystjärvi 2017). In the Mn-hypothesis (Hakala et al. 2005), also called two-step mechanism (Ohnishi et al. 2005), after initial inactivation of OEC, $P680^+$ was assumed to cause additional damage. If OEC could recover from the initial damage (without the repair cycle) and an additional damage, via a P680 related mechanism (or via $^1O_2$), would be needed to solidify the inactivation, a deviation from first order kinetics should be observed. However, photoinhibition of PSII proceeds with first order kinetics (e.g. Sarvikas et al. 2010). Therefore, to comply with the photoinhibition kinetics, the initial damage must already irreversibly inactivate PSII and any secondary damage should inactivate another, still functional PSII unit (Tyystjärvi 2013).

The molecular nature of the event inactivating PSII is not clear, but damage to P680, $Pheo_{D1}$, the quinones (Cleland 1986, Ohad et al. 1990, Hou 2014) or OEC have been suggested. Kale et al. (2017) identified several oxidative modifications in the D1 and D2 proteins with mass spectroscopy. After 15 min of illumination, most oxidized amino acids were found in the vicinity of OEC whereas after 30 min of illumination modifications close to P680 appeared (Kale et al. 2017). This agrees with the finding that, during photoinhibition, the reaction center activity of PSII is less affected than the water splitting capacity (Chaturvedi et al. 1992, Hakala et al. 2005, Zavafer et al. 2015b). Several oxidized histidine and tryptophan residues (Kale et al. 2017) may speak for the involvement of $^1O_2$, however, the authors proposed that HO• produced after H_2O_2 formation during incomplete water oxidation by OEC and O_2•$^-$ formed by O_2 reduction by $Pheo_{D1}^-$ and/or Q_A^- caused the damage.

1.3.3 The repair cycle

Repair of damaged PSII units (for reviews, see Järvi et al. 2015, Theis and Schroda 2016) starts with monomerization of the PSII dimer and in plants with migration of PSII to grana margins or to stroma thylakoids (Yamamoto et al. 1981, Baena-González et al. 1999, Aro et al. 2005, Herbstová et al. 2012, Nagarajan and Burnap 2014). Specific zones for PSII repair may exist also in cyanobacteria (Sacharz et al. 2015). In plants, these steps are, at least partly, controlled by phosphorylation and dephosphorylation of the D1 (and D2) protein (Koivuniemi et al. 1995). PSII is partially disassembled (Christopher and Mullet 1994, Weisz et al. 2019), the D1 protein is degraded by Deg1 and FtsH proteases (Ohad et al. 1984, Lindahl et al. 2000, Silva et al 2003, Kapri-Pardes et al. 2007, Krynická et al. 2015, Knopf and Adam 2018) and a new D1 protein is co-translationally inserted into the PSII complex (Zhang et al. 1999, Zhang et al. 2001). Finally, PSII is reassembled (Bowyer et al. 1992, Rokka et al. 2005).

The repair cycle is sensitive to ROS: H_2O_2, $^1O_2$ and O_2•$^-$ all inhibit synthesis of the D1 protein (but did not affect the rate of the damage; Nishiyama et al. 2001, 2004, Jimbo et al. 2013). In *Synechocystis*, inactivation of the translation machinery is due to oxidation of cysteine residues in two elongation factors, EF-Tu and EF-G (Kojima et al. 2009, Yutthanasirikul et al. 2016). In *A. thaliana*, high light stress may transiently inactivate also the FtsH protease (Zaltsman et al. 2005, Kato et al. 2018). It was recently shown that the enzyme converting zeaxanthin back to violaxanthin upon NPQ relaxation is inactivated during photoinhibition, which possibly protects PSII repair (Bethmann et al. 2019).

As damage proceeds simultaneously with repair, it is often useful to separate the processes in research. In plants and algae, the D1 protein is coded by the chloroplast genome, and inhibitors of bacterial translation, most commonly lincomycin or

chloramphenicol (of these two, lincomycin may be preferred as it is shown not to block nuclear translation and chloramphenicol produces $O_2^{\bullet -}$; Okada et al. 1991, Mulo et al. 2003, Rehman et al. 2016b, Kodru et al. 2020), can be used to block the repair (for an example, see Fig. 4B).

1.3.4 Photoinhibition of photosystem I

PSI too can be damaged in light, specifically at low temperatures (Terashima et al. 1994) and under high (e.g. Huang et al. 2015) or fluctuating light (Tsuyama and Kobayashi 2008, Suorsa et al. 2012). In the absence of (other) electron acceptors, PSI donates electrons to O_2, producing $O_2^{\bullet -}$ (Asada et al. 1974; for the site of the production, see Kale et al. 2020 and discussion in Khorobrykh et al. 2020a). $O_2^{\bullet -}$, or other ROS derived from $O_2^{\bullet -}$ (H_2O_2 via dismutation and HO• via dismutation and Fenton chemistry), may damage PSI (Takahashi and Asada 1988, Sonoike 1996, Tjus et al. 1998, Grieco et al. 2012), possibly the Fe-S clusters (Inoue 1986, Tiwari et al. 2016). In addition, based on e.g. protective effects of scavengers of $^1O_2$, a role for $^1O_2$ has been suggested (Rajagopal et al. 2005, Cazzaniga et al. 2012, Takagi et al. 2016, Kale et al. 2020). As PSI photoinhibition requires O_2 (and electrons from PSII), it does not occur under anaerobicity (Satoh 1970). Contrary to PSII, UV radiation does not damage PSI (Jones and Kok 1966b). Recovery from PSI photoinhibition is costly (Zhang and Scheller 2004, Lima-Melo et al. 2019).

It has been suggested that the function of photoinhibition of PSII is to protect PSI (Sonoike 2011, Tikkanen et al. 2014). So, is the extent of PSII repair regulated (the extent of the PSII damage increases linearly with light, give or take some modulation by the above described protective measures such as NPQ)? Replacement of a cysteine residue in the cyanobacterial EF-Tu with an amino acid not sensitive to ROS rendered EF-Tu more tolerant against high light stress and improved PSII repair (Jimbo et al. 2018). Thus, the susceptibility of the translation machinery to oxidative stress may not be a necessity of its function but a regulation mechanism. However, in *Synechocystis*, transcription for the messenger RNA of the D1 protein increases in high light (Mohamed and Jansson 1989) following the amount of PSII photoinhibition (Tyystjärvi et al. 2002). Also the amount of EF-Tu increases in high light, leading to decreased net photoinhibition (Jimbo et al. 2019). Exposure to high light leads to increased amount and/or activity of FtsH in *C. reinhardtii* (Wang et al. 2017) and in plants (Sinvany-Villalobo et al. 2004), enhancing the repair of PSII (Tyystjärvi et al. 1992, Aro et al. 1994, Serôdio et al. 2017). Therefore, at least in continuous high light, it seems more important to keep PSII functional, rather than to limit the electron flow to PSI (by means of PSII photoinhibition; see also section 1.4.2). Sustained low electron transfer rates of PSII (compared to those of PSI) were proposed to protect PSI during sunflecks in a shade plant (quantitative comparisons

are, however, not possible based on fluorescence data only, as presented by Sun et al. 2020). Measurements of PSI functionality from *C. ohadii*, which can grow under extreme light intensities while PSII seems not to get inhibited (Treves et al. 2016), or from the cyanobacterial mutants in which the normally sensitive translation is made more tolerant against ROS (Jimbo et al. 2018), may be of interest.

1.4 Responses to changing quantity and quality of light

Photosynthetic organisms have evolved multiple mechanisms to cope with constantly changing natural conditions (for an interesting example of a plant experiencing fluctuating light intensities, see Townsend et al. 2018). Synthesis of light screening compounds (e.g. against UV radiation) can reduce the amount of irradiation received by the photosystems. Plants (and other photosynthetic organisms) can optimize light capture by leaf and chloroplast movements and by reorganizing the thylakoid membrane and photosynthetic machinery, for example by modulating antenna sizes (e.g. Kasahara et al. 2002, Khatoon et al. 2009, Betterle 2009, Zhao et al. 2020). If light is, however, absorbed in excess, NPQ can dissipate it as heat (section 1.2.5). Some of the alternative electron transfer pathways may be considered as dissipative, too. Excess light can be also partly utilized by enhancing sinks (e.g. Seemann et al. 1987). If produced in excess, ROS can be detoxified (see section 1.2.5 for $^1O_2$). Finally, damaged cell components can be repaired or replaced (see e.g. Li et al. 2018, Nakamura et al. 2018). Some of these mechanisms are discussed in more detail below.

1.4.1 Balancing the photosystems: roles for plastoquinone redox state

PQ molecules mediate electron transfer from PSII to PSI and, consequently, in low light, the redox state of the PQ pool depends on the relative electron transfer rates of the two photosystems. In high light, electron transfer through Cyt b6f complex becomes the limiting step (Stiehl and Witt 1969; section 1.4.2), which leads to reduction of the PQ pool (note that $^1O_2$ produced by high light can also oxidize PQH_2; section 1.2.5).

State transitions change excitation distribution between PSII and PSI. The STN7 kinase (STT7 in *C. reinhardtii*) is activated by reduction (occupancy of a PQH_2 molecule at the lumenal Qo site of the Cyt b6f complex) of the PQ pool (Vener et al. 1995, Zito et al. 1999). How exactly the lumenal redox message is transferred to the STN7 residing on the stromal side of the thylakoid membrane is unclear (see e.g. Vladkova 2016, Dumas et al. 2017). In any case, LHCII is phosphorylated (Depège

et al. 2003, Bellafiore et al. 2005, Pietrzykowska et al. 2014), which leads to an increase in the absorption cross-section of PSI and/or to a decrease in the absorption cross-section of PSII (state 1 → state 2). Which LHCII (those bound to PSII super-complex, or free, "extra" LHCII, those in grana margins, or all) migrate to PSI, and to which degree (if at all), is not clear (e.g. Delosme et al. 1996, Wientjes et al. 2013a, Grieco et al. 2015, Ünlü et al. 2015, Bos et al. 2019). Oxidation of the PQ pool activates the TAP38 phosphatase (PPH1 in *C. reinhardtii*, where also PBCP may be involved; Cariti et al. 2020), which leads to dephosphorylation of LHCII and back to state 1.

In plants, state transitions optimize light absorption under low light (state 2 is not induced in high light even though the PQ pool may be reduced, probably due to inactivation of STN7 by thioredoxins; Rintamäki et al. 2000, Ancín et al. 2019) or fluctuating light (Bellafiore et al. 2005, Wagner et al. 2008, Tikkanen et al. 2010) and after PSI photoinhibition (Rantala et al. 2020b). The amplitude of state transitions can be much bigger in *C. reinhardtii* than in plants (see e.g. Delosme et al. 1996, Drop et al. 2014), CP29 and CP26 are involved (Kargul et al. 2005) and a part of LHCII may disconnect from any photosystem in state 2 (Nagy et al. 2014). *C. reinhardtii* mutants incapable of state transitions suffer in high light, possibly due to increased production of H_2O_2 (Allorent et al. 2013). Redox state of the PQ pool has been shown to regulate state transitions also in cyanobacteria (Mullineaux and Allen 1990, Mao et al. 2002) but the mechanism and the contribution of the phycobilisome are not resolved (e.g. Ranjbar Choubeh et al. 2018; for a review, see Kirilovsky 2015). In addition, it was recently shown that neither the Cyt b6f complex nor phosphorylation events are involved in cyanobacterial state transitions (Calzadilla et al. 2019).

In addition, the redox state of the PQ pool has been shown to regulate the expression of chloroplast and nuclear genes. For example, LHCII transcription was shown to be affected in the green alga *Dunaliella tertiolecta* (Escoubas et al. 1995, Chen et al. 2004) and in plants (Yang et al. 2001). In cyanobacteria, the stoichiometry of the photosystems was suggested to be under the control of PQ redox state (Allen et al. 1989). However, wider scale transcription studies do not show a clear set of genes regulated by the redox state of the PQ (see e.g. Piippo et al. 2006, Bräutigam et al. 2009, Pesaresi et al. 2009, Bode et al. 2016). Signaling cascades are not well known, but an involvement of the STN7 kinase has been proposed (Pesaresi et al. 2009, Berger et al. 2014, Petrillo et al. 2014). Recently it was shown that self-phosphorylating activity of the chloroplast sensor kinase is inhibited by PQH_2 (Ibrahim et al. 2020). The kinase represses transcription of some chloroplast genes and was proposed to transfer the PQ pool redox signal to gene expression changes (Puthiyaveetil et al. 2008, Ibrahim et al. 2020). In addition, H_2O_2 produced by PQH_2 (Khorobrykh et al. 2015; for a review, see Khorobrykh et al. 2020a) has been

proposed to act as a signaling molecule, regulating e.g. stomatal closure and PSII antenna size (Borisova-Mubarakshina et al. 2015, Wang et al. 2016b).

1.4.2 Photosynthetic control

Oxidation of PQH_2 by the Cyt b6f complex may take several milliseconds (Stiehl and Witt 1969, Laisk et al. 2016), making it the slowest step of the photosynthetic electron transport chain (for discussion, see Hasan and Cramer 2012, Tikhonov 2018). Lumen acidification below pH ~6.5 further slows down oxidation of PQH_2 at the Cyt b6f (Tikhonov et al. 1984, Hope et al. 1994). Other mechanisms decreasing the activity of Cyt b6f may also exist (Heimann et al. 1998, Laisk et al. 2005). This limitation of the electron transfer rate by lumen acidification (commonly due to high light) is termed "photosynthetic control". Photosynthetic control keeps PSI sufficiently oxidized preventing photoinhibition of PSI (Grieco et al. 2012, Suorsa et al. 2012).

1.4.3 Cyclic pathways and other alternative routes

In addition to the linear electron transfer, several alternative routes exist. A cyclic electron transfer (i.e. separate from charge recombinations) within PSII is assumed to function: cytochrome b559 may reduce $P680^+$, possibly via the β-carotene Car_{D2} and/or the chlorophyll $ChlZ_{D2}$ (Knaff and Arnon 1969, Buser et al. 1992, Shinopoulos et al. 2014). Cytochrome b559 is then reduced by electrons from Q_B^- or PQ pool (for a review, see Shinopoulos and Brudvig 2012). Simultaneous proton pumping from Q_BH_2 to lumen has been proposed (Ananyev et al. 2016, 2017) even though, to my understanding, no convincing evidence exists. The pathway protects PSII either by dissipating excess energy when linear electron transfer and NPQ cannot keep the PQ pool sufficiently oxidized, or by preventing accumulation of oxidizing Car_{D2}^+ (Telfer et al. 1991, Barber and De Las Rivas 1993, Miyake and Okamura 2003, Faller et al. 2005, Feikema et al. 2006, von Sydow et al. 2016).

In contrast to the cyclic electron transfer within PSII, cyclic electron transfer from PSI (most probably via reduced Fd) back to the PQ pool conserves energy in the form of ATP (but not NADPH; see Fig. 1C). Two pathways are known to function; the PGR5 and PGRL1 dependent route (Munekage et al. 2002, DalCorso et al. 2008, Hertle et al. 2013) and the NAD(P)H dehydrogenase (or NADH dehydrogenase-like) complex dependent route (e.g. Shikanai et al. 1998). In plants, the PGR5 route seems to be more important (Munekage et al. 2004).

These cyclic pathways may balance the ATP/NADPH ratio. Based on theoretical predictions, 4.7 protons need to go through the ATPase of chloroplasts to produce an ATP molecule (e.g. Rastogi and Girvin 1999, Seelert et al. 2000), however,

measurements rather suggest ~4 (van Walraven et al. 1996, Petersen et al. 2012). Assuming the latter, linear electron transport would produce an ATP/NADPH ratio of 3/2 (=1.5), which exactly matches the ATP/NADPH need of CO_2 fixation reactions. Accordingly, Laisk et al. (2005) did not observe much cyclic electron transfer under low light, suggesting little "ATP deficiency". In addition to CO_2 fixation, ATP is needed for other chloroplast functions, such as nitrogen metabolism and protein import and synthesis. It has been suggested that cyclic electron transfer is important also in supplying ATP for the repair cycle of PSII (Endo et al. 1999, Huang et al. 2018a, Murata and Nishiyama 2018).

Cyclic electron transfer may significantly contribute to the total electron transfer during stress conditions or in the case of sink limitations (e.g. Harbinson and Foyer 1991) and during dark to light transitions (Joliot and Joliot 2002). Importantly, cyclic electron transfer induces lumen acidification, proposed to protect both PSII and PSI from photoinhibition by inducing NPQ and photosynthetic control, respectively (Munekage et al. 2002, DalCorso et al. 2008, Suorsa et al. 2012, Yamori and Shikanai 2016). Accordingly, PSI is damaged in mutants incapable of cyclic electron transfer (Munekage et al. 2004, Suorsa et al. 2012). However, the sensitivity of the *pgr5* mutant (Suorsa et al. 2012) may not be due to the inactivated cyclic electron transfer *per se* but because of impairments in Cyt b6f complex (Buchert et al. 2020) or proton conductivity of the ATPase (Avenson et al. 2005). Upon high light, acidification of lumen is accelerated also by ion channels and decreased proton conductivity of the ATPase (Armbruster et al. 2017, Huang et al. 2018b).

O_2 reduction by PSI or plastid terminal oxidase (for a review, see Nawrocki et al. 2015) may at least in some conditions contribute to oxidation of the PQ pool (Asada 2000, Li et al. 2016, Kambakam et al. 2016, Rantala et al. 2020b). In the dark, NADH dehydrogenase-like complex may reduce the PQ pool (Burrows et al. 1998). In cyanobacteria, some algae and gymnosperm plants, flavodiiron proteins function as efficient electron acceptors of PSI (for a review, see Alboresi et al. 2019).

2 Aims of the study

The present work intends to find answers to basic research questions, related to photosynthetic reactions and their regulation in plants and cyanobacteria, which have so far remained unresolved. More specifically, I aimed:

1. To develop detection methods for $^1O_2$, and to measure $^1O_2$ from photosynthetic organisms in order to understand the origin and role of $^1O_2$ in photosynthesis, especially as a damaging agent,
2. To resolve the mechanism(s) of photoinhibition of PSII, and
3. By directly measuring the redox state of the photochemically active PQ pool from plants, to better understand how the redox state of the PQ pool responds to different light conditions.

3 Materials and methods

3.1 Organisms and growth conditions

The main organisms used in the present work were wild types of the plants *A. thaliana* and *Cucurbita maxima* (pumpkin) and the cyanobacterium *Synechocystis* sp. PCC 6803. Plants were grown under PPFD of 100–200 µmol m^{-2} s^{-1} at ~20 °C with day/night rhythms of 8 h/16 h for *A. thaliana* and 16 h/8 h for *C. maxima*. *Synechocystis* was grown in BG-11 (Rippka et al. 1979; buffered to pH 7.5 with 20 mM Hepes-KOH) as batch cultures with mixing under constant PPFD of 40 µmol m^{-2} s^{-1} at 32 °C. Thylakoid membranes were isolated from leaves as described in Hakala et al. (2005) and stored at -75 °C until use.

3.1.1 Light sources

"White" lights may greatly differ from each other. Spectra of growth lights for *A. thaliana* (growth chamber) and *C. maxima* (greenhouse), as well as spectra of the Sun and several high light sources used here are presented in Fig. 5.

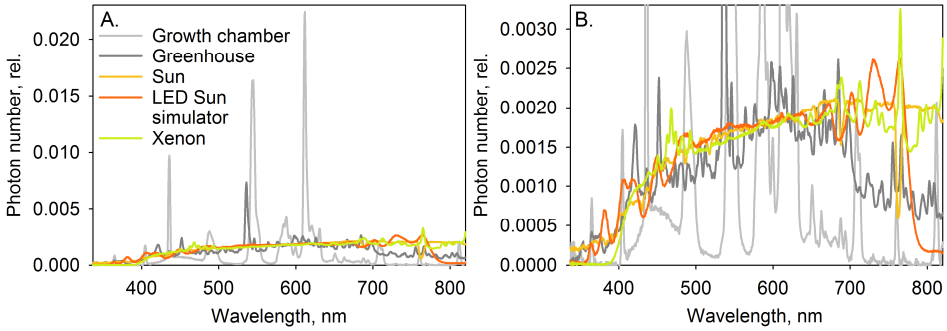

Figure 5. **Spectra of different light sources.** A. Photon number spectra, normalized to equal number of photons at 400–800 nm, from Osram Powerstar HQI-BT lamps (a Weiss Gallenkamp growth chamber), a halogen lamp (Greenhouse), the Sun (measured on 27.2.2020 on a cloudless day in Turku, Finland), an LED (light emitting diode) based sunlight simulator (SLHolland) and a high-pressure xenon lamp (Sciencetech Inc). B. The scale of the Y-axis is reduced for better visualization of the continuous spectra.

Monochromatic illumination was obtained with different LEDs (LED Fedy, China) or defined by filters (e.g. Corion, Newport Corporation), UV radiation with VL-8.LC and VL-8.M lamps (Vilber Lourmat, France) and short flashes with a Nd:YAG laser (532 nm, 4 ns; Continuum, USA) or an FX-200 xenon lamp (3 µs; EG&G, USA). Intensity and spectra were measured with wavelength-calibrated light sensors, LI-189 (LiCor, USA) and STS-VIS (Ocean Optics).

3.2 Chlorophyll *a* fluorescence and P700 signal

Photosynthesis can be studied by chlorophyll *a* fluorescence; fluorescence competes with charge separation and heat dissipation (in PSII). Pulse amplitude modulated (PAM) fluorometry was used to probe the quantum yield of PSII photochemistry (F_V/F_M), electron transfer rate, NPQ, photochemical quenching (qP or qL; Maxwell and Johnson 2000, Kramer et al. 2004) and other PSII parameters. Recombination reactions were measured with thermoluminescence: a thylakoid sample was given a Xenon flash at -10 °C and fluorescence yield was recorded while heating the sample at a constant rate (0.56 °C/s). Absorption cross-sections of PSII and PSI were studied by measuring fluorescence spectra at -196 °C (77 K; in liquid N_2): a dilute thylakoid sample (to prevent re-absorption of fluorescence resulting in an over-estimation of light absorption by PSI) was excited with monochromatic light with a wavelength shorter than those of chlorophyll fluorescence.

Contribution of PSI to variable fluorescence at room temperature is small (Franck et al. 2002). Instead, redox states of P700 and PC were followed by measuring absorbance changes at specific near-infrared (NIR) wavelengths. NIR signals recorded by DUAL-KLAS-NIR fluoro/spectrometer (Walz GmbH) were empirically deconvoluted by Schreiber and Klughammer (2016).

3.3 Quantification of photoinhibition

Photoinhibition of PSII was measured by the fluorescence parameter F_V/F_M (after dark incubation to relax NPQ) or by light-saturated rate of O_2 evolution of thylakoids or *Synechocystis* cells in the presence of artificial electron acceptors. In the case of *Synechocystis* cells or intact leaves, the repair cycle was blocked with lincomycin. In the absence of repair, the rate constant of photoinhibition of PSII (k_{PI}) was calculated by fitting the loss of PSII activity to the first-order reaction equation ($y=A*e^{-kPI*t}$, where y is F_V/F_M or O_2 evolution and t is time. A was fixed to a control value of F_V/F_M or PSII oxygen evolution). In isolated thylakoid membranes, also the rate of PSII inactivation in darkness was measured, and this rate constant was subtracted from the final values.

3.4 Measurements of singlet oxygen

$^1O_2$ was measured from thylakoid membranes with 2,2,6,6-tetramethylpiperidine (TEMP) or histidine. The reaction of TEMP with $^1O_2$ is specific to $^1O_2$ (Zang et al. 1995) and produces 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), which can be detected with EPR (Lion et al. 1976). Here, a method to quantify TEMPO with ultra high-performance liquid chromatography ((U)HPLC; Acquity UPLC, Waters Corporation, USA) combined with an electrospray ionization triple quadrupole mass spectrometer (Xevo TQ, Waters Corporation) was developed. The column used was Acquity UPLC BEH Phenyl (Waters Corporation). Acetonitrile and 0.1 % formic acid were used as solvents. The rate of the reaction of TEMP with $^1O_2$ increases with pH (Houba-Herin et al. 1982), and therefore an alkaline pH 8.7 was used.

Histidine reacts with $^1O_2$, but not with H_2O_2 or $O_2^{\bullet-}$ (Rehman et al. 2013). In a biological system, $^1O_2$ may be deactivated by collisions with water molecules ($O_2 \rightarrow {}^1O_2 \rightarrow O_2$), other quenchers and potential reaction partners (e.g. Merkel and Kearns 1972); in the presence of histidine, less of $^1O_2$ is deactivated by collisions with water. Therefore, a decrease in O_2 concentration in the presence of added histidine reflects $^1O_2$ production by the sample (Telfer et al. 1994, Rehman et al. 2013). O_2 concentration was recorded with an O_2 electrode (Hansatech Instruments Ltd, UK) or an optical sensor (Firesting, PyroScience GmbH). Isolated thylakoid membranes consume O_2 in the absence of histidine (mainly due to O_2 reduction by PSI; Asada et al. 1974) and thus measurements in the presence and absence of histidine are needed. Different stable isotopes of O_2 (^{16}O and ^{18}O; ^{18}O is rare in the nature) were distinguished with membrane inlet mass spectroscopy (MIMS); gases diffused from a sample chamber through a PTFE membrane via vacuum lines to Prima PRO Process Mass Spectrometer (Thermo Scientific™). The dye Rose Bengal was used to chemically produce $^1O_2$ in light with a known yield (Redmond and Gamlin 1999).

3.5 Measurements of plastoquinone

Total amount and the redox state of the PQ pool were measured with HPLC (Agilent; Kruk and Karpinski 2006) as described in Khorobrykh et al. (2020b), from plant leaves. Shortly, leaves were fast (~10 s) ground in ice-cold dry ethyl acetate under treatment conditions, the extract was dried under N_2 flow, dissolved to a solution of methanol and hexane and divided into two parts; the other part was immediately assayed with HPLC. All PQ in the other part was reduced by freshly made $NaBH_4$ (2 mM). Based on these measurements, the ratio of PQH_2 to all PQ in the sample can be calculated. To obtain fully oxidized photochemically active PQ pool as a reference, leaves were illuminated for 4 min with 690 nm light (PPFD 50 μmol m^{-2} s^{-1}), and dark-acclimated leaves were illuminated for 30 s with high light (PPFD 2000 μmol m^{-2} s^{-1}), to obtain fully reduced photochemically active PQ pool.

4 Overview of the results

4.1 Mass spectroscopy could be used to quantify singlet oxygen

A method, combining liquid chromatography with a triple quadrupole mass spectrometer (UHPLC-MS), was developed to quantify TEMPO, the reaction product of TEMP and $^1O_2$. A high concentration of TEMP (here 20 mM was used) is required to detect $^1O_2$ from photosynthetic material. Therefore, to protect the mass spectrometer, samples were diluted 50-fold prior to the analysis. As the concentration of the reaction product, TEMPO, is much smaller than that of TEMP, further dilution should be avoided. Reliable quantification of TEMPO was ensured by directing only TEMPO to mass spectrometer; otherwise the UHPLC flow (including TEMP) went to waste. With the UHPLC-MS method, TEMPO was first quantified from solutions with known concentrations of TEMPO. The response of the signal to the amount of TEMPO was linear (Fig. 4 in Paper I). TEMPO could be detected also from samples where $^1O_2$ was produced by illuminating Rose Bengal or isolated pumpkin (*C. maxima*) thylakoid membranes in the presence of TEMP (Fig. 4 in Paper I). The results correlated well (R^2 = 0.83–0.99; Fig. 7 in Paper I) with quantifications of the same samples with a traditionally used EPR method. The rate of the reaction between TEMP and $^1O_2$ was calculated based on how much the Rose Bengal solution, which absorbed 2.6 % of the light, produced TEMPO. It could be then calculated that thylakoids produced 3.7 x 10^{-7} $^1O_2$ chlorophyll^{-1} s^{-1}.

The newly developed UHPLC-MS method allows fast measurements (one sample takes a bit over seven min) of multiple samples. However, long storage, even at -75 °C under an inert gas, is not advisable, as the signal decreased up to ~30 % during the storage (Fig. 6 in Paper I).

4.2 Nascent oxygen produced by photosystem II was not converted to singlet oxygen

If histidine is added to a sample, it efficiently reacts with $^1O_2$ (mostly originating from gaseous O_2) and thereby decreases the O_2 concentration of the sample; the more $^1O_2$ is produced, the faster is the decrease (Telfer et al. 1994, Rehman et al. 2013;

see Materials and methods for details). Consequently, $^1O_2$ production can be measured by recording O_2 concentration in the presence and absence of histidine. Here, oxygen ($^{16}O_2$) dissolved in the buffer was replaced with a heavier isotope ($^{18}O_2$) and concentrations of the two isotopes were monitored with MIMS. With this method, it is possible to differentiate oxygen evolution ($H_2{}^{16}O \rightarrow {}^{16}O_2$; water splitting by PSII) from oxygen consumption ($^{18}O_2 \rightarrow {}^{18}O_2{}^{\bullet-}$; mostly due to electron donation by PSI). Illumination of isolated thylakoid membranes of pumpkin in the presence of histidine accelerated consumption of $^{18}O_2$ (compared to the control without added histidine) but did not significantly affect the rate of $^{16}O_2$ evolution (Fig. 6A; Fig. 1 in Paper II). The result indicates that the $^1O_2$ histidine reacted with derived from dissolved ambient gas ($^{18}O_2$), but the nascent $^{16}O_2$ produced by PSII was not converted to $^1O_2$ (Fig. 6B). Very similar results were obtained with two different initial oxygen ($^{16}O_2 + {}^{18}O_2$) concentrations (~280 µM in Fig. 6 and 150 µM in Paper II; air-saturated concentration of oxygen at 25 °C in fresh water is 253 µM). In the present experimental setting, all $^{16}O_2$ could not be replaced with $^{18}O_2$; before switching on the light, 15 to 25 % of the ambient oxygen was of the lighter isotope $^{16}O_2$. Theoretically, this would lead to a small decrease (~5 % of the observed $^{16}O_2$ evolution) in apparent oxygen evolution in the presence of histidine, very similarly as observed (Fig. 6A; assuming that all $^1O_2$ derived from ambient dissolved gas and not from nascent oxygen).

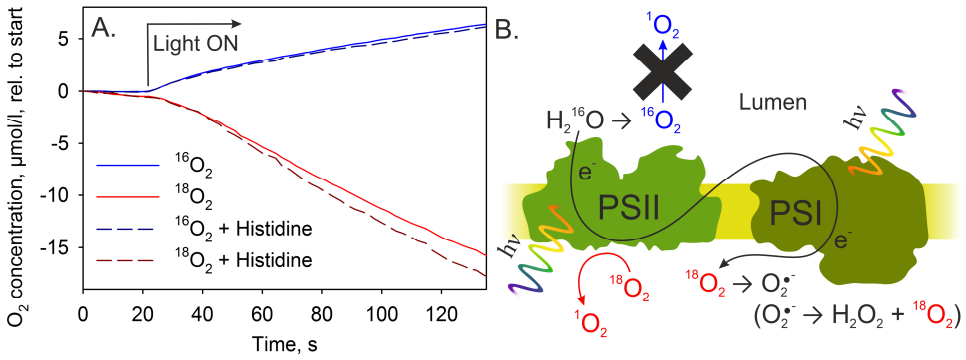

Figure 6. **Origin of singlet oxygen in photosystem II.** A. Concentrations of two oxygen isotopes ($^{16}O_2$ and $^{18}O_2$) upon a high light (PPFD 3000 µmol m^{-2} s^{-1}) illumination at 25 °C in isolated thylakoids of pumpkin in the presence and absence of 20 mM histidine, measured with MIMS. The difference between samples with and without histidine represents production of $^1O_2$. Prior to the measurements, dissolved oxygen in the sample was removed and replaced with $^{18}O_2$. The curves are normalized to the respective oxygen concentrations at the time point zero. The data represent averages from at least three replications. B. A schematic representation showing the proposed origin of $^1O_2$; $^1O_2$, produced in the light by PSII, derives from ambient dissolved gas ($^{18}O_2$ in this case), not from nascent oxygen ($^{16}O_2$) originating from splitting of water ($H_2{}^{16}O$) by PSII.

Next, to test whether active or inactive PSII is needed for $^1O_2$ production, leaves and isolated thylakoid membranes of pumpkin were illuminated for different times to inactivate different proportion of PSII units, after which $^1O_2$ production was measured with the histidine method. In the case of leaves, thylakoid membranes were isolated immediately after the illumination treatment and prior to $^1O_2$ measurements. The results show that the capacity of PSII to evolve O_2 (or the amount of PSII photoinhibition) did not affect the rate of $^1O_2$ production (Fig. 2 in Paper II).

4.3 Photoinhibition of photosystem II

4.3.1 $S_2Q_A^-$ recombination reactions cannot be the reason for photoinhibition caused by visible light or laser pulses

To resolve the mechanism of photoinhibition of PSII in visible light, the temperature dependence of the damaging reaction at 3–40 °C was measured. In white light, the rate constant of photoinhibition, measured *in vitro* from isolated pumpkin thylakoid membranes and *in vivo* in the presence of lincomycin from pumpkin leaves and *Synechocystis* cells, slowly increased with increasing temperature (Fig. 1A in Paper III). Similar response in all the three experimental materials show that the observed positive temperature dependence is a universal feature of PSII photoinhibition.

The temperature dependence was measured also under UV radiation (UV-A, UV-B and UV-C), blue light ($\lambda < 450$ nm) and red light ($\lambda > 650$ nm). Photoinhibition depended on temperature rather similarly in all the cases, however, slightly steeper temperature dependence was observed under visible light than under the tested UV wavelengths (Fig. 1E–F in Paper III). In addition, the temperature depencence steepened towards longer wavelengths also under visible light (Fig. 1E–F in Paper III). The results indicate that more than one dominant photoinhibition mechanism functions under visible light.

$^1O_2$ produced by the slow recombination reactions between the S-states of OEC and the quinone acceptors ($S_{2/3}Q_{A/B}^- \rightarrow S_{1/2}Q_{A/B}$) have been proposed to cause photoinhibition of PSII (e.g. Vass and Cser 2009). Therefore, the rate of $^1O_2$ production in pumpkin thylakoids in white light was measured with the histidine method; the rate of $^1O_2$ production increased with temperature very similarly as the rate constant of photoinhibition (Fig. 2A in Paper III). Next, the temperature dependences of $S_2Q_A^-$ recombination reactions were measured by thermoluminescence (Fig. S4 in Paper III). Compared to photoinhibition and $^1O_2$ production, the rate constants of the $S_2Q_A^-$ recombination reactions very strongly depended on the temperature at 0–40 °C (Fig. 1C in Paper III). *In vivo*, the rate of

the charge recombination depends on the rate constant of the recombination, but also on the amount of the substrate (Q_A^-); in pumpkin leaves Q_A was slightly less reduced at higher temperatures (Fig. 1B in Paper III). However, the temperature response of the rate of the direct $S_2Q_A^-$ recombination reaction in pumpkin leaves, obtained by multiplying the rate constant by the fluorescence parameter 1-qL (reflecting the amount of Q_A^-), did not resemble the observed temperature dependence of photoinhibition nor that of $^1O_2$ production (Fig. 1C in Paper III).

Based on the fact that the efficiency of short laser pulses to damage PSII increases with increasing pulse interval (Fig. 1D in Paper III), it has been suggested that the $S_{2/3}Q_{A/B}^-$ recombination reactions cause laser pulse photoinhibition (as the longer the interval the higher the probability of the recombination; Keren et al. 1997). However, photoinhibition of PSII induced by 4 ns flashes did not have any observable temperature dependence (Fig. 1D in Paper III).

To conclude, the $S_2Q_A^-$ recombination reactions cannot be the main reason for photoinhibition of PSII or $^1O_2$ production.

4.3.2 Singlet oxygen did not affect photoinhibition

To understand the role of $^1O_2$ in photoinhibition, several experiments affecting the rate of production and quenching of $^1O_2$ were conducted. Quenchers of $^1O_2$ did not protect PSII during illumination of thylakoid membranes (Fig. 2B in Paper III). Both a water-soluble quencher (histidine) and a lipid-soluble quencher (α-tocopherol) were tested, as water-soluble quenchers are expected to react only with $^1O_2$ that already has escaped PSII and therefore may not be anymore very dangerous to PSII (Telfer et al. 1994, Stamatakis et al. 2016).

According to Hideg et al. (1994) $^1O_2$ is not produced under anaerobic conditions. However, photoinhibition proceeded under anaerobicity, and the rate was even faster than under aerobic conditions (Fig. 2C in Paper III). In addition, photoinhibition under anaerobic conditions depended on temperature and wavelength of illumination similarly as aerobic photoinhibition (Fig. 2D in Paper III). Previously it has been shown that a reciprocity between duration and intensity of illumination on the amount of PSII damage holds for anaerobic photoinhibition, as it holds for aerobic photoinhibition (Park et al. 1997, Serôdio et al. 2017). These data suggest that the same mechanism(s) causes photoinhibion of PSII both in the presence and absence of oxygen, indicating that $^1O_2$ cannot be the sole inducer of the damage.

Here, anaerobicity was achieved by constantly flushing the sample with nitrogen gas, and low O_2 concentrations may have persisted due to water splitting of PSII (though, in the absence of O_2 that functions as the only terminal acceptor in isolated thylakoid membranes, PSII O_2 evolution markedly diminishes). To eliminate this possibility, the experiments were repeated in the presence of 3-(3,4-dichlorophenyl)-

1,1-dimethylurea (DCMU), which binds to the Q_B site of PSII, blocking O_2 evolution. Anaerobicity did not protect PSII from photoinhibition with DCMU either (Fig. 2C in Paper III). Curiously, photoinhibition in the presense of DCMU did not have any clear temperature dependence.

4.3.3 Photoinhibition proceeded even at -78.5 °C

To probe the role of the recombination of the primary pair ($P680^+Pheo_{D1}^-$) in photoinhibition of PSII, pumpkin thylakoids were frozen and illuminated at -78.5 °C. At this temperature, the only recombination reaction capable of producing $^1O_2$ is that of $P680^+Pheo_{D1}^-$ (Takahashi et al. 1987). Even at -78.5 °C, PSII was photoinhibited, both under visible light and UV radiation (Fig. 4 in Paper III). Similarly to photoinhibition at physiological temperatures, degradation of the D1 protein was induced by illumination at -78.5 °C and took place after melting the sample (Fig. 4B in Paper III). However, contrary to photoinhibition at physiological temperatures, anaerobicity almost completely abolished the damage to PSII (Fig. 4A in Paper III).

4.4 Redox state of the plastoquinone pool

4.4.1 F_0 rise revealed visible light wavelengths favoring photosystem II or photosystem I

When an illumination of moderate intensity ceases, chlorophyll *a* fluorescence yield decreases to a dark level (F_0), but after a few seconds a small bump, called F_0 rise, is often observed (Fig. 7A). The increase in fluorescence level has been proposed to be due to reduction of the PQ pool by the NADH dehydrogenase-like complex (Endo et al. 1997). However, fluorescence probes the redox state of Q_A, not that of the PQ pool, and the big difference in redox potentials between Q_A and PQH_2 (e.g. De Causmaecker et al. 2019) makes direct reduction of Q_A by a plastoquinol molecule an unlikely event. Accordingly, an attempt to record F_0 rise in total darkness (the measuring beam of the PAM fluorometer was switched on every five seconds, only for a short time) resulted in no rise in fluorescence level (Fig. 1 in Paper IV). If, on the top of the chopped measuring beam, low intensity ”activating” light was applied, F_0 rise was restored (Fig. 7A; Fig. 1 in Paper IV). Even with activating light, F_0 rise was not observed in a mutant lacking the NADH dehydrogenase-like complex (Fig. 1B in Paper IV).

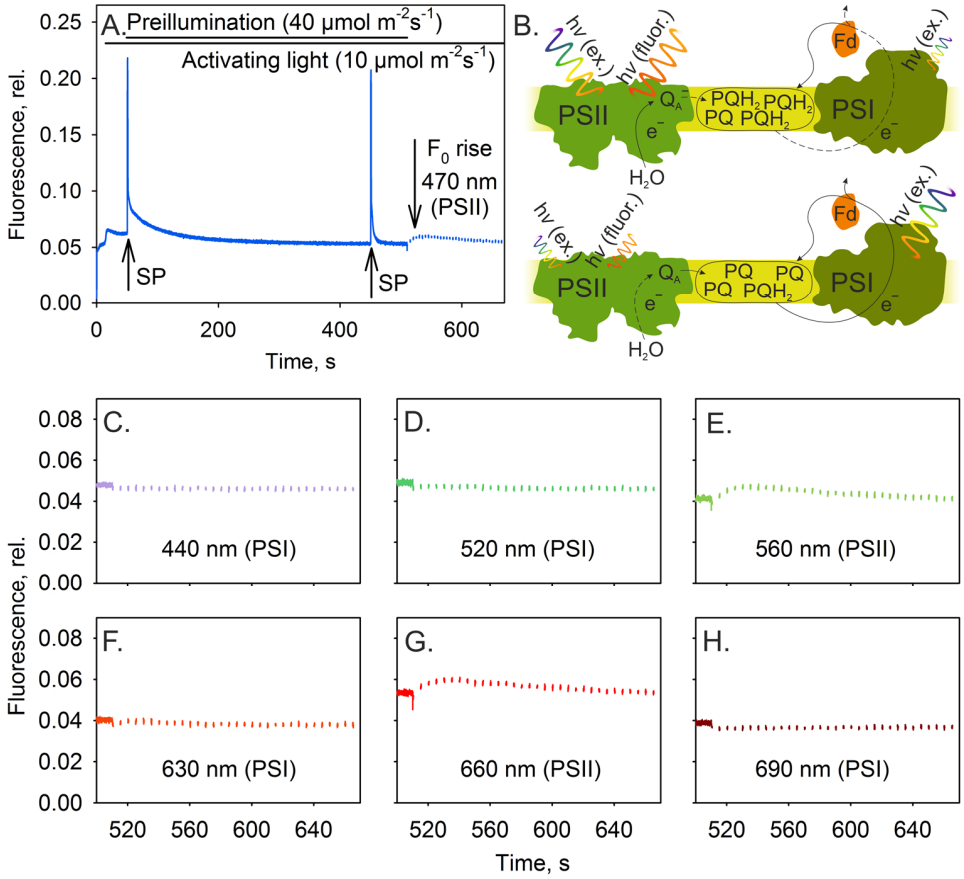

Figure 7. **Activated F_0 rise in leaves of *Nicotiana tabacum*.** A. The measurement protocol. Leaves were illuminated with white preillumination light (from a KL-1500 halogen illuminator), weak activating light and saturating pulses (SP; upward arrows), and chlorophyll *a* fluorescence was measured with a PAM fluorometer. Durations (by horizontal lines) and intensities of illumination are indicated. At the end of the preillumination, the measuring beam of the fluorometer was switched to a chopped mode (on 0.6 s for every five seconds), to record a post-illumination rise in the fluorescence level (F_0 rise; indicated with a downward arrow). The wavelength of the activating light is indicated next to the F_0 rise. PSII or PSI indicates whether the activating light favors PSII or PSI, respectively. B. A model explaining the wavelength-dependence of the appearance of an F_0 rise. If activating light (hv (ex.)) excites PSII more efficiently than PSI, accumulation of PQH_2 (by components of PSI cyclic electron flow) slows down re-oxidation of Q_A^-, thereby increasing fluorescence (hv (fluor.)). Activating light favoring PSI, oxidizes the PQ pool, leading to fast re-oxidation of Q_A^- and low fluorescence. C–H. Inserts showing the post-illumination fluorescence transient. The wavelength of the activating light is indicated. The curves have been averaged from three replications.

Therefore, an F_0 rise occurs when re-oxidation of Q_A^- (reduced by the measuring beam of a fluorometer or by the activating light) slows down when the PQ pool is

reduced. Consequently, activated F_0 rise should be sensitive to the electron transfer balance between PSII and PSI, as activating light favoring PSII over PSI would efficiently reduce Q_A (giving rise to an F_0 rise), whereas activating light favoring PSI would suppress the F_0 rise because PSI would oxidize the PQ pool (Fig. 7B).

Next, the activated F_0 rise method was used to screen visible light wavelengths that would favor PSII or PSI; an action spectrum of F_0 rise in light-acclimated *N. tabacum* leaves was measured with monochromatic illumination for the activating light, with 10 nm intervals. Three wavelength ranges favoring PSII (460–500 nm, 560–570 nm and 650–660 nm) and four favoring PSI (420–440 nm, 520 nm, 630 nm and 680–690 nm) were found, irrespective if the PPFD of the activating light was 0.5 (data not shown), 2.5 (Fig. 2A in Paper IV) or 10 (Fig. 7) μmol m^{-2} s^{-1}. Separate measurements of fluorescence yield, P700 oxidation and 77 K fluorescence (Figs. 2, 4 and 6 in Paper IV) confirmed the PSI or PSII nature of the wavelengths.

4.4.2 The photochemically active plastoquinone pool was reduced by three and oxidized by four visible light wavelengths

As any fluorescence method, including the activated F_0 rise, probes PQ pool indirectly, PQ was next extracted from light acclimated *A. thaliana* leaves and the redox state of the photochemically active PQ pool was measured with HPLC (Fig. 8A). The photochemically active PQ (i.e. PQ molecules taking part in electron transfer from PSII to PSI; defined here as those PQ molecules that can be reduced by high light and oxidized by far-red light) comprised ~50 % of all PQ + PQH_2 of the leaves (Fig. 8B).

Illumination for four min with moderate intensity (PPFD 50 μmol m^{-2} s^{-1}) of light favoring PSII (470 nm, 560 nm or 660 nm) reduced 80–90 % of the photochemically active PQ pool (hereafter PQ pool), and illumination with 440 nm, 520 nm or 690 nm PSI light led to oxidation of 90–100 % of the pool (Fig. 3 in Paper IV). Illumination with 630 nm light reduced 40–60 % of the PQ pool (Fig. 3 in Paper IV). After three h of darkness, ~30 % of the photoactive pool PQ pool was reduced (Fig. 8B; Fig. 3 in Paper IV).

4.4.3 The lights favoring photosystem II or photosystem I induced related physiological changes

Illumination (PPFD 50 μmol m^{-2} s^{-1}) of plant leaves with wavelengths favoring PSII (560 nm or 660 nm) induced phosphorylation of LHCII and illumination with wavelengths favoring PSI (430 nm or 520 nm) induced dephosphorylation (Fig. 10B in Paper IV). Occurence of state transitions was confirmed with 77 K fluorescence

measurements; illumination with PSII wavelengths (470 nm, 560 nm or 660 nm) led to a decrease in the absorbtion cross-section of PSII relative to that of PSI, and illumination with PSI wavelengths (420 nm, 520 nm or 680 nm) to an increase (Fig. 10 in Paper IV). At the begining of the illumination 440 nm, 520 nm, 630 nm or 690 nm light oxidized P700 and PC (oxidation was persistent towards the end of the four min illumination only in 690 nm), whereas in 470 nm, 560 nm and 660 nm light both P700 and PC were almost completely reduced (Fig. 6 in Paper IV). Similarly as in the fluorescence experiments (Fig. 7F; Figs. 2, 4 and 6 in Paper IV), 630 nm light behaved as a PSI light in the PC and P700 measurements. However, 630 nm induced more LHCII phosphorylation than the 430 nm and 520 nm PSI lights, and 77 K measurements revealed state transitions that were somewhere between those induced by extreme PSII or PSI wavelengths, in accordance with the observed 40–60 % reduction of the PQ pool after 630 nm illumination (Figs. 3 and 10 in Paper IV).

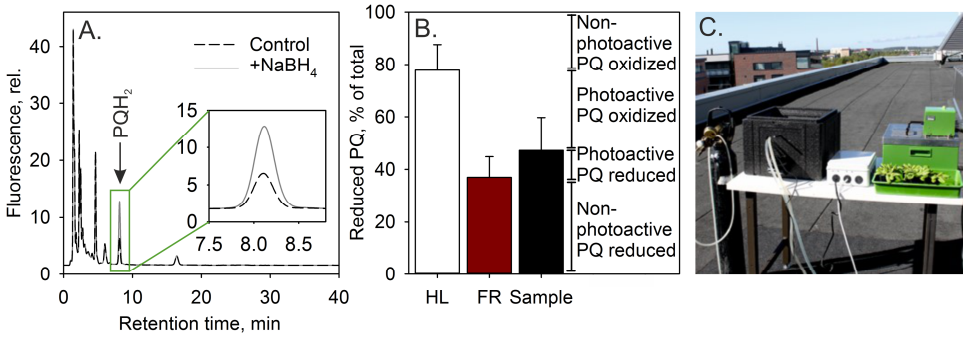

Figure 8. **Redox state of the plastoquinone pool in leaves of *A. thaliana* measured with high performance liquid chromatography.** A. Representative chromatograms from a leaf extract, divided into two parts, one in the absence (black dashed line) and the other in the presence (grey continuous line) of $NaBH_4$ (used to reduce all PQ in the sample). The insert shows an enlarged view of the PQH_2 peak. B. Percentage of reduced PQ (of total PQ + PQH_2) after 30 s high light illumination (PPFD 2000 µmol m^{-2} s^{-1} on dark-acclimated leaves; HL), four min illumination with 690 nm (PPFD 50 µmol m^{-2} s^{-1} on light acclimated leaves; FR) and three h in darkness (Sample). Proportions of oxidized and reduced PQ in non-photochemically active (non-photoactive) and photochemically active (photoactive) PQ pools for the dark sample are indicated. Error bars show SD, calculated from three biological replications. C. The setting for treatments under sunlight.

4.4.4 Redox state of the plastoquinone pool in white light

The experiments so far were carried out with monochromatic light; however, natural light is always polychromatic. Therefore, the redox state of the PQ pool was measured after illumination with various sources of white light. All the white light sources used here, at the PPFD of 50 µmol m^{-2} s^{-1}, oxidized more than 50 % of the photochemically active PQ pool. Next, an empirical model, based on the observed

effects of 25 monochromatic and polychromatic light sources on the redox state of the PQ pool, calculating how much a single wavelength of light affects the reduction or oxidation of the PQ pool, was developed (Fig. 7 in Paper IV). The analysis showed that green and red PSI wavelengths, in addition to far-red, often contributed significantly to oxidation of the PQ pool (Fig. 8 in Paper IV). Green and red PSII wavelengths were often equally important in reducing the PQ pool, and in natural sunlight also blue PSII wavelengths contributed significantly to the reduction (Fig. 8 in Paper IV).

In addition to the wavelength of light, intensity also affects the redox state of the PQ pool. Next, intensity responses of a PSII light (470 nm), a PSI light (440 nm) and natural sunlight (Fig. 8C) were measured. 50 % of the PQ pool was already reduced at the PPFD of ~25 μmol m^{-2} s^{-1} of the PSII light, whereas the intensity response of the PSI light was much weaker; at the PPFD of 200 μmol m^{-2} s^{-1}, only 30 % of the PQ was reduced (Fig. 9A in Paper IV). Similarly to all the other studied white light sources, sunlight slightly favored PSI and a gradual reduction of the PQ pool with increasing intensity, faster than in 440 nm PSI light but clearly slower than in 470 nm PSII light, was observed (Fig. 9A in Paper IV).

Finally, the rate of the change in the PQ pool redox state was measured. Alternating one min of high light (PPFD 500 μmol m^{-2} s^{-1}) with four min of low light (PPFD 45 μmol m^{-2} s^{-1}) resulted in a similarly alternating redox state of the PQ pool (Fig. 11A in Paper IV). Faster changes were probed with the activated F_0 rise fluorescence method. It was found that 10 s was enough to induce or remove an F_0 rise (Fig. 11B in Paper IV), suggesting that the redox state of the PQ pool can change very rapidly, in a few seconds.

4.4.5 Validation of the liquid chromatography method

Here, PQ was extracted by grinding a leaf in a mortar, which takes 10 to 15 s. To ensure that the method reflects the redox state of the PQ pool *in vivo*, the grinding was performed under treatment conditions. However, the pestle may cause a shadow during grinding, and therefore, we repeated a set of experiments with a transparent mortar, illuminated from the bottom. The results were almost identical with those obtained with the ceramic mortar (Table S1 in Paper IV), indicating that even though grinding of the whole leaf takes some time, individual chloroplasts are destroyed immediately. In addition, far-red illumination ($\lambda \geq 700$) resulted in similar oxidation of the PQ pool (Fig. 7A in Paper IV) as the 690 nm light used here as a control in most of the experiments.

5 Discussion

5.1 Improved detection methods for singlet oxygen are needed

It seems clear that photosynthetic materials produce significant amounts of $^1O_2$ (see e.g. Telfer et al. 1994, Rehman et al. 2013, Hakkila et al. 2014). Here, the rate of $^1O_2$ production by pumpkin thylakoid membranes at the PPFD of 3000 μmol m^{-2} s^{-1} was roughly 15 % of the maximum electron transfer rate in the same thylakoids (i.e. for every ~6 electrons transferred by PSII, a molecule of $^1O_2$ was born; Fig. 6; Papers I and II). Clearly, $^1O_2$ is an important molecule (in good and bad) for photosynthetic organisms. Unfortunately, very different $^1O_2$ yields, differing by four orders of magnitude, are presented in the literature (Paper I, Telfer et al. 1994, Tomo et al. 2012), indicating that to fully understand the role of $^1O_2$, better detection methods are still needed.

Here, the detection methods for $^1O_2$, based on TEMP and histidine, were developed further (Papers I and II). Some limitations, however, remain. For example, both methods are difficult to use for leaves *in vivo* (for the use of TEMP with leaves, see Mor et al. 2014). SOSG (Fig. 3), which has been widely used *in vivo* (e.g. Ramel et al. 2013, Ballottari et al. 2014), on the other hand, may be used only with red light (Ragás et al. 2009). Furthermore, to achieve even distribution of SOSG into leaf material may be challenging (Prasad et al. 2018).

5.2 Singlet oxygen and photoinhibition

Even though more than 60 years of research and vast amount of data, no consensus exists about the molecular mechanism of photoinhibition of PSII, mainly because none of the present hypotheses completely explain all the published results. Here, an attempt is made.

5.2.1 Photosystem II is protected against nascent oxygen

OEC lays only ~20 Å away from P680 (Umena et al. 2011), possibly exposing the PSII reaction center to high O_2 concentrations. Nonetheless, $^1O_2$ measurements

with histidine combined with MIMS (Fig. 6) show that the $^1O_2$ produced by isolated plant thylakoid membranes during illumination derives from ambient dissolved O_2, not from the nascent O_2 produced by the water splitting reactions of OEC. Of course, (practically) all atmospheric oxygen on Earth originates in O_2 evolution by PSII. The present results, however, indicate that the nascent O_2 does not immediately (in the seconds to minutes timescale of the present experiments) travel to (triplet) P680 to become converted to $^1O_2$. It should be noted that with the present signal to noise ratio, it cannot be stated with absolute certainty that none of the nascent O_2 molecules diffused to ^{3}P680 and became $^1O_2$. However, clearly the majority, if not all, of the observed $^1O_2$ derived from ambient dissolved O_2.

The results (Fig. 6 and Fig. 2 in Paper II) show that the ability to evolve O_2 *as such* does not render PSII vulnerable to oxidative damage (it may be added that the reactions leading to O_2 evolution are energetically demanding and incomplete water oxidation, for example, may induce damage and/or production of ROS as suggested e.g. by Kale et al. 2017). How is PSII reaction center protected from nascent O_2? Both experimental data and structural analysis suggest that channels exist in PSII to divert nascent O_2 out of OEC (as well as to bring protons out and water in), possibly into the lumen (e.g. Murray and Barber 2007, Gabdulkhakov et al. 2009, Frankel et al. 2012, Vassiliev et al. 2013). As proposed already by Anderson (2001), the channel(s) seem to efficiently divert nascent O_2 out of the reach of the PSII reaction center. The fact that nascent O_2 is not converted to $^1O_2$ would have been even more important during the evolution of oxygenic photosynthesis, when all the protective measures against $^1O_2$ of extant organisms were not yet fully functional (for discussion, see Fischer et al. 2016b, Cardona et al. 2019). Furthermore, it has been calculated that nascent O_2 quickly equilibrates with the environment (Kihara et al. 2014), further diminishing $^1O_2$ production in the early anaerobic (or microaerobic) conditions of Earth.

The rate of $^1O_2$ production did not depend on the O_2 evolution capacity of PSII (Fig. 2 in Paper II). The observation agrees with the previous measurements showing that the cumulative amount of $^1O_2$ production increases linearly with illumination time (even though the high light intensities used are expected to lead to some degree of photoinhibition of PSII) (Nishiyama et al. 2004, Alboresi et al. 2011). High light should also induce NPQ, which should decrease $^1O_2$ production (see e.g. Dall'Osto et al. 2012). Accordingly, some experiments show that the rate of $^1O_2$ production is fastest at the beginning of the illumination (Poulson and Thai 2015), but even in this case, linear increase in $^1O_2$ with time was observed at later time points.

Hideg et al. (1998), on the other hand, reported a correlation of *in vivo* $^1O_2$ production and the amount of inactive PSII units (with the D1 protein not yet degraded), up to ~60 min of illumination, after which the correlation was lost. The discrepancy with the present results may be partly explained due to increased $^1O_2$

production by lincomycin treatment (used by Hideg et al. 1998), as it has been suggested that impaired repair reactions of PSII lead to increased $^1O_2$ production (Wang et al. 2016a).

5.2.2 Photoinhibition and misses of oxygen evolving complex: an unexpected resemblance

Temperature dependences of PSII photoinhibition, $^1O_2$ production and the slow recombination reactions between OEC and the quinone acceptors of PSII were measured, in order to understand the mechanism(s) behind photoinhibition. Clearly, $S_{2/3}Q_{A/B}^- \rightarrow S_{1/2}Q_{A/B}$ recombination reactions cannot explain the slow increase from 5 to 35 °C either in the $^1O_2$ production rate or in the rate constant of photoinhibition of PSII (Figs. 1 and 2 in Paper III). Recombination of the primary pair, on the other hand, occurs even at cryogenic temperatures (Takahashi et al. 1987), and should therefore have a negligible temperature response at physiological temperatures.

On the contrary, the temperature dependence of misses of PSII, measured by Isgandarova et al. (2003) from spinach thylakoids, is almost identical to the temperature dependence of photoinhibition measured here in white light from pumpkin thylakoids (Fig. 2A in Paper III). A "miss" is a failure of a photon to induce stable charge separation and to advance the S-state of OEC. Misses are rather common (occurring usually with 5 to 10 % probability, per flash), dampening the famous period of four oscillation in O_2 evolution, induced by single turnover saturating flashes, of PSII (Kok et al. 1970). It is not clear whether misses are induced by charge recombination reactions or if the recombination occurs because an S-state of OEC fails to advance, leading to an inability of PSII to reduce $P680^+$ (or TyrZ•) (e.g. de Wijn and van Gorkom 2002b, Han et al. 2012). In the latter case, misses would be an intrinsic property of OEC, possibly due to the demanding chemistry of water splitting (Han et al. 2012). The finding that the "miss-recombination" takes only 100–200 μs (Reinman and Mathis 1981, de Wijn and van Gorkom 2002a) suggests that the origin of misses lay within OEC (as water splitting with its millisecond kinetics would be outcompeted by the much faster recombination). In addition, miss frequency is not affected by the redox properties of Q_A/Q_A^- pair (Han et al. 2012), but depends on the S-state of OEC, being highest in either $S_2 \rightarrow S_3$ or $S_3 \rightarrow S_0$ transition (de Wijn and van Gorkom 2002b, Han et al. 2012, Suzuki et al. 2012, Pham and Messinger 2016).

5.2.3 Long-lived $P680^+$ damages photosystem II

The similarity of the temperature dependences of PSII photoinhibition, $^1O_2$ production and misses obviously suggest a connection between these phenomena.

Also literature data support a connection between $^1O_2$ and photoinhibition (e.g. Jung and Kim 1990, Fischer et al. 2006, Rehman et al. 2013). Does $^1O_2$, produced mainly by the miss-recombination, inactivate PSII? The present experiments do not agree with $^1O_2$ being the major agent of the photoinhibitory damage to PSII. Quenchers of $^1O_2$ or anaerobicity did not protect PSII from damage (Fig. 2 in Paper III), in line with previous studies (e.g. Satoh 1970, Chaturvedi et al. 1992, Nishiyama et al. 2001, 2004, Hakala et al. 2005). Based on the results presented here (Fig. 6; Paper II), PSII is not expected to produce $^1O_2$ in the absence of ambient dissolved O_2. Accordingly, Hideg et al. (1994) could not detect $^1O_2$ under anaerobic illumination of isolated spinach thylakoids.

The data presented here strongly suggest that $^1O_2$ production in thylakoids mainly originates in the recombination reaction occurring after a miss. However, if a miss occurs, but no recombination reaction follows (especially probable if the electron has already travelled to Q_B), long-lived $P680^+$ (or TyrZ•) is produced. $P680^+$ is very oxidizing and could potentially extract an electron from its surroundings, oxidizing a vital component of PSII and inactivating PSII. Here, a model is proposed where a miss induces both photoinhibition and production of $^1O_2$, however, $^1O_2$ is not the major damaging agent, but a side product of the recombination reaction that removes the dangerous $P680^+$ (Fig. 9A–B).

The results where increased redox potential of the Q_A/Q_A^- pair coincides with decreased rates of photoinhibition and $^1O_2$ production (due to increased rate of those recombination reactions that do not produce $^1O_2$) have been explained to indicate that $^1O_2$ is the main damaging agent in photoinhibition (Fufezan et al. 2002, Vass 2012, Treves et al. 2016). However, a decrease in the redox gap between Q_A and Q_B is expected also to increase the overall rate of recombination reactions (as also observed by Roach et al. 2013 and Takegawa et al. 2019), at the expense of forward electron transfer. This would decrease the lifetime of the miss induced $P680^+$, protecting PSII.

5.2.4 Direct light absorption of the manganese ions contributes to photoinhibition also in visible light

UV radiation damages PSII by direct light absorption of the Mn ions of OEC. Temperature dependences of photoinhibition caused by UV radiation and visible light resembled each other (Fig. 1F in Paper III). However, the slowly increasing activation energy of photoinhibition from UV radiation to blue light and further to red light (Fig. 2E in Paper III) suggest that direct light absorption by OEC functions also in visible light but becomes gradually less important towards longer wavelengths. Accordingly, release of Mn ions from OEC occurs faster at blue (460 nm) than in red (660 nm) light (Zavafer et al. 2017).

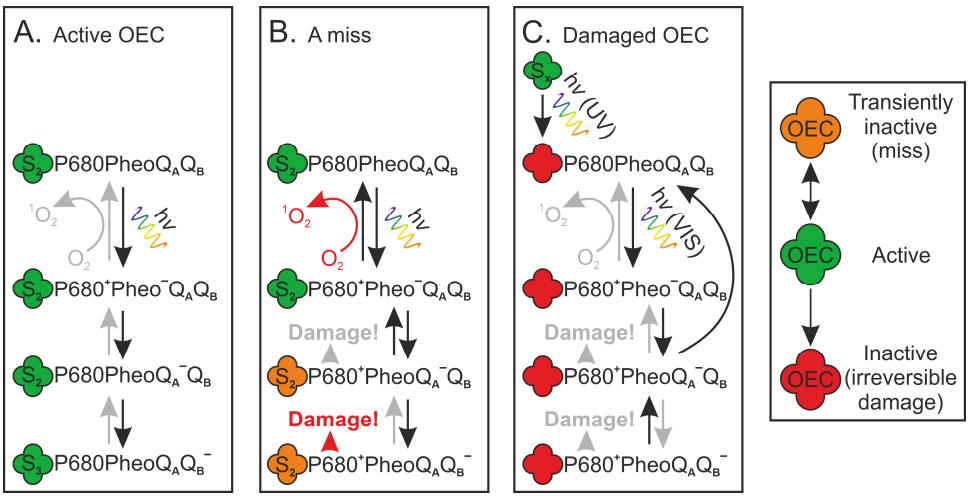

Figure 9. **A photoinhibition hypothesis.** A. When OEC of PSII is functional, forward electron transfer dominates over recombination reactions. B. When OEC is transiently inactive (a miss), the lifetime of $P680^+$ increases. A recombination reaction removes $P680^+$ but may produce $^1O_2$. If the recombination does not occur (more probable if the electron has gone to Q_B), $P680^+$ lives long enough to oxidize a vital component of PSII. C. UV radiation, but also visible light (VIS) with decreasing efficiency towards longer wavelengths, can directly damage OEC. When OEC is unable to donate an electron, further damage may arise due to $P680^+$ or $^1O_2$, however, a conformational change has been proposed to occur, increasing probabilities of such recombination reactions that do not repopulate $P680^+Pheo^-$ and thus do not produce $^1O_2$. Damage by $P680^+$ or $^1O_2$ does not induce further photoinhibition, as PSII is already irreversibly inactivated, unless (in the case of $^1O_2$) the damage is directed to another, still functional, PSII unit.

Originally, TyrZ• and $P680^+$ were suggested to cause damage in PSII centers without functional OEC (Jegerschöld et al. 1990, Chen et al. 1992, Anderson et al. 1998). After OEC is directly damaged by UV radiation or visible light, does $P680^+$ or $^1O_2$, produced via a recombination reaction, cause further damage (see also Hakala et al. 2005, Ohnishi et al. 2005)? A less negative, compared to active PSII, value for the redox potential of the Q_A/Q_A^- pair has been measured in PSII units with non-fuctional (Cl-depleted PSII) or missing Mn-cluster (during PSII assembly or due to chemical removal; Johnson et al.1995, Krieger and Rutherford 1997, Rova et al. 1998, Ido et al. 2011, Brinkert et al. 2016), favoring a recombination that does not produce $^1O_2$ (Spetea et al. 1997, Brinkert et al. 2016) and also shortening the lifetime of $P680^+$. However, 680 nm light damages the reaction center activity with high yield, if OEC has been inactivated by UV radiation prior the 680 nm illumination (Ohnishi et al. 2005). Anaerobicity slightly protects the reaction center activity of PSII, but not the water splitting activity (Chaturvedi et al. 1992), suggesting a role for a ROS in damaging PSII after (light-induced) inactivation of OEC. Electron acceptors are also able to protect PSII reaction center from photodamage (Chen et

al. 1992, Krieger and Rutherford 1997, Zavafer et al. 2015b). The data suggest that long-lived $P680^+$ or $^1O_2$ or both are able to damage PSII reaction center after inactivation of OEC (note that the PSII unit is already irreversibly damaged, net photoinhibition increases only if the damage targets a nearby still functional PSII unit; Fig. 9C).

5.2.5 Why do carotenoids protect photosystem II?

The photoinhibition hypothesis presented above can explain most of the results presented in the literature (protection by NPQ, evidence for involvement of the antennae, general lack of effect by $^1O_2$ quenchers, anaerobic photoinhibition and the effects of Q_A redox changes). However, protection against PSII damage by carotenoids (e.g. Hakkila et al. 2014), is difficult to explain with the present model. If $P680^+$ accumulates, photobleaching of a carotenoid and an accessory chlorophyll has been observed to occur (Telfer et al. 1991). However, whether cyclic electron transfer within PSII via cytochrome b559 and the β-carotene Car_{D2} is fast enough to significantly protect against photoinhibition (Thompson and Brudwig 1988, Buser et al. 1992, Barber and De Las Rivas 1993), remains to be solved. It has been proposed, however, that *C. ohadii* tolerates (and grows in) extreme high light intensities by utilizing (yet to be identified form of) cyclic electron flow within PSII (Ananyev et al. 2017).

Contrary to photoinhibition at 5–35 °C, photoinhibition at -78.5 °C was practically abolished by removal of oxygen, suggesting an involment of ROS. Recombination of the primary pair occurs readily even at 10 K (-263 °C), producing $^3P680$ with 80 % yield (Takahashi et al. 1987). In a purple bacterium (*Rhodobacter sphaeroides*) a strong external magnetic field decreases $^1O_2$ production by the recombination of the primary pair (Liu et al. 2005). However, in plants the rate of the damaging reaction is not slowed down in the presense of a magnetic field, even though the repair cycle appears to function faster during illumination in a strong magnetic field than in the weak ambient field (Hakala-Yatkin et al. 2011), speaking against an important role of $^1O_2$ produced by the recombination of the primary pair in photoinhibition of PSII at physiological temperatures. These data suggest that $^1O_2$ may be more dangerous when its diffusion is limited, as in a frozen state. Alternatively, if the amount of $^1O_2$ is very high, damage may be inevitable, as observed by Hideg et al. (2007). Similarly, lethal amounts of H_2O_2 accelerated PSII damage (in addition to slowing down the repair reactions) in a *Synechococcus* species, but a H_2O_2 concentration that could be tolerated by the cells did not affect the rate of the damage to PSII (Blot et al. 2011). A protecting effect of NPQ against PSII photoinhibition was also observed in elevated O_2 but not in an N_2 atmosphere (Roach et al. 2020).

5.2.6 Sustained quenching of fluorescence may affect photoinhibition measurements

Previously, Lazarova et al. (2014) reported a temperature response of UV-B induced photoinhibition between 4 and 22 °C that was rather similar to what was observed here (Fig. 1E in Paper III). The reports about the temperature dependence of visible-light-induced photoinhibition, on the other hand, are variable. Photoinhibition was reported to decrease with decreasing temperature in pumpkin thylakoids (Tyystjärvi et al. 1994, Tyystjärvi 2013), similarly as here. Also in cyanobacteria, the rate of the damage slowed down (Ueno et al. 2016), or did not change much (Allakhverdiev and Murata 2004, Vonshak and Novoplansky 2008), with temperature. On the contrary, Tsonev and Hikosaka (2003) and Kornyeyev et al. (2003) observed higher rates of damage at lower temperatures in the leaves of *Chenopodium album* and *Gossypium hirsutum*. In the latter two studies, photoinhibition was quantified by chlorophyll fluorescence and, therefore, oxygen evolution and fluorescence assays were compared here (Fig. S3A in Paper III). In pumpkin leaves, the decline in both the fluorescence parameter F_V/F_M and oxygen evolution capacity of PSII depended similarly on temperature. However, if the 30 min dark incubation after the illumination and prior to the fluorescence measurement was conducted at 5 °C, instead of 22 °C, photoinhibition appeared faster (Fig. S3B in Paper III). Tsonev and Hikosaka (2003) performed the dark incubation at the illumination temperature. Thus, it may be possible that the discrepancy between some literature and the present results originate in low-temperature-induced quenching of chlorophyll fluorescence, which does not relax during a typical dark acclimation time (usually 20 to 60 min). Indeed, a form of chlorophyll fluorescence quenching, which does not relax fast, has been observed to develop in *A. thaliana* in high light after short cold treatments (Malnoë et al. 2018). However, to reveal the molecular players behind the slow reversion of the decline in F_V/F_M in darkness (presumably due to slow relaxation of NPQ) in pumpkin leaves at low temperatures, more research would be needed.

Differences in observed temperature responses of photoinhibition in the literature may also reflect real differences in the rates of damage to PSII, as the miss frequency may depend on temperature differently in different species. Isgandarova et al. (2003) measured more misses at low than at high temperature in the thermophilic cyanobacterium *Synechococcus elongatus*. The observed low rate of photoinhibition at their optimal growth temperature in *Arthrospira platensis* (Vonshak and Novoplansky 2008) may be due to small number of misses at that temperature. On the contrary, in *Synechocystis* here, photoinhibition proceeded relatively fast at their growth temperature (Fig. 1A in Paper III).

5.3 Redox state of the plastoquinone pool senses both the imbalances between the photosystems and the intensity of sunlight

The redox state of the PQ pool regulates gene expression and photoacclimation responses (section 1.4.1), but direct measurements of the sizes of the photochemically active PQ pool and the pool of the rest of the PQ molecules or of their redox states are surprisingly rare (so far!). Here, the redox state of the PQ pool under various illumination conditions was measured with HPLC from *A. thaliana* leaves.

5.3.1 Delicate balance between the photosystems

Three wavelength ranges of visible light were found to favor PSII and four wavelength ranges were found to favor PSI (Fig. 2A in Paper IV). Also previous measurements show that PSII and PSI absorb visible light differently (Canaani and Malkin 1984, Wientjes et al. 2013b, Laisk et al. 2014). The most obvious difference between the photosystems is that PSI + LHCI binds less chlorophyll *b* than PSII + LHCII (ee e.g. Caffarri et al. 2014, Mazor et al. 2017). Accordingly, the PSII wavelengths of 460–500 nm and 660 nm coincide with absorption peaks of chlorophyll *b*, and at the PSI wavelengths below 450 nm and above 680 nm chlorophyll *a* absorbs better than chlorophyll *b* (Frigaard et al. 1996, Cinque et al. 2000). Indeed, comparison of the present results with chlorophyll *b* to *a* absorbtion ratio in methanol (calculated based on the data from Frigaard et al. 1996) reveals peaks and deeps at very similar positions as those observed in the F_0 rise spectrum (reflecting PSII/PSI absorption; Fig. 2B in Paper IV). In the F_0 rise spectrum the peaks above 550 nm are red-shifted compared to the spectrum of chlorophyll *b* to *a* absorption ratio, most probably due to the protein environments of both pigments (Thomas and Van Der Wal 1963, Cinque et al. 2000).

Illumination at the peak and deep wavelegths of the F_0 rise spectrum almost completely reduced or oxidized, respectively, the PQ pool (Figs. 2A and 3 in Paper IV). A relatively low intensity of light (PPFD 50 µmol m^{-2} s^{-1}) was used to prevent photosynthetic control (i.e. slowing down of the electron transfer rate of the Cyt b6f complex due to lumen acidification; Tikhonov et al. 1984). 630 nm behaved as PSI light in fluorescence and absorbance measurements but reduced ~50 % of the PQ pool (Figs. 2 and 3 in Paper IV) suggesting that 630 nm light may have steeper light intensity response than the other PSI wavelengths tested here (see Fig. 9A in Paper IV).

The absorption differences between PSII and PSI were very small (Figs. 2, 4 and 5 in Paper IV), even though large effects were exerted on the PQ pool. The data indicate that in the course of evolution, absorption properties of the two

photosystems have been so well balanced that small differences such as state transitions can readjust the balance (it seems that under almost any conditions, LHCII serves as an antenna also for PSI; Wientjes et al. 2013b, Grieco et al. 2015). All polychromatic white light sources tested here, including, importantly, the Sun, oxidized the PQ pool at the PPFD of 30–50 μmol m^{-2} s^{-1} (Fig. 9A in Paper IV). Oxidation of the PQ pool by low intensity sunlight in combination with the mild intensity response enable plants to sense the intensity of sunlight via PQ redox state.

Enrichment of far-red light in canopy shade has been believed to oxidize the PQ pool. However, low intensity white light already favors PSI, oxidizing the PQ pool (Fig. 7A in Paper IV). Accordingly, photosynthetic parameters measured from plants grown under either neutral or canopy shade were rather similar (Pons and de Jong van Berkel 2004). In addition, green PSII light is also proportionally increased under canopy (Fig. 9B in Paper IV; a spectrum of light under canopy is available, for example, also in a maize field in Hirth et al. 2013). In field grown maize, canopy light induced more phosphorylation of LHCII than artificial light enriched in far-red (Hirth et al. 2013), which may suggest that the green PSII light in canopy shade antagonizes the effects of far-red light. Indeed, green light, especially when other wavelengths of light are lacking, can be used to drive photosynthesis (Zhang et al. 2011). On the other hand, green light (as well as far-red light) induces shade avoidance signaling (for a review, see Smith et al. 2017).

5.3.2 Implications for state transitions

The redox state of the PQ pool correlated well with state transitions (quantified as the ratio of PSI to PSII fluorescence at 77 K), after short illumination with selected wavelenghs favoring either PSII or PSI (Fig. 10C in Paper IV). The relationship was curvilinear, and state 2 was reached already after moderate reduction (~30 % reduction of the PQ pool was needed to induce 50 % of the maximal state transition). This is in agreement with the observation that only a single PQH_2 molecule at the Qo site of Cyt b6f is needed to activate the STN7 kinase (Zito et al. 1999), and with the previous research where state 2 has been reached with illumination with “PSII light” which is predicted to reduce 30–80 % of the PQ pool (Table S2 and Fig. S5 in Paper IV; Tullberg et al. 2000, Pesaresi et al. 2002, Adamiec et al. 2008, Wagner et al. 2008).

Contrary to what sometimes is assumed, photochemically active PQ pool is not completely oxidized in the dark in *A. thaliana* (Kruk and Karpinski 2006, Szechyńska-Hebda et al. 2010, Yoshida et al. 2010); here, about 26 % of the PQ pool was reduced after 2 to 3 h of darkness (Fig. 8). In the dark, the PQ pool is probably reduced by NADH dehydrogenase-like complex (Burrows et al. 1998). The light state, defined through the PSI/PSII fluorescence ratio at 77 K, diligently followed

the redox state of the PQ pool also in darkness (Fig. 10C in Paper IV). The STN7 kinase is able to phosphorylate LHCII in darkness (though, for the full activity, *flux* of electrons through the Cyt b6f may be needed; Rintamäki et al. 2000, Hou et al. 2003, Tikkanen et al. 2010). Accordingly, usually (but not always; see e.g. Rintamäki et al. 2000), a small amount of phosphorylated LHCII is detected in darkness, as well as the accumulation of the "state transition complex" consisting of LHCII, LHCI and PSI (e.g. Fristedt et al. 2010, Tikkanen et al. 2010). Different LHCII phosphorylation levels observed may be due to different dark acclimation times used in the different studies, as the light history as well as the age of the plants have been shown to affect LHCII phosphorylation (Trotta et al. 2016, Schwarz et al. 2018, Longoni et al. 2019). On the contrary, during far-red illumination, STN7 is degraded (Willig et al. 2011, Trotta et al. 2016). In the light as well as in darkness, a threonine residue of STN7 is phosphorylated, which seems to protect STN7 from degradation (Trotta et al. 2016).

During low intensity illumination with monochromatic light, the phosphorylation level of LHCII linearly correlated with the extent of state transitions (Fig. 10A). When moving to white lights of different intensities, the situation becomes more complicated. Even though the phosphorylation level of LHCII decreases from low to high light (presumably due to inactivation of STN7; Rintamäki et al. 2000, possibly also due to oxidation of PQH_2 by $^1O_2$; Kruk and Szymańska 2012), almost no state transitions were observed (Tikkanen et al. 2010). The lack of state transitions was observed under sunlight (Wientjes et al. 2013b) and in field-grown leaves (Hirth et al. 2013). Also here, illumination with sunlight of different intensities did not lead to different light states (Fig. 10B). However, sometimes more considerable state transitions, upon changes in light intensity, following rather nicely LHCII phosphorylation, have been reported (Pesaresi et al. 2009, Wood et al. 2018). Interestingly, here, the light state in the samples taken directly from growth chamber followed LHCII phosphorylation (Fig. 10A). It is not yet completely clear, why, despite changes in phosphorylation of LHCII and in the accumulation of PSI-LHCII, state transitions are not (sometimes) observed. It was proposed that state transitions are modulated also by PSII core phosphorylation, which under different intensities of white light mirrors LHCII phosphorylation (Tikkanen et al. 2010, Mekala etal. 2015). Also, why does STN7 phosphorylate LHCII under low light (e.g. Rintamäki et al. 2000, Tikkanen et al. 2010) when the PQ pool should be rather oxidized (Figs. 7 and 9 in Paper IV)? One possibility is that plants compensate the imbalance between electron transfer rates of PSII and PSI (see e.g. Wagner at al 2008), which after a while is expected to lead to a more optimal oxidation state of the PQ pool. At least in mutants lacking active STN7, also STN8 (PSII core kinase) can to a degree phosphorylate LHCII (Longoni et al. 2019).

Acetylation may also be important in state transitions (Koskela et al. 2018). Clearly, the mechanistics of state transitions are not yet fully understood.

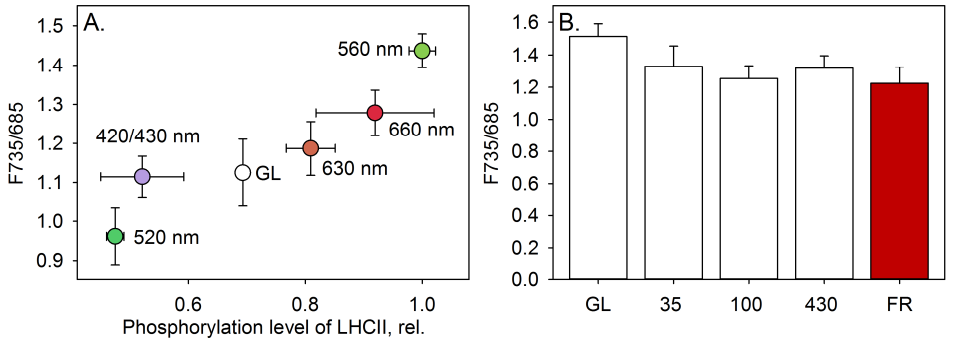

Figure 10. **State transitions and phosphorylation of LHCII in *A. thaliana* leaves.** A. Phosphorylation of LHCII (relative to the level of the 560 nm sample) after 8-min illumination with indicated wavelengths (PPFD 50 µmol m^{-2} s^{-1}) or in growth light (GL) plotted against the ratio of PSI (735 nm) to PSII (685 nm) fluorescence, measured at 77 K from thylakoids isolated from leaves illuminated with the indicated lights for 1 h or taken directly from growth light. Data are from Fig. 10 in Paper IV. B. The ratio of PSI to PSII fluorescence, measured at 77 K from thylakoids isolated from leaves illuminated with sunlight of indicated intensity (PPFD 30, 100 or 430 µmol m^{-2} s^{-1}) or far-red (FR; PPFD 50 µmol m^{-2} s^{-1}) or taken directly from growth light. Fluorescence was excited with 470 nm light. Error bars show standard error of the mean (A) or SD (B) from at least three biological replications.

Expectedly, the PSI to PSII fluorescence ratio was higher when fluorescence was excited with light favoring PSI compared to excitation with a PSII light (Figs. 2 and 10D in Paper IV). Accordingly, if values of PSI to PSII fluorescence from literature are compared, the effect of the excitation wavelength should be kept in mind. In addition, the finding that a larger apparent amplitude of state transitions (i.e. larger relative difference in PSII to PSI fluorescence levels between states 1 and 2) was observed when fluorescence was excited with a PSII light, rather than with a PSI light (Fig. 10D in Paper IV, see also calculations in Paper IV), supports the view that during state 1 to 2 transition a proportion of LHCII serving PSII moves to serve PSI (e.g. Bassi et al. 1988, Iwai et al. 2008).

5.3.3 Redox state of the plastoquinone pool can and should be measured

So far direct HPLC measurements have revealed important roles for PQ at least in plants' tolerance to high light (Kruk and Szymańska 2012, Ksas et al. 2015, Ferretti et al. 2018, Pralon et al. 2019; see also section 1.2.5), heat (Pshybytko et al. 2008) and salinity (Wiciarz et al. 2018). In addition, Yoshida et al. (2010) showed that

besides the PQ pool, illumination affects the redox state of the ubiquinone pool (carriers of electrons from complex I or II to complex III in mitochondria) in *A. thaliana*. Worries about the redox state of the PQ pool possibly changing during the required extraction prior HPLC analysis, therefore not reflecting the situation *in vivo*, may be a reason for the relatively small number of published HPLC measurements. However, the redox state of the PQ pool, measured with HPLC, correlated well with redox states of other components of the photosynthetic electron transport chain (Figs. 4–6 in Paper IV) and acclimation responses known to be regulated by the redox state of the PQ pool (Fig. 10 in Paper IV). We also confirmed that grinding a leaf in a ceramic mortar did not affect the results (Table S1 in Paper IV); once in an organic solvent, PQH_2 is rather stable (Kruk and Strzałka 1999). In addition, comparison with literature shows that the method gives rather consistent results (about e.g. the size of the photoactive pool and the redox state in darkness; Kruk and Karpinski 2006, Yoshida et al. 2010, Szechyńska-Hebda et al. 2010, Ksas et al. 2015).

However, there are important points to note in order to achieve successful PQ measurements with HPLC. Extraction of the sample should be done under treatment conditions, as fast as possible and right before the HPLC analysis. Solutions of $NaBH_4$ (used to reduce PQH_2) cannot be stored. In addition, good controls should be performed for 100 % oxidation and reduction of the photochemically active pool (also the non- photochemically active PQ pool consists of both reduced and oxidized fractions; Fig. 8). These matters may explain why Schuurmans et al. (2014) failed to observe changes in the redox state of the PQ pool under different conditions in *Synechocystis*, contrary to Khorobrykh et al. (2020b).

Compared to HPLC, fluorescence measurements are fast and (seemingly; see e.g. Schansker et al. 2014) simple, and therefore, fluorescence methods have often been used to estimate (even stronger words are sometimes used in the literature) the redox state of the PQ pool. A fluorescence parameter developed to measure the proportion of closed reaction centers (i.e. amount of Q_A^-), 1-qP (in general, and also particularly in this case, instead of qP, qL that takes into account connectivity between PSII units, may be preferable; Kramer et al. 2004), was found to correlate well with HPLC measurements of the redox state of PQ pool, during different time points of high light illumination after darkness (Yoshida et al. 2010). On the contrary, under monochromatic light of moderate intensity, as used here, the correlation was rather poor ($R^2 = 0.74$; Fig. S3 in Paper IV). Fluorescence parameters reflect the reduction state of Q_A, and it is not surprising that the reduction of Q_A does not necessarily follow the reduction of PQ; the difference between the redox potentials of the Q_A/Q_A^- and Q_B/Q_B^- pairs have been measured to be over 200 mV (in intact PSII), and between Q_B^-/PQH_2 and PQ/PQH_2 ~70 mV (Golbeck and Kok 1979, Krieger at al. 1995, Brinkert et al. 2016, Kato et al. 2016, De Causmaecker et al. 2019), making it hard for an electron from PQH_2 to climb to Q_A. Absence of an F_0

rise in the absence of light (using a chopped measuring beam of the fluorometer; Fig. in Paper IV), also shows that PQH_2 does not directly reduce Q_A. In addition, the redox state of the PQ pool in darkness cannot be estimated based on these fluorescence parameters (both qP and qL are by definition 0 in the dark).

Tóth et al. (2007) developed a proxy for the redox state of the photochemically active PQ pool, based on fluorescence rise curve (called OJIP rise), which may be more reliable than qP or qL (however, if light acclimated leaves are used, or if severe PSII damage exists, control values for 100 % oxidation and reduction may not be possible to obtain). The method has not been compared, to my knowledge, with an HPLC method. Such a comparison may, however, be of importance, as the theory behind the method of Tóth et al. (2007) assumes, for example, that binding constants of PQ and PQH_2 to an empty Q_B site are the same (which is probably not the case; Zobnina et al. 2017, De Causmaecker et al. 2019).

To conclude, direct measurements of the redox state of the PQ pool are advisable, especially when the redox state is of importance for interpretation of the results.

6 Conclusions and future perspectives

For plants and all other photosynthetic organisms, light is a source of both energy and problems. To absorb light is easy but to safely convert it to useful forms of energy is not so. To split water, for example, it is required to generate the strongest of the known biological oxidants ($P680^+$). How is the photosynthetic electron transport chain regulated and protected, damaged and repaired? How do plants and other photosynthetic organisms achieve the relatively high efficiencies in the environment that is all the time changing? These are fascinating topics to do research on.

In the present work, methodology was expanded (Papers I, II and IV) and then utilized to solve basic reasearch questions in the field of photosynthesis (all Papers). One may hope that increased understanding could produce increased appreciation to our one-of-a-kind (probably) world, which is, at least for the present human population, irreplaceable and should therefore be taken great care of.

Acknowledgements

This work was conducted in the laboratory of Molecular Plant Biology. Financial support from Academy of Finland, Turku University Foundation, Doctoral Programme of Molecular Life Sciences, Väisälä Foundation, FinCEAL, The Finnish Concordia Fund, Emil Aaltonen Foundation and University of Turku is appreciated.

I thank Esa Tyystjärvi for teaching me how to do solid science and also for letting me quite freely play with whatever things I wanted to. I especially appreciate that you have always kept your door open for questions. For constructive criticism and comments, I wish to thank Saijaliisa Kangasjärvi, Yagut Allahverdiyev-Rinne and Albert Porcar-Castell, the members of my advisory committee, and the pre-examiners professor Shunichi Takahashi and assistant professor Alizée Malnoë. My sincere thanks go also to associate professor Pavel Pospíšil for agreeing to be my opponent and to Eevi Rintamäki to be the custos. Taina I would like to thank for being a very nice and supporting person. I want to thank also all my collaborators.

I have enjoyed my years with plant research. Thanks to Dimitar, Mariel, Sergey, Satu, Sujata, Susa, Tinja and all the other people I have worked with! I hope that this unit will continue to be one where help can always be found when needed. Thank you, Kurt, Tapio Mika, Anniina and all the other office and laboratory personnel, and sorry for the trouble… When asked what I liked most in my work, the coffee breaks, I once answered. For the Pharmacity coffee culture, I want to thank Daniel, Duncan, Hari, Janne, Juha, Pasi, Olli and everybody else visiting the coffee room. Thank you, Gayathri and Pooneh for sports and gossip (for serious discussions too). Special thanks to everybody organizing and attending Shitty Movie Nights.

I want to thank my family for support in all times, for fun discussions or activities and, importantly in this respect, for teaching me critical thinking since birth.

Last, I try to express my gratitude to Vesa, for peer-support during these years, for giving credit (too much, I have no doubt) to my scientific achievements and simply for being (and being wonderful).

9.11.2020

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UNIVERSITY OF TURKU

Painosalama Oy, Turku, Finland 2020

ISBN 978-951-29-8269-1 (PRINT)
ISBN 978-951-29-8270-7 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)