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PHOTODAMAGE TO OXYGEN EVOLVING COMPLEX

An Initial Event in Photoinhibition of Photosystem II

by

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals in the text.

I Tyystjärvi, E., King, N., Hakala, M. and Aro, E.-M. (1999) Artificial Quenchers of Chlorophyll Fluorescence Do Not Protect Against Photoinhibition. Journal of Photochemistry and Photobiology B: Biol. 48: 142-147.

II Hakala, M., Tuominen, I., Keränen, M., Tyystjärvi, T. and Tyystjärvi, E. (2005) Evidence for the Role of the Oxygen-Evolving Manganese Complex in Photoinhibition of Photosystem II. Biochimica et Biophysica Acta - Bioenergetics 1706: 68-80.

III Sarvikas, P., Hakala, M., Pätsikkä, E., Tyystjärvi, T. and Tyystjärvi E. (2006) Action Spectrum of Photoinhibition in Leaves of Wild Type and *npq1-2* and *npq4-1* Mutants of *Arabidopsis thaliana*. Plant and Cell Physiology 47:391-400.

IV Hakala, M., Rantamäki, S., Puputti, E.-M., Tyystjärvi, T. and Tyystjärvi, E. (2006) Photoinhibition of Manganese Enzymes: Insights into to the Mechanism of Photosystem II Photoinhibition. Journal of Experimental Botany 57: 1809-1816.

V Hakala, M., Khriachtchev, L., Keränen, M., Tyystjärvi, T. and Tyystjärvi E. (2007) Two Photoreactions Are Involved in Laser-Pulse-Induced photoinhibition of Photosystem II. Manuscript.

ABBREVIATIONS

BLC	Bovine liver catalase
Chl	Chlorophyll
$Cytb_{559}$	Cytohrome b_{559}
$Cytb_6/f$	Cytochrome b_6/f complex
DBMIB	Dibromothymoquinone
DCBQ	2,6-dichlorobenzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DLGA	DL-glyceraldehyde
DMBQ	2,6-dimethylbenzoquinone
DNB	*m*-dinitrobenzene
DPC	Diphenylcarbazide
Fd	Ferredoxin
FNR	Ferredoxin-NADP reductase
H_2O_2	Hydrogen peroxide
k_{PI}	Rate constant of photoinhibition
LHCII	Light harvesting complex II
MnCat	Manganese containing catalase
MV	Methyl viologen
NPQ	Energy dependent non-photochemical quenching
OEC	Oxygen evolving complex
P_{680}	Reaction center chlorophyll of PSII
PC	Plastocyanin
Pheo	Pheophytin
PPBQ	Paraphenylbenzoquinone
PFD	Photon flux density
PPFD	Photosynthetic photon flux density
PSI	Photosystem I
PSII	Photosystem II
Q_A/Q_B	Primary/secondary plastoquinone acceptor of PSII
RC	Reaction center
ROS	Reactive oxygen species
S_0-S_4	S-states of the oxygen evolving complex
SOD	Superoxide dismutase
UV	Ultraviolet
Tyr_Z	Tyrosine Z
VIS	Visible

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ABSTRACT

Photosystem II (PSII) of oxygenic photosynthesis is susceptible to photoinhibition. Photoinhibition is defined as light induced damage resulting in turnover of the D1 protein subunit of the reaction center of PSII. Both visible and ultraviolet (UV) light cause photoinhibition. Photoinhibition induced by UV light damages the oxygen evolving complex (OEC) via absorption of UV photons by the Mn ion(s) of OEC. Under visible light, most of the earlier hypotheses assume that photoinhibition occurs when the rate of photon absorption by PSII antenna exceeds the use of the absorbed energy in photosynthesis. However, photoinhibition occurs at all light intensities with the same efficiency per photon. The aim of my thesis work was to build a model of photoinhibition that fits the experimental features of photoinhibition. I studied the role of electron transfer reactions of PSII in photoinhibition and found that changing the electron transfer rate had only minor influence on photoinhibition if light intensity was kept constant. Furthermore, quenching of antenna excitations protected less efficiently than it would protect if antenna chlorophylls were the only photoreceptors of photoinhibition. To identify photoreceptors of photoinhibition, I measured the action spectrum of photoinhibition. The action spectrum showed resemblance to the absorption spectra of Mn model compounds suggesting that the Mn cluster of OEC acts as a photoreceptor of photoinhibition under visible light, too. The role of Mn in photoinhibition was further supported by experiments showing that during photoinhibition OEC is damaged before electron transfer activity at the acceptor side of PSII is lost. Mn enzymes were found to be photosensitive under visible and UV light indicating that Mn-containing compounds, including OEC, are capable of functioning as photosensitizers both in visible and UV light.

The experimental results above led to the Mn hypothesis of the mechanism of continuous-light-induced photoinhibition. According to the Mn hypothesis, excitation of Mn of OEC results in inhibition of electron donation from OEC to the oxidized primary donor P_{680}^{+} both under UV and visible light. P_{680} is oxidized by photons absorbed by chlorophyll, and if not reduced by OEC, P_{680}^{+} may cause harmful oxidation of other PSII components. Photoinhibition was also induced with intense laser pulses and it was found that the photoinhibitory efficiency increased in proportion to the square of pulse intensity suggesting that laser-pulse-induced photoinhibition is a two-photon reaction. I further developed the Mn hypothesis suggesting that the initial event in photoinhibition under both continuous and pulsed light is the same: Mn excitation that leads to the inhibition of electron donation from OEC to P_{680}^{+}. Under laser-pulse-illumination, another Mn-mediated inhibitory photoreaction occurs within the duration of the same pulse, whereas under continuous light, secondary damage is chlorophyll mediated. A mathematical model based on the Mn hypothesis was found to explain photoinhibition under continuous light, under flash illumination and under the combination of these two.

1. INTRODUCTION

1.1. Photosynthesis

Photosynthetic organisms are miracles of the nature: they have the ability to use sunlight, carbon dioxide and water to provide the Earth with energy-rich carbohydrates and to release oxygen as a byproduct for us to breathe. Light is absorbed by pigments; chlorophylls (Chls) and carotenoids in higher plants, and phycobilins, Chls and carotenoids in cyanobacteria (for review, see Ke 2001). Chls *a* and *b*, found in higher plants, absorb blue and red light and carotenoids absorb blue-green light. The physical energy of the absorbed photons is chemically stored in photosynthesis in two consequent reaction series: the light reactions and the Calvin-Benson cycle (for review, see Ke 2001). The light reactions convert excitation energy into chemical energy: NADPH and ATP. In the Calvin-Benson cycle, NADPH and ATP are utilized in carbon assimilation to synthesize sugar compounds. This thesis concentrates on the light reactions of photosynthesis and especially on the light-induced damage of PSII, i.e. photoinhibition.

Inside the plant chloroplasts thylakoid membranes form piles called grana stacks and the more spaciously arranged membranes are called stroma thylakoids (Fig. 1). The space inside the thylakoid membranes is called lumen and the space surrounding the thylakoids is called stroma. Four protein complexes function in the thylakoid membranes: Photosystem I (PSI), Photosystem II (PSII), Cytochrome b_6/f complex ($Cytb_6/f$) and ATP synthase. In PSII water is split, oxygen is released and plastoquinone is reduced and protonated to plastoquinol. $Cytb_6/f$ catalyzes the oxidation of plastoquinol and the reduction of plastocyanin. Plastocyanin (PC) is a soluble electron carrier that functions between $Cytb_6/f$ and PSI (Joliot & Joliot 1992). PC and cytochrome c_{552} are interchangeable electron carriers in the photosynthetic electron transfer chains in some cyanobacterial and green algal species (Wood 1978). PSI reduces ferredoxin that reduces $NADP^+$ to NADPH via ferredoxin-NADP reductase (FNR). In the light, a proton gradient is developed across the thylakoid membrane due to proton release into lumen in water splitting and due to proton transfer into lumen by plastoquinone that is protonated on the stromal side and deprotonated by $Cytb_6/f$ on the lumenal side. The proton gradient is the driving force for ATP synthesis.

The protein complexes participating in light reactions are not homogeneously distributed in the thylakoid membranes: PSIIs are mostly located in the grana thylakoids and PSIs and ATP syntheses mostly in the stroma exposed thylakoid membranes (Anderson and Andersson 1982; Chow et al. 1991; Albertsson 2001; Danielsson et al. 2004; Chow et al. 2005). $Cytb_6/f$ complexes are evenly distributed throughout the thylakoid membranes (Albertsson 2001).

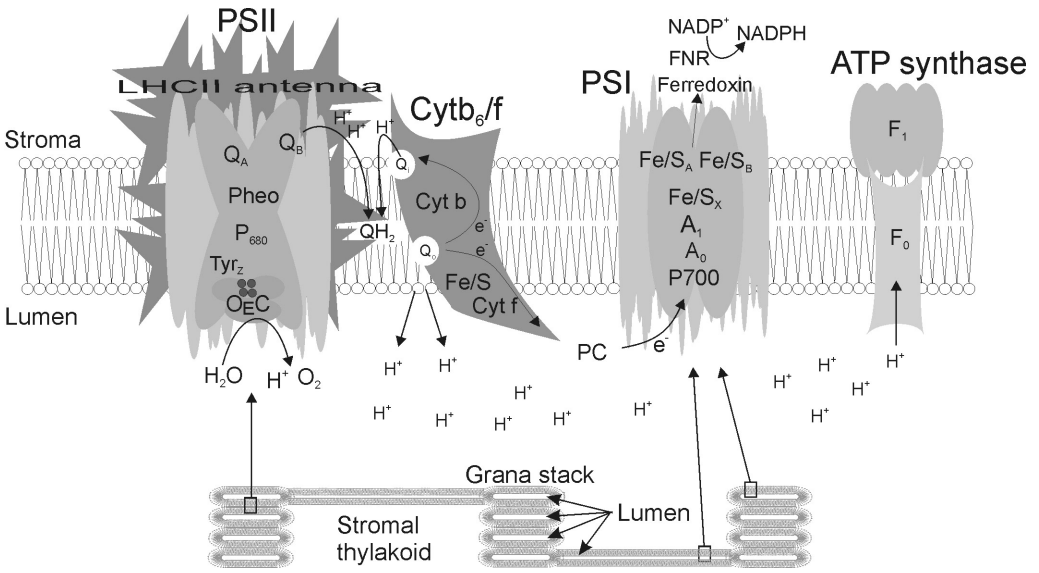

Fig. 1. The four protein complexes of the thylakoid membrane. Plant PSII complexes are mainly present as dimers in grana stack areas and PSI complexes as monomers in stroma exposed areas. The electron transfer components of PSII are: Tyr_Z, P_{680}, Pheophytin (Pheo) and plastoquinones Q_A and Q_B. Water is split in the OEC, and the double reduced final electron acceptor of PSII, plastoquinol (QH_2), binds to the Cyt b_6/f complex at Q_0 site. One of the two electrons from plastoquinol continues from Cyt b_6/f to reduce plastocyanin (PC). The other electron participates in the Q-cycle where plastoquinone at the Qi site of Cyt b_6/f is re-reduced and protonated, which leads to transport of additional protons to the lumen. PC donates electrons to $P700^+$, the oxidized primary donor of PSI. The components of PSI electron transfer are: Chl a (A_0), phylloquinone (A_1) and three [4Fe-4S] clusters. PSI reduces ferredoxin and then ferredoxin-NADP reductase (FNR) catalyzes the formation of NADPH after two consecutive reducing reactions. The ATP synthase is composed of F_0 and F_1 subunits.

1.2. Photosystem II

1.2.1. Structure of PSII

PSII is a protein-pigment complex of plant and cyanobacterial thylakoid membranes. Under natural sunlight conditions approximately half of the absorbed photons in higher plants are absorbed by PSII where the water oxidation, oxygen evolution and reduction of plastoquinone occur (for review, see Melis 1999). The detailed structure of PSII (Zouni et al. 2001; Ferreira et al. 2004; Iwata and Barber 2004; Loll et al. 2005) has been elucidated recently by X-ray crystallography. The structure gives insight into the evolution of photosynthetic organisms showing existence of common ancestors of both photosystems (Grotjohann et al. 2004; Nelson and Ben-Shem 2005). The detailed structure of PSII also gives more understanding to the function of each photosystem protein, while the functions of many photosystem proteins are known mainly from mutant studies. The PSII core contains at least 20 protein subunits, 36 Chl molecules and 11 carotenoid

molecules (Ferreira et al. 2004; Loll et al. 2005). The crystallized PSII supercomplex with PSII dimer and light harvesting complex (LHC) II contains all together 190 Chl molecules (Nield and Barber 2006). The crystallized PSII does not contain as many LHC trimers as PSII *in vivo*, as the Chl per PSII ratio is estimated to be between 200 and 300, varying considerably according to the growth light conditions (Melis et al. 1999; for review, see Dekker and Boekema 2005). PSII electron transfer components are bound by the reaction center (RC) proteins D1 and D2.

1.2.2. Linear Electron Transfer and Charge Recombination Reactions in PSII

The core of PSII electron transfer is the RC Chl P_{680}, to which the excitation energy is ultimately transferred from the PSII antenna systems. The time constants of PSII electron transfer reactions can be seen in Fig. 2A. P_{680} is actually composed of two pairs of Chl molecules, P_{D1}/P_{D2} and Chl_{D1}/Chl_{D2}, and the excitation may be localized in any of the four core Chls (Fromme et al. 2006). The cation radical P_{680}^+ can only be found in P_{D1} and the occasionally produced triplet excited state $^3P_{680}$ is most probably located in Chl_{D1} (Durrant et al. 1990; van Mieghem et al. 1991; Fromme et al. 2006). The primary electron acceptor pheophytin (Pheo) (Klevanik et al. 1977) is reduced by excited P_{680} (P_{680}^*). The primary quinone acceptor, Q_A, is reduced by $Pheo^-$ and reduces the secondary electron acceptor, Q_B. Q_B is double reduced and protonated to plastoquinol (Q_BH_2). Plastoquinol reduces $Cytb_6/f$ and a new oxidized Q_B binds to PSII. The oxidized primary donor P_{680}^+ is highly oxidizing (for review, see Ke 2001) and attracts an electron from the tyrosine residue 161 of D1 protein (Tyr_Z) which is thereafter reduced by the oxygen evolving complex (OEC). The great oxidizing power of P_{680}^+ fuels the oxidation of the Mn ions of OEC. The consecutive oxidations of OEC whirl the S-state cycle and four successive oxidation reactions of OEC result in the splitting of water. Electrons have always a certain probability to move backwards from Q_A or Q_B and charge recombine with the positive charge from the donor side, with S_2 or S_3 state of OEC (Fig. 2B).

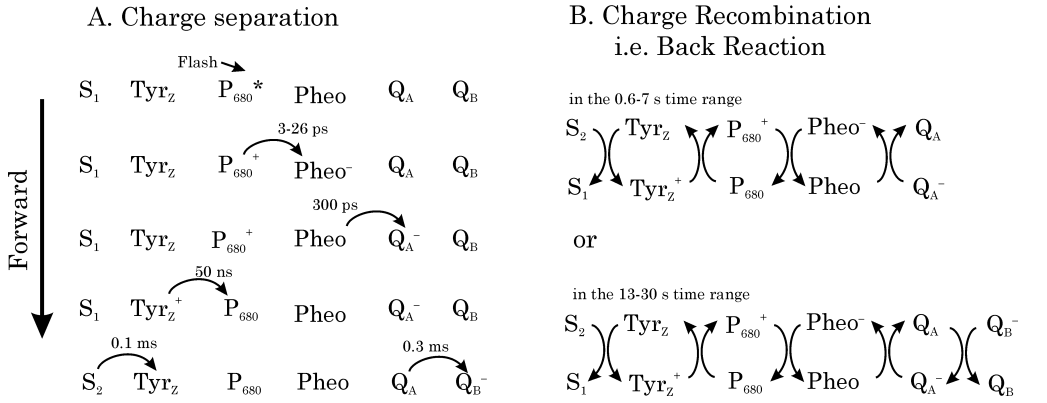

Fig. 2. Forward (A) and backward (B) electron transfer in PSII. (A) The time constants of the forward electron transfer reactions were obtained from Renger and Holzwarth (2005), Diner and Britt (2005) and Holzwarth et al. (2006). Electron transfer from Tyr_Z to P_{680}^+ has minor, slower phases in the microsecond time domain (Schlodder et al. 1984; Christen et al. 1998). The electron transfer time constant from Q_A to Q_B is highly dependent on the reduction state of Q_B and the presence of Q_B in Q_B site. It is 0.3 ms when Q_B is present in Q_B site, 0.7 ms when Q_B is reduced and 2.5 ms when Q_B site is empty and has to be replaced by a new plastoquinone molecule before electron transfer may occur (de Wijn and van Gorkom 2001). (B) The charge recombination $S_2/Q_A^- \rightarrow S_1/Q_A$ predicted from thermoluminescence experiments has a 5-7 s half-time (Keren et al. 1997) and 0.6-1 s half-time from fluorescence relaxation experiments (Vass et al. 1999; Cser and Vass 2007). The charge recombination reaction $S_2/Q_B^- \rightarrow S_1/Q_B$ has a half-time of 30 s, as predicted from thermoluminescence experiments (Rutherford et al. 1984; Keren et al. 1997) and a half-time of 13 s as obtained from fluorescence relaxation experiments.

1.3. Oxygen Evolving Complex

1.3.1. Structure of OEC

OEC of PSII is composed of four Mn ions, one Ca^{2+} ion and one Cl^- ion. The most up to date X-ray structure of OEC has been solved with 3.0 Å resolution (Loll et al. 2005). 3.0 Å resolution, however, is not enough to refer to the exact places of the atoms. X-ray spectroscopy studies combined with electron paramagnetic resonance spectroscopy suggest that Mn ions are bridged by oxygen atoms and arranged as a tetramer with Mn-Mn separations of 2.7 Å, 3.3 Å and 3.4 Å (Loll et al. 2005). A schematic view of the Mn cluster of OEC with the distances between the Mn ions and Ca^{2+} ion and their ligands in the lumenal side of PSII are presented in Fig. 3. The ions of the plant OEC are covered by three extrinsic proteins of PSII: 33 kDa PsbO, 23 kDa PsbP and 13 kDa PsbQ. OEC of cyanobacteria has the corresponding PsbO but instead of P and Q they have 15 kDa PsbV and 12 kDa PsbU (for review, see Barber 2006). The Mn stabilizing protein PsbO is thought to function in optimizing the levels of Ca^{2+} and Cl^- in OEC (Debus 1992). PsbO has been suggested also to play an important role in the repair cycle of PSII, because of its Ca^{2+} and GTP binding properties (de las Rivas and Barber 2004). The PsbP protein has been suggested to be involved in retaining Cl^- and Ca^{2+} ions (Ifuku et

al. 2005a), but also a role as a Mn storage during the repair cycle of PSII has been shown (Bondarava et al. 2005). The PsbQ protein of higher plants seems to be less important under normal growth conditions (Ifuku et al. 2005b), but under low light conditions PsbQ is essential in PSII assembly and stability (Yi et al. 2006).

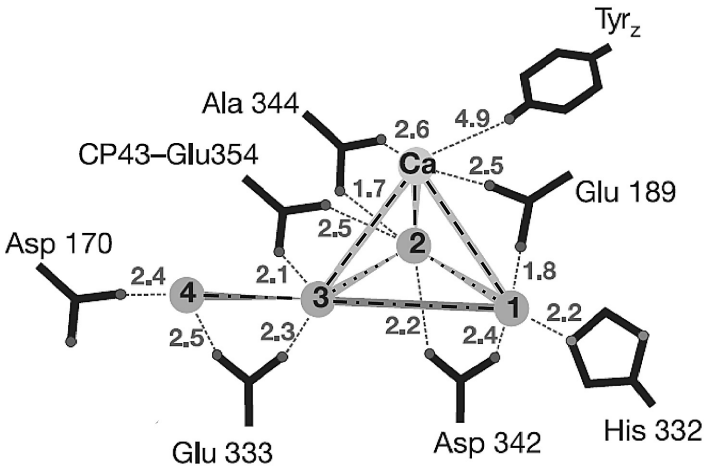

Fig. 3. Schematic view of OEC from *Thermosynechococcus elongatus*. The ligands are amino acid residues of D1 except Glu354 which is an amino acid residue of CP43. The distances between Mn ions (numbered from one to four) and the Ca^{2+} ion (Ca) are indicated by the connecting lines (dotted line, 2.7 Å; dash-dotted line, 3.3 Å; dashed line, 3.4 Å). The decimal numbers are distances between the ions and their ligands, given in Ångströms. Modified from Loll et al. 2005.

1.3.2. Function of OEC

OEC evolves one molecule of oxygen after four oxidations of the Mn ions. OEC has five states: S_0, S_1, S_2, S_3, S_4; the numbers refer to the number of oxidations accumulated in OEC (Fig. 4; Haumann et al. 2005). Illumination of photosynthetic systems with short flashes was studied in the 1970's by Barbieri et al. (1970) and by Kok et al. (1970); the latter was the first to suggest that accumulation of four oxidizing equivalents in the so called S-state cycle can explain the flash-number-dependent variation of oxygen yield per flash. The oxidation steps of OEC are motored by light via the formation of P_{680}^+ and Tyr_Z^+. During the $S_0 \rightarrow S_1$ step, an electron release (reduction of Tyr_Z^+) is followed by proton release; during the $S_1 \rightarrow S_2$ step, only an electron transfer occurs; during the $S_2 \rightarrow S_3$ step, first a proton and then an electron is released from OEC; and during the $S_3 \rightarrow S_4 \rightarrow S_0$ step, an electron is released first and later in the transition two more protons and oxygen are released (Dau and Haumann 2006). Recently, the details of the step from S_4 to S_0 have been characterized and a new intermediate state S_4' was described (Haumann et al. 2005; Dau and Haumann 2007). The relaxation of OEC from the intermediate state S_4' into the most reduced state, S_0, results in the release of one molecule of oxygen. The time constants for different S-state advancements are in the order of 0.03-1 ms (Fig. 4). Ca^{2+} and Cl^- ions in OEC are essential, but their roles in oxygen evolution remain unclear. The Ca^{2+} ion has been suggested to play

a structural role in early S-states and to have a functional role in the oxygen bridge formation at the later S-states (Lee et al. 2007). Cl^- ion has been shown to be needed for $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions and its role in electron transfer between Mn ions is suggested (Wincencjusz et al. 1997). Bicarbonate has been suggested to be a prerequisite both for the function of OEC and for the acceptor side electron transfer of PSII (van Rensen et al. 1999, Klimov and Baranov 2001). However, the presence and role of bicarbonate in OEC is still under debate.

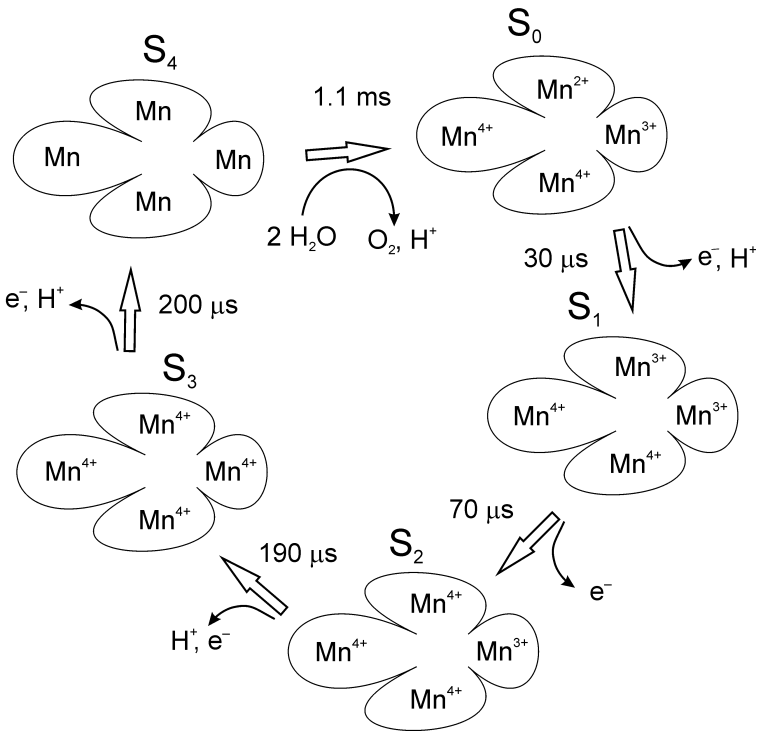

Fig. 4. The S-state cycle of OEC. Each photon absorbed by PSII induces the advancement of one step in the S-state cycle, and oxygen is released during the conversion of S_3 to S_0. It is highly probable that the water molecules are bound at early S-states (for review, see Britt et al. 2004). The time constants of Tyr_Z reduction by intact OEC were obtained from Dau and Haumann (2007). The Mn valences at the transient S_4-state cannot be determined.

The valence states of the Mn ions at different S-states have been detected first by UV absorption difference spectroscopy at different S-states (Pulles et al. 1976; Dekker et al. 1984; Kretschmann et al. 1988) and lately X-ray absorption spectroscopy has showed absorption changes that are interpreted as showing the oxidation of Mn during S-state advancement (Haumann et al. 2005). The valence states of Mn ions during the S-state cycle have not been undoubtedly solved yet, but the following distributions have been suggested: S_0 $Mn(II,III,IV,IV)_4$ or $Mn(III,III,IV,IV)_4$, S_1 $Mn(III, III,IV,IV)_4$, S_2 $Mn(III,IV,IV,IV)_4$ and S_3 $Mn(IV,IV,IV,IV)_4$ (Haumann et al. 2005). By combining the valence state information and the data from Fourier transform infrared spectroscopy, it has been possible to suggest the

localization of the oxidation states to the individual Mn ions (See fig. 3). Mn1 and Mn3 can be either Mn(III) or Mn(IV) in the S_1-state. Mn2 changes from Mn(III) to Mn(IV) in the $S_1 \rightarrow S_2$ transition and the valence of Mn4 ion is Mn(IV) at all S-states (Loll et al. 2005). Under continuous light, the distribution of the four S-states is equal: 25% of OEC is in states S_0, S_1, S_2 and S_3. In the dark, states S_0 and S_1 are stable, whereas states S_2 and S_3 are relaxed via charge recombination reactions so that approximately 75% of the OEC are finally in S_1-state and the remaining 25% are in S_0-state (Kok et al. 1970).

Short single-turnover flashes are used in the study of OEC function to advance the S-states ($S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and so on). Only at the time when a flash causes advancement from S_3 to S_0 via S_4, an oxygen molecule is released. Thus, repetitive single turnover flashes fired at dark adapted thylakoids induce period four oscillations in oxygen evolution in OEC (Kok et al. 1970). Due to the highest concentration of the S_1-state in the dark, the biggest amount of oxygen is released after the third flash. However, sometimes P_{680}^+ is not reduced by OEC, but gets an electron either via charge recombination from Q_A^- or from an alternative electron source within PSII, thus causing a miss in the S-state cycle (Kok et al. 1970). The probability of misses has been shown to be different for each S-state (Shinkarev and Wraight 1993) and in average more than 10% of charge separations fail to advance S-states (Naber et al. 1993). In addition to the misses, single turnover flashes may sometimes cause double hits, i.e. one flash causes two turnovers of PSII and thus two advancements in S-state cycle. With xenon flashes there is a probability of approximately 5 % for double hits, but the probability becomes higher when the length of the flash is increased and vice versa, with nanosecond pulses the probability of the double hits is zero (for kinetics of OEC, see Fig. 2). Under illumination with single-turnover flashes, both misses and double hits disturb the regularly oscillating cycle of OEC (Kok et al. 1970; Shinkarev 2005).

1.3.3. Photoactivation of OEC

During chloroplast development, OEC is constructed by oxidation of Mn^{2+} ions via P_{680}^+ in a light driven reaction called photoactivation (for review, see Burnap 2004). The same process occurs during the repair of PSII when the photosystem is re-assembled after light induced damage. Photoactivation can also be induced after OEC has been chemically washed away from PSII, if the required ions are added. Photoactivation occurs in weak light and requires the presence of bicarbonate and Mn^{2+}, Ca^{2+} and Cl^- ions (Tamura and Cheniae 1987).

Mn ions are oxidized in a sequence of light-activated steps which are separated by light-independent molecular rearrangements. The light-independent steps are apparently necessary for creating the coordination environment for the incoming metal ions. Initially, only one high-affinity Mn binding site exists in the preliminary OEC (Tamura and Cheniae 1987; for review, see Burnap 2004). In the first photoreaction, one Mn^{2+} is oxidized to Mn^{3+}, which is the first intermediate. The Mn4 ion has been shown to be the first Mn bound during photoactivation

(Campbell et al. 2000). The first intermediate is transformed into a second intermediate possibly by binding of a Cl^- ion (Riva et al. 1996), which is needed before the second photoreaction can occur. In the second photoreaction a second Mn^{2+} ion is oxidized to Mn^{3+} and bound to OEC. After this, the photoactivation process cannot decay backwards anymore. The second photoreaction is followed by the light-independent oxidation of two more Mn^{2+} ions. The oxidation of Mn2 is the finalizing step of photoactivation (Cser et al. 2005). During photoactivation the midpoint potential of Q_A/Q_A^- shifts from +110 to -80 mV, indicating a long-range interaction between OEC and Q_A across the thylakoid membrane (Johnson et al. 1995). The roles of Ca^{2+} and Cl^- ions in photoactivation are not fully understood and also various roles for the three extrinsic proteins of OEC (PsbO, PsbP and PsbQ proteins) have been suggested.

1.4. Photoinhibition

1.4.1. Interplay between Damage and Repair

Light is an energy source for photosynthesis, but light also damages PSII in the process called photoinhibition. Photoinhibition in this thesis is defined as light-induced damage to RC of PSII. Under natural conditions photoinhibition results in the degradation and repair of the D1 protein of PSII (Ohad et al. 1984; Aro et al. 1993). The concept defined here as photoinhibition has also been called photodamage (for example, Nishiyama et al. 2006) or photoinactivation (for example, Chow and Aro 2005). Sometimes, the word photoinhibition refers to the decrease of the quantum yield of PSII when the rate of damage is faster than the rate of repair (for example, Nishiyama et al. 2006).

Photoinhibition of PSII follows first order kinetics and the rate constant of photoinhibition (k_{PI}) can be defined as the initial rate of loss of PSII activity when the initial PSII activity is one. Photoinhibition occurs in any light intensity and the rate constant of photoinhibition is directly proportional to the light intensity (Jones and Kok 1966; Tyystjärvi and Aro 1996; Nishiyama et al. 2005; Santabarbara et al. 2002). The direct proportionality leads to reciprocity in photoinhibitory efficiency between the duration of the illumination and the intensity of light (Park et al. 1996; Lee et al. 1999). UV light has much higher photoinhibitory efficiency than visible light (Jones and Kok 1966; Renger et al. 1989; Jung and Kim 1990).

Photoinhibited PSII centers are constantly repaired by *de novo* D1 protein synthesis (for review, see Aro et al. 1993; Melis 1999; Andersson and Aro 2001). In the repair process the PSII dimer with a damaged and phosphorylated D1 protein is monomerized and migrates to the stroma thylakoids where damaged D1 protein is dephosphorylated and then degraded by proteases. The FtsH protease alone has been shown to be capable of D1 degradation (Huesgen et al. 2006), but it has been proposed that several redundant D1 protein degradation pathways might exist involving the DegP2 protease (Nixon et al. 2005; Cheregi et al. 2007). In plants, D1 re-synthesis is regulated at the levels of translation initiation and elongation (Zhang and Aro 2002). The D1 protein is co-translationally incorporated into the

PSII center (Zhang and Aro 2002) and the removal of the C-terminal extension of the pre-D1 protein is needed before photoactivation can take place (Taylor et al. 1988; Diner et al. 1988). The reassembled PSII migrates back to the grana membranes where it forms a functional dimer of PSII. The re-synthesis of D1 protein alone is needed and the other components of PSII are recycled. The rate constant of repair is close to its maximum already at low light intensity and when light intensity exceeds the growth light of the plant, the rate constant starts to decline moderately (He and Chow 2003). However, the rate of repair is fastest under relatively high light intensity, where the damage occurs fast (Ohad et al. 1984). The repair rate increases with temperature (Chow and Aro 2005).

Photoinhibition does not lead to loss of PSII activity unless the rate of damage is higher than the rate of repair. The rate of photoinhibition can only be measured in the absence of the concomitant repair. After isolation of thylakoids, the repair machinery is not functional and thus illumination of thylakoids expresses directly the accumulation of the damage. In intact leaves the repair can be inhibited by translation inhibitors such as lincomycin, streptomycin or chloramphenicol. Lincomycin is a specific inhibitor of chloroplast translation (Mulo et al. 2003). Fig. 5 illustrates the relationship between photoinhibition and repair.

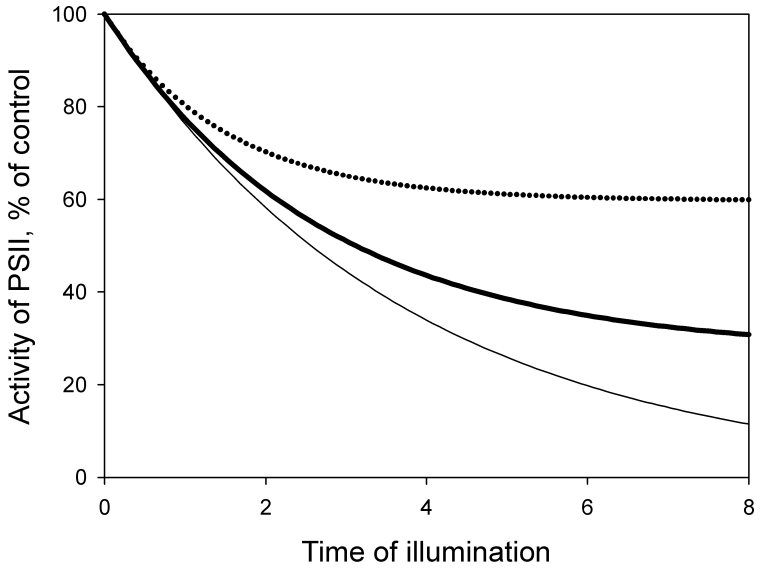

Fig. 5. Simulation of the effects of photoinhibition and recovery on PSII activity of plant leaves under three different conditions. The rate constant of damage was assumed to be 0.25 h^{-1}, which corresponds to illuminating pumpkin leaves at 1800 µmol $m^{-2}s^{-1}$, calculated according to Aro and Tyystjärvi (1996). The thin line simulates the PSII activity decrease in an illuminated lincomycin treated leaf where the rate of repair was assumed to be zero. The dotted line simulates PSII activity of a non-lincomycin treated leaf under illumination at growth temperature, where the rate constant of repair was assumed to be 0.4 h^{-1}. The thick line simulates PSII activity decrease in non-lincomycin treated leaf illuminated at low temperature, which retards the repair process. The rate constant of repair at low temperature was assumed to be 0.1 h^{-1}.

Many hypotheses have been presented about the mechanism of photoinhibition. The most popular earlier hypotheses are summarized below. The manganese hypothesis, which is the topic of my thesis will not be described here, but will be discussed in detail in the Results and Discussion part of this thesis.

1.4.2. Acceptor-side Photoinhibition Hypothesis

According to the acceptor-side photoinhibition hypothesis (Vass et al. 1992), the acceptor side of PSII becomes over-reduced in excess light. It is suggested that in anaerobic conditions under high light, the Q_A electron acceptor becomes stably reduced and possibly protonated (Vass et al. 1992). Further excitations make Q_A double reduced and protonated, which leads to the release of Q_A and thus to irreversible inhibition of PSII. Acceptor-side photoinhibition leads to production of singlet oxygen (Hideg et al. 1998) because the lifetime of the charge pair $P_{680}^+/Pheo^-$ increases when Q_A is protonated or lost. Increase in the lifetime of the $P_{680}^+/Pheo^-$ charge pair increases the probability of electron spin change in this pair. If the spin is turned, the following charge recombination produces triplet Chl (Durrant et al. 1990). The triplet Chl, in turn, can react with molecular oxygen producing harmful singlet oxygen, which can damage the D1 protein (Telfer et al. 1999). Triplet chlorophyll production in the light has been seen in the isolated reaction center complex $D1D2Cytb_{559}$ (Macpherson et al. 1993). However, double reduction of Q_A has not been seen under aerobic conditions (Vass et al. 1993). Vass and Aro (2007) have recently suggested a new form of acceptor-side hypothesis, which is based on the fact that singlet oxygen is produced during photoinhibition (Hideg et al. 1994b, 1998, 2001). It is suggested that in the presence of oxygen, Q_A is stably reduced producing singlet oxygen via charge recombination reactions between $Pheo^-$ and P_{680}^+, which leads to the damage of PSII (Vass and Aro 2007).

1.4.3. Low-light Photoinhibition Hypothesis

Not only continuous light but also illumination with short flashes of light causes photoinhibition. The low-light hypothesis (Keren et al. 1995; Keren et al. 1997; Keren et al. 2000) was designed to explain the observation that flash illumination with a long interval between the flashes causes more photoinhibition per flash than frequent flashes of light. This mechanism has been thought to play an important role especially under low light and under illumination with short flashes of light, when the excitations in PSII are rare events. The hypothesis is based on the function of the charge recombination reactions (van Mieghem et al. 1995). In low light, charge recombination reactions occur mainly between the S_2 state of OEC and the Q_B^- electron acceptor with a constant rate under constant light. Under low excitation rate, Q_B^- is not immediately double reduced by a subsequent turnover of PSII, and thus the quantum yield of the charge recombination reactions per PSII excitation increases. The increased quantum yield of charge recombination increases the quantum yield of singlet oxygen production, which is proposed to cause damage to the D1 protein. The role of charge recombinations in low-light hypothesis was supported by the finding that the increase in the photoinhibitory efficiency when the delay between fired flashes was increased is related to the half-time of the charge recombination reactions (Keren et al. 1997).

1.4.4. Donor-side Photoinhibition Hypothesis

Donor-side photoinhibition occurs when electron donation from OEC is disrupted. When OEC is unable to donate electrons, the highly oxidizing species P_{680}^+ and Tyr_Z^+ cause damage by oxidizing the surrounding molecules. Donor-side photoinhibition has been directly observed after chemical inactivation of OEC (Callahan et al. 1986, Eckert et al. 1991, Chen et al. 1992). It has also been proposed that there is always certain probability that P_{680}^+ fails to be reduced by OEC, which causes donor-side type of photoinhibition (Anderson et al. 1998). Electron donation can be disrupted when the lumen pH decreases in the light and Ca^{2+} is partly released from OEC (Krieger and Weis 1993; Johnson et al. 1995) and in accordance, low pH has also been shown to increase the rate of photoinhibition (Spetea et al. 1997). Furthermore, the OEC-less mutant of *Scenedesmus* has been shown to be very sensitive to light (Keren et al. 1995) and a high concentration of copper or cadmium *in vitro* increases the susceptibility to photoinhibition, probably by deteriorating the donor side of PSII (Pätsikkä et al. 2001; Pagliano et al. 2006).

1.4.5. Singlet Oxygen Hypotheses

Jung and Kim (1990) suggested that photoinhibition is caused by singlet oxygen produced by iron-sulphur centers or cytochromes. Later it was shown that the Rieske center of the $Cytb_6/f$ complex produces singlet oxygen in the light, which was considered as a reason for photoinhibition (Suh et al. 2000). However, the isolated $Cytb_6/f$ complex may contain free Chl, which is an efficient source of singlet oxygen in light. Due to the low efficiency protection against photoinhibition by quenching of excitations of antenna Chl, Santabarbara et al. (2001a) suggested that Chl molecules that are weakly energetically coupled to PSII are responsible for photoinhibition by producing singlet oxygen in the light. The involvement of weakly coupled Chls was suggested to be supported by the finding that the action spectrum of photoinhibition in the red region is blue-shifted compared to the action spectrum of photosynthesis (Santabarbara et al. 2001b; Santabarbara et al. 2002). Santabarbara et al. (2007) also studied triplets produced by PSII and they found triplets, apart from the recombination triplet, produced by weakly coupled Chl in PSII. Those triplets may more readily react with oxygen producing singlet oxygen due to their vicinity to molecular oxygen, compared to the recombination triplets produced within grana membranes. However, the weakly coupled Chls have not been identified. The singlet oxygen hypotheses are able to explain why the quantum yield of photoinhibition is independent of light intensity, but do not explain the photoinhibition occurring in the absence of oxygen.

1.4.6. Photoinhibition under UV Light

Photoinhibition occurs under visible light (400-700 nm), UVA (320-400 nm), UVB (280-320 nm) and UVC (220-280 nm) light. The light from the Sun reaching the Earth contains almost no UVC and very little UVB due to the protective layer of ozone in the atmosphere, while UVA is only weakly absorbed by ozone (for review, see Madronicha et al. 1998). UV photons are highly energetic and are known to

cause various harm to organisms (Sinha and Hader 2002). In plants, UV light causes photoinhibition more efficiently than visible light (Jones and Kok 1966; Jung and Kim 1990). Greenberg et al. (1989) found that the action spectrum of D1 degradation resembles the absorption spectrum of plastosemiquinone. Furthermore, plastoquinone degradation has been observed during UVB illumination (Melis et al. 1992; Barbato et al. 1995). Afterwards it was shown, nonetheless, that photoinhibition proceeds in the absence of the quinones, which indicates that plastosemiquinone is not the main photoreceptor of photoinhibition under UV light (Spetea et al. 1996). There is a moderate consensus that the primary target site of UV light induced damage is the Mn complex of OEC (Renger et al. 1989; Barbato et al. 1995; Larkum et al. 2001; Vass et al. 2002). Absorption of UV light by Mn of OEC leads to the inhibition of OEC and later also the acceptor side of PSII becomes inhibited (Vass et al. 2002), but the molecular mechanism how UV light damages Mn complex of OEC is not known.

1.5. Light and Photosynthesis

In photosynthesis, light is absorbed by antennas and each RC is served by approximately 200 Chl molecules (Melis 1999). The energy transfer reactions from the antenna to the RCs occur in the time scale of femto to pico seconds (for review, see van Grondelle and Novoderezhkin 2006) whereas the electron transfer reactions have slow elements in ms time scale (Fig. 2). Thus plants often receive more excitation energy than they can use in photosynthesis. After absorption of a photon by an antenna pigment molecule, the excited state decays in three competing pathways: the excitation energy is used as reducing power in electron transfer reactions, emitted as fluorescence or dissipated as heat. The distribution of energy between these competing reactions is flexible (Genty et al 1989; Krause and Weis 1991; Kramer et al. 2004a). Under low light intensity, most of the excitation energy is used in photosynthesis and a small fraction of energy is emitted as fluorescence (for review, see Govindjee 2005; Gruszecki et al. 2006; Lazar 2006) or dissipated as heat (for review, see Niyogi et al. 1998; Szabó et al. 2005). When light intensity increases, an increasing fraction of absorbed energy is directed to heat dissipation. This flexibility is necessary due to the changing light environment that a photosynthetic organism has to cope with (Külheim et al 2002). Light intensity varies daily between full sunlight and darkness and in addition, clouds and shading vegetation move in the wind causing abrupt changes of light intensity. Due to the competition between fluorescence, photosynthesis and heat dissipation, the yield of fluorescence at each light environment is indicative of the function of electron transfer and heat dissipation reactions and thus provides a non-invasive method for measuring the photosynthetic reactions *in vivo*.

1.6. Photoprotection

Plants cannot seek for shelter when they are exposed to bright light. Therefore, plants have developed various mechanisms to tolerate, avoid or protect themselves against the harmful effects of light (Demmig-Adams and Adams 1992; Niyogi et al.

1998). Although the quantum yield of photoinhibition has been shown to be constant under different light intensities (Tyystjärvi and Aro 1996), other harmful reactions may be specific to high light intensities: For example, the retardation of the repair cycle of PSII occurs under high light intensity.

1.6.1. Physical Photoprotection

Avoidance mechanisms of excitation energy include alteration of whole-leaf light absorption by paraheliotropic leaf orientation and leaf folding (Öquist and Huner 1991; Jiang et al. 2006), enhanced reflectance through leaf hairing (Ripley at al. 1999), reflective epicuticular wax layers (Robinson et al. 1993), and other morphological adaptations, for example small leaf size, thick leaves and compact growth habit found in plants growing in sunny conditions. Internal measures that reduce light absorption by the photosynthetic machinery are for example chloroplast movements. Chloroplast movements towards the anticlinal cell walls can occur in sudden high light exposure within minutes (Chow et al. 1988; Park et al. 1996; Briggs and Christie 2002, Wada et al. 2003). Mutant plants lacking the chloroplast movement response are more susceptible to photoinhibition than wild-type plants (Kasahara et al. 2002). Light absorption by the photosynthesis machinery is also decreased by accumulation of screening compounds like nonphotosynthetic pigments such as anthocyanins, betalains and rhodoxanthin (Weger et al. 1993; Smillie and Hetherington 1999; Steyn et al. 2002). The plant cuticle has flavonoids and hydroxycinnamic acids that act as UV screening compounds (Kolb et al. 2001; Markstädter et al. 2001).

1.6.2. Non-photochemical Quenching

Non-photochemical quenching provides a fast and flexible means to increase heat dissipation of absorbed photons. There are three types of regulated non-photochemical quenching mechanisms: energy-dependent quenching (qE), state-transition quenching (qT) and photoinhibitory quenching (qI) (Quick and Stitt 1989).

The energy-dependent nonphotochemical quenching, qE (hereinafter referred to as NPQ) is a means for plants to regulate energy transfer to the RC under changing light environment (Niyogi et al. 1998). After transfer from dark to light, NPQ is induced in the time scale of seconds while the Calvin-Benson cycle starts up more slowly. After Calvin-Benson cycle runs at high speed, NPQ relaxes within minutes. The quenching mechanism and identity and location of the quenching pigment are still being discussed (Holt et al. 2004; Cogdell 2006). The accumulation of protons in the lumen of the thylakoid membranes is the initial event in the induction of NPQ (Briantais et al 1979; Horton et al. 2005; Szabó et al. 2005). Low pH causes the protonation of the PsbS protein of PSII (Li et al. 2000) and activation of the violaxanthin de-epoxidase that converts violaxanthin into zeaxanthin (Demmig et al. 1987). Both protonated PsbS and zeaxanthin enhance NPQ. Zeaxanthin, compared to violaxanthin, has two additional conjugated carbon-carbon double bonds, which has been suggested to be the reason why zeaxanthin, but not

violaxanthin, is an efficient absorber and quencher of Chl excitons (Berera et al. 2006). However, a mutant lacking violaxanthin de-epoxidase still shows NPQ although to a smaller extent (Niyogi et al. 1998), whereas a PsbS deficient mutant has very little NPQ although the zeaxanthin levels are normal (Li et al. 2000). It has been shown that under constant high light an NPQ deficient *Arabidopsis* mutant is able to compensate the lacking NPQ by increasing photosynthetic capacity and by decreasing light harvesting (Golan et al. 2006).

State transition (Murata 1969; Bonaventura and Myers 1969) is based on the interchange of part of LHCII between PSII and PSI to balance the absorbed energy between the two photosystems (for review, see Dekker and Boekema 2005). In the dark, plant thylakoids are in State 1, where LHCII is bound to PSII. When the plastoquinone pool becomes reduced in the light, a conformation change in Cyt b_6/f activates LHC kinases, and the LHCII of PSII becomes phosphorylated. Phosphorylated LHCII units have decreased affinity to PSII and they move to PSI resulting in State II (for review, see Bennett 1983; Aro and Ohad 2003; Kanervo et al. 2005; Dekker and Boekema 2005). Transition to State II causes lowering of a Chl fluorescence yield because PSII is the main source of fluorescence. Therefore, a fluorescence quenching parameter, qT, has been assigned for state transitions. Mainly the LHCII complexes in the grana margins may be phosphorylated (for review, see Dekker and Boekema 2005). The primary kinase phosphorylating LHCII has been shown to be STN7 in *Arabidopsis* (Bellafiore et al. 2005). When the $Cytb_6/f$ is oxidized, LHCIIs are dephosphorylated and attach again to PSII. Arabidopsis mutants deficient in qT have been shown to be impaired in growth and have a more reduced plastoquinone pool under changing light environment (Bellafiore et al. 2005), although at non-varying light conditions a mutant lacking qT seems to grow normally (Tikkanen et al. 2006). It has also been suggested that qT is related to change from linear to cyclic electron transfer under conditions of limiting CO_2 and a high reduction level of the chloroplast electron transfer chain. In this case, qT would increase ATP production at the expense of NADPH production rather than balance the excitations between PSII and PSI (Finazzi et al. 1999; for review, see Szabó et al. 2005).

The third form of non-photochemical quenching is called photoinhibitory quenching (qI) and it is less studied than qE or qT. qI is a product of photoinhibition but also considered as a photoprotective process (Stroch et al. 2004). Recently it has been proposed that residual active PSII units have an especially important photoprotective role in functioning as strong excitation energy quenchers when most of the PSII centers are photoinhibited (Chow et al. 2002; Sun et al. 2006). At a late point of photoinhibition, photon energy absorbed by active PSII complexes is efficiently transferred to these photoinhibited neighbors. A very short lifetime of Chl fluorescence has been shown to appear late during photoinhibition revealing a troop of strongly quenching inactive PSII centers (Sun et al. 2006). The qI quenching has been suggested to be vital in the eventual recovery of the whole pool of PSIIs (Sun et al. 2006). The protection can be seen as

a deviation from first order kinetics at the point when activity has decreased to approximately 20 % (Chow et al. 2002). However, the divergence from first order kinetics at the late state of photoinhibition can also be interpreted to reflect a reversible phase in photoinhibition.

1.6.3. Photochemical Quenching

Photochemical quenching is quenching of fluorescence by photochemical reactions. Plants are able to increase the proportion of electrons directed to the photosynthetic electron transfer chain. Alternative electron acceptors mitigate the over-reduction of the acceptor-side of PSII (Kanervo et al. 2005). One of the alternative electron acceptors is oxygen and especially under high excitation pressure of PSII, oxygen is an important electron acceptor, although it also induces oxidative stress in plants (Polle 1996). Photorespiration has been shown to alleviate the excitation pressure of PSII (Kozaki and Takeba 1996; Jiang et al. 2006). Interestingly, mitochondrial electron transport is also able to decrease the excitation pressure in photosynthetic electron chain, as inhibition of mitochondrial pathways, either via the cytochrome c oxidase pathway or via alternative oxidase, has resulted in increased excitation pressure in PSII (Yoshida et al. 2006).

1.6.4. Cyclic Electron Transfer

Cyclic electron transfer around PSI changes the ratio between ATP and NADPH production. In cyclic electron transfer only ATP is formed and no net accumulation of NADPH occurs. Cyclic electron transport is suggested to play a role in providing ATP for activation of Calvin-cycle enzymes during the light induction period, and in general cyclic electron transfer is a means to increase ATP production when needed (for review, see Bukhov and Carpentier 2004; Finazzi and Forti 2004). At least two different cyclic electron transfer routes exist. The route from reduced ferredoxin to plastoquinone is catalyzed by a putative ferredoxin-plastoquinone reductase and assisted by PGR5 protein (Munekage et al. 2002). The second cyclic electron transfer route occurs via NADPH dehydrogenase complex that donates electrons to plastoquinone and it has been shown to be essential in photoprotection (Endo et al. 1999). The physiological roles and the functions of these two routes of cyclic electron transfer are not fully known, but it has been shown that cyclic electron transfer is essential for photosynthesis (Munekage et al. 2004).

Cyclic electron transfer has been suggested to occur also around PSII when PSII acceptor side is reduced and OEC is inefficient in electron donation (Arnon et al. 1988). Under these conditions, electrons are suggested to flow around PSII from plastoquinone through Cyt b_{559} via PSII peripheral Chl_Z to P_{680}^+. The role of the cyclic flow around PSII was suggested to be similar than the water-water cycle in dissipating excitation energy under high light intensities (Miyake et al. 2002). In the latest structural models the Cyt b_{559} has been located near Q_B, thus lending support to the idea that Cyt b_{559} functions in cyclic electron transfer in PSII. Cyclic electron transfer via Cyt b_{559} has also been suggested to play a role in re-reduction of β-carotene thus preserving its ability to quench singlet oxygen (Telfer 2005).

2. OBJECTIVES OF STUDY

The main objective of this study was to describe the mechanism of PSII photoinhibition occurring under different light conditions. The aim was to propose a model that could explain most of the experimental results of photoinhibition. The more specific objectives were:

- to describe the continuous-light-induced, xenon-flash-induced, and laser-pulse-induced photoinhibition
- to find the photoreceptor and mechanism of photoinhibition that would be in agreement with the majority of experimental results

3. METHODOLOGY

3.1. Plant Material

The bean (*Phaseolus vulgaris* L.) plants were grown under 300 µmol photons m^{-2} s^{-1} (Paper I), pumpkin (*Cucurbita pepo* L.) and pea (*Pisum sativum* L.) plants (Paper II) under 150 µmol photons m^{-2} s^{-1}, all in a 12-h day/night rhythm. *Arabidopsis thaliana* were grown under 100 µmol photons m^{-2} s^{-1} in 8/16 hours day/night rhythm (Paper III). Thylakoid isolation was done using the method described in Paper II and thylakoids were stored after isolation at -70°C until use. The *in vivo* experiments were done using pea (Paper II), *Arabidopsis* (Paper III) or pumpkin (Paper IV) leaves. The *in vivo* experiments in Paper II were performed with pea leaves, because pea leaves were known to efficiently soak up DL-glyceraldehyde (DLGA), which was added to inhibit Calvin-Benson cycle. *The in vivo* action spectra in Paper III were performed with *Arabidopsis* leaves, because two NPQ deficient *Arapidopsis* mutant lines were available. The *npq1-2* mutant lacks a functional violaxanthin de-epoxidase enzyme and the *npq4-1* mutant lacks a functional PsbS protein (Niyogi et al. 1998; Li et al. 2000). Pumpkin leaves were used due to their suitability for *in vivo* photoinhibition experiments, because pumpkin leaves are big and homogenously active.

3.2. Photoinhibitory Illumination

In the experiments of this thesis work, photoinhibition was induced with multiple wavelengths and intensities of continuous and pulsed light. A slide projector (Paper I) and a 300 W high-pressure ozone-free xenon lamp through a Schott GG400 UV-blocking filter (Papers II, III and IV) were used as light sources for continuous visible-light experiments. The different visible wavelength regions were obtained by using pairs of low-pass and high-pass filters (Corion, USA) to define 50 nm wavelength bands. 10 nm filters (Corion, USA) were used to define narrow wavelength bands. An ENF-280C lamp (Spectronics, USA) was used as a light source for continuous UVC and UVA light illuminations (Papers II and IV). For the UV illumination under different redox conditions (Fig. 9 in this thesis) a UV emitting xenon arc lamp was used as a light source and the sample was illuminated through a 7-54 filter (Corning Inc.) that blocks visible and IR wavelengths. Microsecond-range short flashes were obtained from FX-200 xenon flash lamp (EG&G, USA) through a Schott GG400 UV-blocking filter. The 5-ns laser pulses were given by an optical parametric oscillator (Sunlite, Continuum) pumped by an Nd:YAG laser. Laser wavelength could be tuned from short UV to near infrared regions. The 4-ns laser pulses were provided by an Nd:YAG laser (Powerlite, Continuum) that can be used to produce visible 532 nm pulses and 266 nm UV pulses depending on the crystal used in the laser. The laser energy densities used in 532 nm experiments were between 36 to 173 mJ cm^{-2}, and each sample contained 2 µg Chl in 27 µl (concentration 67 µg Chl/ml). The ratios of pulse energy to the sample size in these experiments are thus comparable with the

laser pulse photoinhibition experiments of Keren et al. (1997) who applied 532 nm Nd:YAG pulses of 300 mJ cm^{-2} energy to thylakoid samples with 400 µg Chl/ml.

3.3. Measurements of Photon Flux Density

The photosynthetic photon flux density (PPFD) of the continuous visible light experiments was measured with a quantum sensor (LiCor, USA). The photon flux density (PFD) of the continuous UV light, xenon flashes and laser pulses cannot be measured with the LiCor quantum sensor. Actinometry is the only way to measure the number of photons that actually enter the sample cuvette. The PFD of UV light was measured actinometrically using a UV-light-sensitive chemical, $K_3[Fe(C_2O_4)_3]$, that undergoes photochemical reduction from Fe^{3+} to Fe^{2+} upon absorption by UV (Hatchard and Parker 1956). The Fe^{2+} is released to the illumination medium. In actinometry, the concentration of the free ferro ions was measured by adding o-phenanthroline that forms a colored complex with Fe^{2+}. To measure visible light xenon flashes, another light sensitive substance, *meso*-diphenylhelianthrene (Brauer et al. 1983), was illuminated in the photoinhibition cuvette. Increase of the absorbance at 429 nm is proportional to the number of photons absorbed by *meso*-diphenylhelianthrene. *Meso*-diphenylhelianthrene is sensitive to illumination with wavelengths between 475 and 610 nm, and the emission in the 400-700 nm range was obtained by correcting with the emission spectrum of the lamp. The energy of laser pulses was measured with a laser energy meter (Molectron Detector Inc, Portland, OR).

3.4. Chemicals Used in Photoinhibition Studies

Lincomycin was used in the *in vivo* experiments to inhibit the repair cycle of PSII (Papers II, III and IV). At moderate concentrations (0.5-3 mM) lincomycin fully inhibits chloroplast protein synthesis but has no other direct effect on PSII (Mulo et al. 2003). In an alga *Dunaliella salina*, the degradation of the D1 protein in the light has been found to proceed similarly in the presence and absence of lincomycin (Vasilikiotis and Melis 1995). Very high lincomycin concentrations (25 mM) in leaves have been found to slow down the recovery of NPQ after illumination (Bachmann et al. 2004). However, such high concentrations are not used in photoinhibition studies.

Quenchers of Chl fluorescence were used to study the importance of chlorophyll absorbed photons in photoinhibition (Paper I). The following quenchers were used: 0.19 mM *m*-dinitrobenzene (DNB), 0.4 mM dibromothymoquinone (DBMIB) and 1 mM paraphenylbenzoquinone (PPBQ). All the quenchers were assumed to be dynamic quenchers that are not destroyed in the quenching process. DNB has been shown to have different quenching properties depending on its concentration: at low micromolar concentrations DNB quenches photochemically (Neubauer and Schreiber 1988), but in general DNB quenches both excitations both in the RC and in the antenna (Etienne et al. 1974). DBMIB and reduced PPBQ function as non-photochemical quenchers. PPBQ, in addition to being a fluorescence quencher,

works also as an electron acceptor of PSII, but only in oxidized state. For that reason, PPBQ was first allowed to get reduced. Oxidized PPBQ was also used as on electron acceptor in oxygen evolution measurements.

The pea petioles were incubated in DLGA solution and it was found that DLGA treatment inhibited linear electron transfer in PSII and PSI almost completely (Paper II). DLGA is an inhibitor of phosphoribulokinase that functions in the Calvin-Benson cycle catalyzing the ATP-dependent phosphorylation of ribulose-5-phosphate to ribulose-1,5-biphosphate (for review, see Miziorko 2000). Methyl viologen (MV), an electron acceptor of PSI, was used to increase the electron transfer rate in PSII in isolated thylakoids (Paper II). When MV is reduced from MV^{2+} to MV^{+} it may react with oxygen, producing superoxide, which is dismutated to hydrogen peroxide. Catalase was added to the reaction medium to avoid damage by hydrogen peroxide.

Anaerobic conditions in Paper II were obtained by bubbling the photoinhibition buffer first with argon and then adding 8 U/ml glucose oxidase and 6 mM glucose to maintain the anaerobicity. 800 U/ml catalase was added to protect the sample from the harmful effects of H_2O_2 produced in the oxidation of glucose. As a side effect, the enzymatic removal of oxygen with glucose oxidase may lower the redox potential of the reaction medium (Nedbal et al. 1992). After the anaerobic photoinhibition treatment, thylakoids were washed twice with the O_2 measurement medium before measuring O_2 evolution. The anaerobic conditions in Fig. 9 in this thesis were obtained by bubbling the photoinhibition buffer with argon prior to the experiment and by applying argon flow on the surface of it during the experiment. In these experiments, oxidizing conditions (above +90 mV) of photoinhibition medium were maintained by 10 µM 1,2-naphtoquinone and 1 mM potassium ferricyanide and reducing conditions (below -300 mV) by adding 10 µM 2-hydroxy-1,4-naphtoquinone and 0.3 mM dithionite. In the photoinhibition experiments of metal-containing enzymes, anaerobic conditions were obtained by nitrogen bubbling (Paper IV).

3.5. Measuring Photoinhibition

To measure the rate of photoinhibition, the light-saturated activity of oxygen evolution was measured before and after photoinhibition treatment with a Clark type oxygen electrode (Hansatech, King's Lynn, UK) from isolated thylakoids. A slide projector was used as a source of saturating light and 0.5 mM PPBQ (Paper I), 0.5 mM 2,6-dichlorobenzoquinone (DCBQ) (Papers II and V) or 0.5 mM 2,6-dimethylbenzoquinone (DMBQ) (Papers III and IV) were used as artificial electron acceptors. DCBQ oxidizes plastoquinol pool more efficiently than DMBQ, which is seen as higher oxygen evolving activity when DCBQ is used as an acceptor (Srivastava et al. 1995). k_{PI} was calculated by fitting the photoinhibitory loss of PSII activity to a first-order reaction equation and subtracting the rate constant of eventual dark inactivation. The quantum yield of photoinhibition was calculated by dividing k_{PI} by the incident PPFD and multiplying by the initial number of PSII

centers in the sample. For this calculation, the number of PSII centers in the sample was determined by measuring the maximum flash-induced rate of oxygen evolution from a similar thylakoid preparation.

Light-saturated oxygen evolution is a good measure of the number of remaining active PSII centers in photoinhibition experiments because it is not based on any assumption on how photoinhibition proceeds. However, oxygen evolution measurement cannot separate the damage in OEC from damage in other parts of the electron transfer chain of PSII. To distinguish between OEC independent and OEC dependent electron transfer rates, oxidized dichlorophenolindophenol was used as electron acceptor and reduction of dichlorophenolindophenol was measured in the presence and absence of an artificial electron donor, diphenylcarbazide (DPC) (Paper II).

3.6. Chl *a* Fluorescence

One common method to study Chl *a* fluorescence is the pulse amplitude modulated (PAM) fluorometry (Schreiber et al. 1986). In PAM measurements the measuring beam is modulated and only the modulated fluorescence signal is detected while the illumination applied during the fluorescence measurement to influence the PSII function is filtered from the signal.

The *in vitro* fluorescence quenching induced by the addition of artificial quenchers was seen as immediate decrease in the level of variable fluorescence during illumination (Paper I). The induction of NPQ during the measurement was taken into account when estimating the chemical induced quenching. NPQ of the wild-type and *npq1-2* and *npq4-1* mutants of *Arabidopsis* leaves was determined as $(F_M-F_M')/F_M'$ (for review, see Kramer et al. 2004a) and in *npq1-2*, NPQ was very small and in *npq4-1*, NPQ was almost negligible. The quantum yield of PSII electron transport was determined by the Genty parameter, $(F_M'-F_S)/F_M'$ (Genty et al. 1989). For the photochemical quenching, two parameters were used ($q_P = (F_M'-F_S)/(F_M'-F_0')$) (Schreiber et al. 1986) and $q_L = qP * F_0'/F_S$ (Kramer et al. 2004b)) and both yielded qualitatively similar conclusions about the differences between mutants and wild type. Both quantum yield of PSII activity and the photochemical quenching parameter were smaller in the mutants than in the wild type. The F_0' value was defined by an extrapolation method that takes into account both the increase in fluorescence due to relaxation of NPQ and the decrease in fluorescence due to reopening of PSII RCs after illumination in the dark.

The fluorescence excitation spectrum was measured from the wild type and the two different NPQ deficient mutant plants. The fluorescence excitation spectrum at 77K corresponds to the action spectrum of electron transfer reactions at different wavelengths of light. However, this method only gives signals from the surface of the leaf, because light penetrates inefficiently through the leaf and the signal from the deeper cell layers is absorbed by the leaf. NPQ does not significantly develop during the measurement of fluorescence excitation spectrum.

Annihilation of singlet excitations caused by the laser pulses was measured as decrease in the yield of Chl fluorescence (Fig. 8). Fluorescence was measured from thylakoid samples with S2000 spectrophotometer during short bursts of laser pulses in the presence of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at 688 nm (Ocean Optics, Dunedin, FL). The fluorometer was protected from the laser light by measuring at 90° angle with regard to the laser beam, and by placing two high-pass filters (LS-600 and LS-650, Corion, Franklin, MA) between the sample and the fluorometer probe.

3.7. Activity Measurements of the Enzymes

Catalase catalyzes a reaction breaking hydrogen peroxide into water and oxygen. Catalase activity was measured with an oxygen electrode as oxygen production in the presence of 15 mM hydrogen peroxide (Paper IV). The activity of superoxide dismutase (SOD) was measured spectrophotometrically as a slow down of superoxide-dependent nitro blue tetrazolium reduction. Superoxide reduces nitro blue tetrazolium, which can be seen as increase in absorbance at 560 nm. The activity of glucose oxidase was measured as the rate of decrease in oxygen concentration with oxygen electrode when glucose was added to the measurement medium.

3.8. Changes during the Photoinhibition Damage

The Mn content of the lumen before and after photoinhibition of isolated thylakoids under visible and UV light was determined by plasma emission spectroscopy (Paper II). The content of the lumen from control and illuminated samples was obtained by a Yeda press treatment of the thylakoid sample. This was done on ice to avoid D1 degradation. In Yeda press, the sample is forced through a tiny hole by 100 bar pressure. When the sample passes through the hole, the pressure gradient breaks the thylakoids into small pieces thus releasing the lumen into the medium.

The concentration of the D1 protein before and after photoinhibition under visible and UV light was determined immunologically with SDS-page method (Paper II). Degradation of D1 protein gives an estimation of the proceeding of photoinhibition. However, the degradation of D1 protein occurs with a delay after decrease in PSII activity (Paper II).

4. RESULTS AND DISCUSSION

4.1. Quenchers of Chl Excitations Have a Small Protective Effect on Photoinhibition

At the time when I started my PhD studies, the acceptor-side, donor-side and low-light hypotheses of photoinhibition were generally thought to be responsible for all photoinhibition under various conditions (for review, see Aro et al. 1993). Especially over-reduction of the acceptor side of PSII in bright light was believed to cause photoinhibition. I wanted to further study the relationship between photoinhibition and electron transfer rate under fixed light intensity.

The effect of quenching PSII excitations on photoinhibition was studied by illuminating thylakoids in the presence of three different artificial quenchers of Chl fluorescence: DNB, DBMIB and PPBQ (Paper I). The quenching properties of each artificial quencher were tested by measuring Chl fluorescence. The yield of chlorophyll *a* fluorescence is proportional to the excitation density of the antennae, and therefore excitation quenching lowers the quantum yield of all antenna-mediated photochemical reactions. The addition of DBMIB decreased the yield of fluorescence by 25% and addition of PPBQ by 50%. Neither of the two artificial quenchers had any effect on the rate of photoinhibition. When DNB was added and the yield of fluorescence decreased by 88 %, slight protection against photoinhibition was seen. These results were the first pieces of evidence showing that antenna excitations are not the main damaging agents in photoinhibition (Paper I). Decreasing the excited state population of PSII by adding quinones or increasing spillover by unstacking thylakoids was earlier found to have only a minor effect on photoinhibition (Santabarbara et al. 1999). The inefficiency of antenna Chl quenching by DNB to protect against photoinhibition *in vitro* and *in vivo* was later seen also by Santabarbara et al. (2001b; 2002).

The susceptibility to photoinhibition was measured from wild type *Arabidopsis* and two *Arabidopsis* mutants, *npq1-2* and *npq4-1* (Paper III). The most NPQ deficient mutant, *npq4-1*, was found to be only 25% more susceptible to photoinhibition than wild type in visible light. In *npq1-2* mutant, the level of susceptibility to photoinhibition was between that of the wild type and of the *npq4-1* mutant, as was its level of NPQ too. Grasses et al. (2002) found that another PsbS deficient *Arabidopsis* mutant was more susceptible to photoinhibition, but only during the first two hours in high light. They suggest that the main role of NPQ is not to protect against photoinhibition, but rather to fine-tune the energy distribution. Faster photoinhibition in NPQ-deficient mutants compared to the wild type shows that photons absorbed by Chl do participate in photoinhibition *in vivo*. However, the protective effect of NPQ deficiency on photoinhibition was smaller than would be predicted if photoinhibition is assumed to be caused by excitations absorbed by antenna Chls alone (Tyystjärvi et al. 2005; Paper III). Different hypotheses of PSII photoinhibition mechanisms predict different protection levels that NPQ can provide against photoinhibition (Tyystjärvi et al. 2005; Santabarbara et al. 2007).

NPQ would protect against formation of stable or double reduced Q_A as much as NPQ decreases the quantum yield of PSII, i.e. maximally by 35% (Kramer et al. 2004b). Singlet oxygen production via charge recombination between $Pheo^-/P_{680}^+$ would slow down maximally by 75% due to NPQ (Kramer et al. 2004b; Tyystjärvi et al. 2005; Santabarbara et al. 2007).

Plants that are deficient in NPQ have earlier been shown to have lower fitness in outdoor conditions suggesting that NPQ is ecophysiologically important in protecting the plant under changing light environments (Külheim et al. 2002). Although NPQ does not efficiently protect against photoinhibition, NPQ may have important roles in other regulatory and protective mechanisms (for review, see Demmig-Adams and Adams 2006).

4.2. Action Spectrum of Photoinhibition Resembles the Absorption Spectra of Mn-Containing Model Complexes of OEC

NPQ was shown to provide only partial protection against photoinhibition (Paper I; Santabarbara et al. 2001b; Paper III) suggesting that photoinhibition in major part is not caused by the photons absorbed by antenna Chls. To find other photoreceptor(s) of photoinhibition, the action spectrum of photoinhibition was studied. Photoinhibition follows first order kinetics (Jones and Kok, 1966; Tyystjärvi and Aro, 1996) and the determination of the rate constant of photoinhibition induced by illumination with different wavelengths of light gives a comparable measure for the photoinhibitory efficiency of each wavelength, i.e. action spectrum of photoinhibition.

To find possible differences between the anaerobic and aerobic action spectrum of photoinhibition and to obtain clues about the photoreceptors involved under these two conditions, a low resolution *in vitro* action spectrum of photoinhibition was measured from pumpkin thylakoids. 50 nm wide low-pass and high-pass filter pairs were used under visible light from 400 to 700 nm and UVC and UVA lamps were used to provide two UV regions peaking at 254 and 365 nm, respectively (Paper II). The spectrum was measured under anaerobic and aerobic conditions and both of these spectra were found to have the same form; UVA light had approximately six times higher photoinhibitory efficiency than visible light. The decrease from higher photoinhibitory efficiency of the UV light to the lower photoinhibitory efficiency of the visible light occurs between 400 and 450 nm. The visible range of the spectrum was relatively flat; no clear peaks were recognizable. Detailed *in vitro* action spectra of photoinhibition of higher plant thylakoids have earlier been measured by Jones and Kok (1966) from 220 to 700 nm, Renger et al. (1989) from 250 to 350 nm, Jung and Kim (1990) from 360 to 700 nm and Santabarbara et al. (2002) from 640 to 720 nm. Also, after the publication of Paper II, an *in vitro* action spectrum of *Thermosynechococcus elongatus* thylakoids measured between 300 and 700 nm was published (Ohnishi et al. 2005).

The action spectrum was also measured with 50 nm wavelength bands of visible range and two UV wavelengths from thylakoids from which OEC had been chemically removed (Paper II). This measurement showed that OEC-less thylakoids were very tolerant to UV illumination and also the spectrum had clear peaks in the blue and red parts of the spectrum, indicating that photoinhibitory damage in the absence of functional OEC is mediated by Chl. The pronounced influence of photons absorbed by Chl in photoinhibition of OEC-less thylakoids was confirmed by Ohnishi et al. (2005).

The *in vivo* action spectrum of photoinhibition was measured from 254 to 700 nm from young *Arabidopsis* leaves using 10 nm narrow band filters in the visible range and 254 and 365 nm UV wavelength bands (Paper III). This detailed spectrum showed pronounced susceptibility of PSII to UV light, compared to the low photoinhibitory efficiency of all visible wavelengths (Paper III). The visible range of the *in vivo* spectrum was relatively flat, similarly as in the *in vitro* spectrum (Paper II). The *Arabidopsis* leaves were very sensitive to both UVA and UVC illumination. That may be because these experiments were done with young leaves grown in a growth chamber where light is almost exclusively in the visible range. Illumination with UV light induces the formation of UV protective compounds like flavonoids (Kolb et al. 2001) and thus the young leaves used in the experiments may have been poorly protected against UV light. Before our *in vivo* spectrum, only one higher plant *in vivo* action spectrum of photoinhibition was measured from *Spirodela* between 254 and 731 nm using D1 degradation as a measure of photoinhibition (Greenberg et al. 1989). An *in vivo* spectrum of photoinhibition in the cyanobacterium *Synechocystis* sp. PCC 6803 was measured earlier using 50 nm wavelength bands between 400 and 700 nm (Tyystjärvi et al. 2002).

The *in vitro* action spectrum of photoinhibition of Jones and Kok (1966) has peaks in the blue and red regions where Chls absorbs strongly (Soret and Q_y bands respectively). They suggested that light absorbed by Chl is involved in photoinhibition damage. The *in vivo* action spectrum of *Synechocystis* shows a low peak of photoinhibitory efficiency in orange light where phycobilisomes absorb, but clearly the highest photoinhibitory efficiency is seen at 400–450 nm where PSII activity was shown to be very low (Tyystjärvi et al. 2002). In the *in vitro* action spectra of Santabarbara et al. (2001a; 2001b; 2002) a clear peak is shown in the red region, but the spectrum was measured only above 640 nm, which makes it difficult to compare the height of the peak with the action spectra measured by others. Santabarbara et al. concluded, however, that the red peak of their detailed action spectrum of photoinhibition resembles the absorption spectrum of free Chl molecules but not the absorption spectrum of the antenna Chl of PSII (Santabarbara et al. 2002). As in our spectra (Paper II and Paper III), no prominent red peak is present in the spectrum of Greenberg et al. (1989) and Jung and Kim (1990). In the spectrum of Ohnishi et al. (2005) no clear red peak can be seen. All action spectra show many times lower susceptibility to photoinhibition in the visible range than in the UV range.

I performed a literature study and searched for absorption spectra of different components of PSII that have been suggested to function as photoreceptors of photoinhibition (Fig.6). The candidates were plastoquinone and plastosemiquinone, Chl *a* and the aromatic amino acids (tyrosine, tryptophan and phenylalanine). Plastosemiquinone was suggested to be a photoreceptor of photoinhibition by Greenberg et al. (1989) and later by Rodrigues et al (2006), but none of the measured photoinhibition action spectra show any peak around 320 nm, which would be indicative of plastosemiquinone-induced damage. Furthermore, photoinhibition occurs even in the absence of plastosemiquinone (Spetea et al. 1996). From the other candidates, the absorption spectra of plastoquinone, aromatic amino acids and Chl do not show resemblance to the action spectrum of photoinhibition (Fig. 6 and Paper II).

The absorption spectra of the Mn ions of OEC are not known, because OEC is tightly surrounded by highly absorbing Chl molecules. The published absorption spectra of Mn gluconate (Bodini et al. 1976), Mn(III)-O-Mn(IV)-(N,N-bis(2pyridylmethyl)-N'-salicyliden-1,2-diaminoethane)$_2$ (Horner et al. 1999) and [Mn_2(III,IV)(μ-O)$_2$(2,2':6,2"-terpyridine)$_2$(CF_3CO_2)$_2$]$^+$ (Baffert et al. 2002) were used to approximate the absorption spectrum of Mn in OEC. The absorbance of Mn complexes is high in the UV region and decreases at 400-450 nm (Fig. 4C in Paper II). Although the absorption spectra of these different model compounds of the OEC differ substantially from each other in both UV and visible ranges, it can be concluded that Mn compounds are the only constituents of PSII that have absorbance spectra that resemble the complete action spectrum of photoinhibition. Mn ion(s) of OEC have earlier been suggested to function as photoreceptors of photoinhibition under UV light (Renger et al. 1989; Barbato et al. 1995; Larkum et al. 2001; Vass et al. 2002). Consequently, the Mn ion(s) would function as photoreceptors of photoinhibition under visible light, too, according to their absorbance spectra. A rough estimation, taking into account the extinction coefficients and concentrations of chlorophylls and manganese in PSII (Paper II), indicates that manganese absorption could easily account for photoinhibition even at the red peak of the absorption spectrum of chlorophyll *a*. The role of Mn absorption in photoinhibition explains why quenching of antenna excitations does not efficiently protect against photoinhibition (Paper I; Santabarbara et al. 2001; Paper III). The fact that the action spectrum of photoinhibition resembles the absorption spectra of Mn complexes does not, however, exclude the possibility that at some wavelengths some other additional damage may occur.

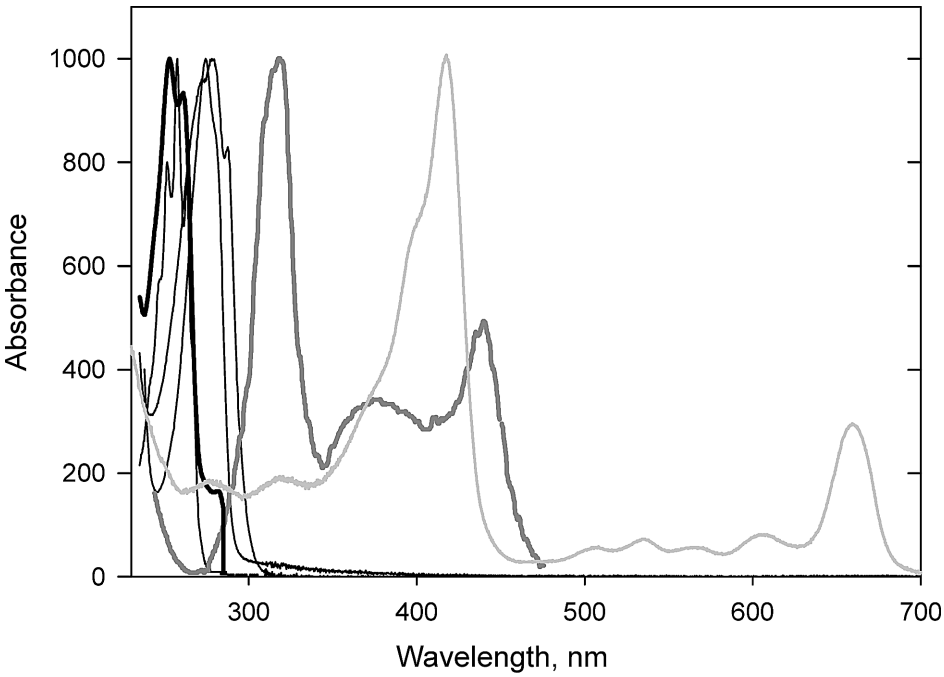

Fig. 6. Absorption spectra of candidates for photoreceptors of photoinhibition. The candidates from the right are: Chl *a* (in MeOH, light gray line), plastosemiquinone (dark gray line), tryptophan, tyrosine, phenylalanine (three thin black lines) and plastoquinone (black line). The spectra of amino acids and Chl *a* were obtained from PhotochemCAD database (http://omlc.ogi.edu/spectra/PhotochemCAD). The spectrum of plastoquinone is from Patzlaff and Barry (1996) and the spectrum of plastosemiquinone from Bensasson and Land (1973). The maximum absorbance value of each compound was normalized to 1000. The maximum extinction coefficients are 15700 (plastoquinone), 11500 (plastosemiquinone), 195 (phenylalanine), 1405 (tyrosine), 5579 (tryptophan) and 110905 (Chl *a*).

According to the inefficiency of NPQ to protect against photoinhibition and the resemblance between the absorbance spectra of Mn model complexes and the action spectrum of PSII photoinhibition, we have come to suggest that Mn ion(s) of OEC have a role in photoinhibition both under visible and UV light. We suggest that a reaction triggered by light absorption by the Mn ion(s) makes OEC incapable of donating electrons to P_{680}^+ The probability of the Mn absorption is dependent on the wavelength and UV light is more photoinhibitory than visible light, because UV light is better absorbed by Mn. When OEC has lost its ability to reduce P_{680}^+, secondary damage is suggested to be caused by harmful oxidations by P_{680}^+. The secondary damage is triggered by light absorbed by Chl. This suggestion is known as the Mn hypothesis of PSII photoinhibition.

4.3. Factors Affecting the Rate of Photoinhibition

4.3.1. PSII Electron Transfer Rate and Photoinhibition

According to the acceptor-side hypothesis (Vass et al. 1992), photoinhibition is caused by the over-reduction of the acceptor side of PSII. Thus it was important to test how varying the excitation pressure of PSII under constant light intensity affects photoinhibition in pea leaves (Paper II). The excitation pressure was increased by inhibiting the Calvin-Benson cycle with DLGA. Lincomycin was used in both control and DLGA-treated leaves to inhibit the synthesis of D1 protein. In the presence of DLGA, the PSII acceptor side of the pea leaves stayed almost fully reduced in the light. It was found that increased excitation pressure did not increase the rate of photoinhibition, but vice versa, had a small protective effect.

Two types of PSII electron transfer inhibitors attach to the Q_B site in PSII: urea/triazine type (such as DCMU) and phenolic inhibitors (such as bromoxynil). The presence of DCMU has either no effect (Nedbal et al. 1986; Mishra et al. 1994) or has a protective effect on the rate of photoinhibition (Kyle et al. 1984; Jegerschöld et al. 1990; van der Bolt and Vermaas 1992; Kirilovsky et al. 1994; Zer and Ohad 1995; Komenda and Masojidek 1998), which is in agreement with the protection against photoinhibition provided by DLGA. Furthermore, a Cyt b_6/f-less mutant of *Chlamydomonas reinhardtii* was shown to be more resistant to photoinhibition than the wild type both in the absence and presence of the repair cycle (Shochat et al. 1990; Gong and Ohad 1991). The protection associated with a reduced acceptor side of PSII contradicts with the hypothesis that over-reduction of the acceptor side is responsible for photoinhibition, but can be explained by the Mn hypothesis. When OEC has suffered the primary damage due to absorption of light by Mn and is dysfunctional in electron donation, the reduced acceptor side, in the absence of functional electron donation, supplies electrons to the highly oxidizing P_{680}^+ and protects against the secondary damage. The presence of DLGA protects against photoinhibition in the same way as DCMU, by increasing charge recombination reactions. This type of protection occurs although recombination reactions may produce the triplet state of the primary donor P_{680} (Cser and Vass 2007).

Phenolic inhibitors of PSII, on the other hand, were shown to speed up photoinhibition in spinach thylakoids (Nakajima et al. 1996) and in *Scenedesmus* (Komenda 1998) and *Synechococcus* cells, but not in *Synechocystis* (Komenda and Masojidek 1998). The reason for the increase in the rate of photoinhibition was suggested to be the reactive oxygen species (ROS) produced in the presence of phenolic inhibitors, not the increase in reduction state of PSII acceptors. The suggestion was based on the finding that phenolic inhibitors do not exacerbate photoinhibition in the absence of oxygen (Komenda et al. 2000). Furthermore, an additional exacerbation of the photoinhibition was seen when azide, an inhibitor of catalase, was added. However, later the increase of photoinhibition by phenolic inhibitor and decrease by DCMU was explained by changes in Q_A/Q_A^- redox potential (Fufezan et al. 2002; Fufezan et al. 2007). These authors suggested that

bromoxynil decreases and DCMU increases the midpoint potential of Q_A/Q_A^-, which influences the type of charge recombination reactions: DCMU increases the slow direct pathway and bromoxynil increases the singlet oxygen producing indirect nonradiative pathway (Cser and Vass 2007).

In addition to decreasing the rate of forward electron transfer through PSII, I also tested the effect of enhancing electron flow through PSII in isolated thylakoids. *In vitro* photoinhibition was induced with white light in the presence and absence of MV, a PSI electron acceptor. Fluorescence measurements showed that MV ameliorated the over-reduction of PSII acceptors. Although MV protected against over-reduction of PSII acceptor side, photoinhibition proceeded faster in the presence than in the absence of MV. The increase in PSII electron transfer by addition of the electron acceptor ferricyanide has also been shown to increase the rate of photoinhibition (Kirilovsky et al. 1994). The increased rate of photoinhibition when electron transfer rate is increased is in line with the results showing that slowing down of electron transfer by DLGA or DCMU protects against photoinhibition.

4.3.2. Other Factors Influencing the Rate of Photoinhibition

Decrease of PSII activity is observed only when the rate of photoinhibition exceeds that of the repair. It is therefore important to distinguish the repair from the photoinhibition damage. The damage to PSII has been intensively studied under natural conditions without inhibiting the repair cycle. The rate of repair is decreased by many factors that in most cases have no effect on the rate of damage (for review, see Giardi et al. 1997; Allakhverdiev and Murata 2004). Such factors are low temperature (Bertamini et al. 2006), oxidative stress (Nishiyama et al. 2001; Nishiyama et al. 2004), drought (Yin et al. 2006) and salt stress (Neale and Melis 1989; Sharma and Hall 1991; Lu and Zhang 1999; Allakhverdiev et al. 2002). If a stress or mutation decreases the PSII activity, it may be that actually it rather affects by retarding the repair cycle and not by exacerbating photoinhibition itself (Nishiyama et al. 2006). For example, it was suggested based on experiments performed without lincomycin that inhibition of the Calvin-Benson cycle increases photoinhibition (Hollinderbäumer et al 1997), but recently we showed that inhibiting Calvin-Benson cycle actually protects against the photoinhibition damage (Paper II). Furthermore, Takahashi and Murata (2005) showed that indeed inhibition of the Calvin-Benson cycle indirectly inhibits the repair cycle. They suggested that many stresses target the damage to the Calvin-Benson cycle thus slowing down the repair process. Lately, four *Arabidopsis* mutants impaired in different photorespiratory pathways were found to be more photosensitive than the wild type. However, it was shown that the translation machinery was suppressed in the mutants and that there were no differences in photosensitivity of the leaves when the repair was inhibited by chloramphenicol (Takahashi et al. 2007). PSII electron transport inhibitors have been also shown to inhibit the repair cycle (Komenda 1998). Nishiyama et al. (2006) showed that ROS produced in the light damage the repair cycle of photoinhibition by inhibiting the translation.

Stress factors or mutations may lead to decrease in the Chl content of the leaves, as a side-effect. A low Chl content increases the light intensity inside the leaf. This causes an increase in the rate of photoinhibition due to the direct proportionality between the light intensity and photoinhibitiory efficiency. An example of this phenomenon is the faster photoinhibition in light green leaves compared to dark green leaves (Pätsikkä et al. 2002). The increased susceptibility to photoinhibition of mildly Cu stressed leaves was also shown to be due to the decrease in the Chl concentration of the leaves (Pätsikkä et al. 1998), because the same Cu concentration *in vitro* caused no effect on photoinhibition (Pätsikkä et al. 2001). Kato et al. (2003) grew *Chenopodium album* leaves under low and high light intensities in the presence of low or high nitrogen availability. They found that the reduction state of Q_A correlated with the photoinhibitory efficiency in the presence of lincomycin. However, the amount of pigments was significantly lowered in the most photosensitive plants and thus may explain the difference in susceptibility to photoinhibition.

In a few studies done in the presence of the repair cycle, a site-mutation in D1 or D2 protein has led to an increased rate of photoinhibition (Kirilovsky et al. 1989; Ohad et al. 1990; van der Bolt and Vermaas 1992), but that may be due to an effect of the mutation on the repair cycle. However, two different *Synechocystis* mutants with few amino acid long deletions in the DE-loop of D1 protein were found to be more tolerant to photoinhibition than the wild type (Mäenpää et al. 1993; Mulo et al. 1998). The PD mutant has slower electron transfer from Q_A to Q_B than the wild type (Mulo et al. 1998) and thus its lower sensitivity to photoinhibition is consistent with our experiments where the lowered electron transfer rate protected against photoinhibition (Paper II). A *Synechocystis* mutant with decreased midpoint potential of the Pheo/Pheo$^-$ pair and with slower rate of charge recombination had higher rate of photoinhibition *in vitro*. Furthermore, a mutant with an increased midpoint potential of the Pheo/Pheo$^-$ pair had a slower rate of photoinhibition (I. Vass, personal communication) similarly as seen in the presence of DCMU (Komenda and Masojidek 1998). Increased sensitivity to photoinhibition *in vivo* in the presence of lincomycin was found by Constant et al. (1996) in their *Synechocystis* DM35 mutant that has two point mutations in the Q_B pocket of D1 protein. This mutant also had a slower rate of charge recombination reactions. Recently, a mutant of *Thermosynechococcus elongatus* that has a decreased midpoint potential of Q_A/Q_A^- pair had faster photoinhibition (Fufezan et al. 2007). However, in this mutant the charge recombination reactions were faster than in the wild type. Fufezan et al. (2007) concluded that the midpoint potential of the PSII electron acceptors affects the rate of photoinhibition by changing the amount of singlet oxygen produced in the charge recombination reactions. The Mn hypothesis does not directly predict differences in the sensitivity to photoinhibition in PSII with changed midpoint potentials in the acceptor side. However, the state of the donor side of PS II has been shown to have a direct influence on the properties of the acceptor side (Johnson et al. 1995) and the secondary damaging reaction

may be hypothesized to be influenced by the redox midpoint potentials of PSII acceptors.

4.4. Role of Reactive Oxygen Species in Photoinhibition

The role of oxygen is different in each PSII photoinhibition hypothesis. Double reduction of Q_A has only been seen under anaerobic conditions (Vass et al. 1992) but singlet oxygen production is only possible in the presence of oxygen. In low-light photoinhibition, singlet oxygen is produced in the presence of oxygen after charge recombination between OEC and Q_A^-/Q_B^- under illumination with low light or short flashes. Neither can singlet oxygen production by weakly coupled Chl (Santabarbara et al. 2001a) occur in the absence of oxygen. The donor-side type of damage induced by the oxidizing agents P_{680}^+ and Tyr_Z^+ seems to be independent of the presence of oxygen. However, superoxide has been shown to contribute to donor-side photoinhibition of Mn-depleted PSII-membranes (Chen et al. 1995).

Oxygen is a good electron acceptor and can easily accept an electron from PSI (Mehler 1951). This reaction produces superoxide O_2^-. Superoxide itself is not very harmful but dismution of superoxide to reactive hydrogen peroxide (H_2O_2) may lead to the Fenton reaction in which a very reactive hydroxyl radical is formed in the presence of Fe^{2+} or Cu^+ ion. Ascorbate peroxidase converts hydrogen peroxide to water. The series of reactions that starts with water splitting in OEC, continues with oxygen reduction by PSI and ends in dissipation of superoxide via hydrogen peroxide to water is called the water-water cycle (for review, see Asada 2006). The water-water-cycle has been suggested to function as a protective valve in the photosynthetic electron transfer chain (Polle 1996) and it was shown that even in the absence of stress, chloroplastic SOD is important for plan survival, as a mutant lacking an active chloroplastic CuZnSOD was suppressed in growth (Rizhsky et al. 2003). Ascorbate is essential for water-water cycle, but it also protects against PSII photoinhibition by increasing the production of zeaxanthin (Forti et al. 1999). Recently superoxide, hydrogen peroxide and hydroxyl radical have been suggested to be involved in the initial damaging reaction of photoinhibition (Song et al. 2006; Liu et al. 2004). Liu et al. (2004) suggested that plastoquinone produces superoxide and that its derivatives, hydrogen peroxide or the hydroxyl radical, cause the damage to PSII. Song et al. (2006) suggested that superoxide and its derivatives cause damage to OEC and thereafter the damage spreads to whole PSII. However, these experiments are not conclusive about the role of superoxide in photoinhibition, because they have been done in the presence of repair.

Singlet oxygen has been experimentally detected during both visible and UV light induced photoinhibition (Hideg et al. 1994a; Hideg et al. 1994b; Hideg et al. 2002; Barta et al. 2004). Singlet oxygen may be detected directly by its near-infrared emission (Telfer et al. 1999) or by tracing its existence by spin trapping EPR spectroscopy or by fluorescing molecules that react with singlet oxygen (Hideg et al. 2001). Singlet oxygen is produced when the excitation energy of a triplet chromophore is transferred to triplet-state oxygen. The observed production of

singlet oxygen in the light supports the acceptor-side photoinhibition hypothesis (Hideg et al. 2001). However, production of singlet oxygen does not seem to require the presence of D1 protein (Tyystjärvi et al. 2005). The production of singlet oxygen has also been seen under UV light, which cannot be explained by the acceptor-side mechanism (Barta et al. 2004). Singlet oxygen is known to be quenched by the carotene pigments of plants (for review, see Telfer 2005) and by α-tokopherol (Kruk et al. 2005). Photoinhibition was found to be faster in a mutant that is deficient in tocopherol production and it was suggested that some tocopherols are located near PSII to quench the singlet oxygen that may cause the D1 protein degradation (Grasses et al. 2001; Trebst et al. 2002; Havaux et al. 2005; Kruk et al. 2005; Krieger-Liszkay and Trebst 2006). In none of these studies, however, the repair was inhibited and the target of singlet oxygen may be the repair cycle. In one recent study, chemically induced singlet oxygen production in tobacco leaves caused loss of PSII activity in the presence of lincomycin showing that singlet oxygen may have direct damaging effect on PSII, too (Hideg et al. 2007).

The effect of molecular oxygen on the rate of photoinhibition is controversial. Anaerobicity has been seen either to speed up photoinhibition (Kirilovsky et al. 1994; Mor et al. 1997; Paper II), to have no effect on photoinhibition (Paper IV) or protect against photoinhibition (Kirilovsky and Etienne 1991). Krause et al. (1985) and Gong et al. (1993) also showed that photoinhibition *in vivo* is faster in a nitrogen atmosphere than in air, but these experiments were done in the presence of the repair cycle. When we removed oxygen by the glucose oxidase enzyme, photoinhibition *in vitro* proceeded faster under anaerobic conditions than under aerobic conditions (data not shown). A special type of reversible photoinhibition has been found under anaerobic conditions (Hundal et al. 1992; Sundby and Schiött 1992; Mor et al. 1997). Such photoinhibition recurs without D1 protein re-synthesis and it was suggested that the acceptor-side photoinhibition in anaerobic conditions is reversible until the formation and release of double reduced Q_A. On the other hand, *in vivo* recovery from photoinhibition without D1 re-synthesis was seen only in the presence of oxygen (Leitsch et al. 1994).

The D1 protein has been shown to be damaged by the action of both hydrogen peroxide (Miyao et al. 1995) and singlet oxygen (Chung and Jung 1995) in the dark or in low light. On the other hand, no D1 degradation is observed in anaerobic conditions (Mishra et al. 1994). D1 protein was found to have regions that are specifically susceptible to cleavage by active oxygen species (Miyao et al. 1995). Quenchers of ROS have also been shown to protect against photoinhibition *in vitro* (Tschiersch and Ohmann 1993; Mishra et al. 1994). Although ROS are produced during photoinhibition and they have been shown to cause D1 degradation, there is no direct evidence that ROS would be the initial cause of damage in photoinhibition. ROS are shown to be important factors in cell signaling and regulation (Strizh et al. 2005). With regard to photoinhibition, ROS often have more influence on the repair cycle of photoinhibition than to the damage itself

(Allakhverdiev et al. 2002; Allakhverdiev and Murata 2004; Nishiyama et al. 2005; Nishiyama et al. 2006). Singlet oxygen has been shown to inhibit the repair of photosystem II by suppressing the translation elongation of the D1 protein in *Synechocystis* sp. PCC 6803 (Nishiyama et al. 2004).

4.5. Mn-Containing Enzymes are Photosensitive

Because the Mn hypothesis suggests that absorption of light by Mn plays a crucial role in photoinhibition, it was of interest to get insight into the effects of light on other manganese-containing structures. Only a limited number of Mn containing enzymes have been characterized. The sensitivity to UVC, UVA and visible light of two manganese containing enzymes, Mn catalase (MnCat) and MnSOD was measured and compared with photosensitivity of two enzymes with other metal cofactors, the heme-containing bovine liver catalase (BLC) and the CuZnSOD (Paper IV). Glucose oxidase was studied as an example of an enzyme that does not have a metal cofactor. To be able to study the effect of light on these enzymes, the experiments were done under anaerobic conditions to avoid possible effects of ROS. Bubbling with nitrogen was used to make the enzyme solutions anaerobic.

The enzymes containing a Mn cofactor or a heme cofactor were found to be sensitive to light-induced damage under anaerobic conditions. MnCat is composed of a catalase enzyme with two oxygen-bridged Mn ions (Barynin et al. 2001) and can be considered to be the closest natural model of OEC. The finding that MnCat and MnSOD were sensitive to visible and UV light indicates that photosensitivity is not a unique property of OEC but probably a universal phenomenon in Mn containing enzymes. The photosensitivity of BLC and CuZnSOD under visible and UV light may signify that all metal enzymes are light sensitive at wavelengths absorbed by the metal complex. Earlier, heme catalase has been found to be sensitive to visible light (Mitchell and Anderson 1965; Aronoff 1965) and a copper containing ascorbate oxidase sensitive to UVC light (Maccarrone et al. 1993), but only in the presence of oxygen (Mitchell and Anderson 1965; Cheng et al. 1981; Maccarrone et al. 1993).

Enzymes may also experience UV light induced damage because the aromatic amino acids absorb in the UVB and UVC ranges and thus can act as photosensitizers (reviewed by Davies and Truscott 2001). However, glucose oxidase was found to be very stable under both UV and visible light and glycolate oxidase, an enzyme also without a metal cofactor, has been shown to be stable under visible light (Schafer and Feierabend 2000). These data suggest that enzyme damage mediated by aromatic amino acid residues under UV light is of minor importance in photoinhibition of metal enzymes.

Photoinhibition of MnCat was also compared with photoinhibition of pumpkin leaves under aerobic and anaerobic conditions. Photoinhibition of both MnCat and PSII of pumpkin leaves was insensitive to oxygen in visible and UV light (Fig. 5 in Paper IV). The similarity of the oxygen response of the inhibition of MnCat activity

and of PSII activity of pumpkin leaves supports the Mn hypothesis of photoinhibition of PSII. Photosensitivity of Mn enzyme shows that Mn-mediated damage may occur despite of the low absorbance of visible light by Mn compounds.

4.6. Short Flashes Induce Photoinhibition Independently of Electron Transfer

Photoinhibition occurs also under illumination with short flashes (Keren et al. 1995; Szilárd et al. 2005). Short flashes provide a way to control light intensity and the rate of electron transfer independently during illumination. Pumpkin thylakoids were photoinhibited with saturating 5-µs xenon flashes and every flash was strong enough to induce one charge separation in each PSII center (Paper II). Varying the energy of the flashes showed that doubling the energy of the saturating flashes doubled their photoinhibitory efficiency. Thus, the direct proportionality, i.e. more light, more photoinhibition, known from continuous light (Tyystjärvi and Aro 1996), is preserved when photoinhibitory illumination is done with xenon flashes. Furthermore, no saturation of photoinhibition was seen even though flash intensity was increased up to 10 times above saturation of photosynthesis reactions.

It was seen earlier in *Chlamydomonas* and *Scenedesmus* cells that photoinhibitory efficiency of xenon flashes is dependent on charge recombination reactions: the consecutive groups with an odd number of flashes cause more photoinhibition than groups with an even number of flashes when the groups of flashes were separated with a 30 s dark period (Keren et al. 1995). This result led to the concept of the low-light hypothesis of photoinhibition. The low-light hypothesis was supported by the finding that very low intensity of continuous light caused relatively more D1 degradation in *Chlamydomonas* cells than higher intensities of light (Keren et al. 1995). Szilárd et al. (2005) found that the greater number of xenon flashes are placed together in a series, the less photoinhibition each flash causes, although no oscillations were found in this study. We were unable to see in our xenon flash experiments any difference in photoinhibitory efficiency of flashes fired in pairs compared to single flashes, but this may be due to longer duration of the strong xenon flashes used in our experiments and also due to relatively short (5 s) dark interval between the flashes when the flashes of the pair were separated with 1 s. Our results indicate that the flash-induced damage is mainly dependent on flash intensity and not on forward nor backward electron transfer reactions. The low-light hypothesis does not explain why photoinhibition increases with increasing flash intensity, when the number of recombination reactions induced per flash is not changed.

4.7. Laser-Pulse-Induced Photoinhibition Gives Insight into the Mechanism of Photoinhibition under Continuous Light

In addition to continuous light and xenon flashes, also very short 4-ns laser pulses cause photoinhibition. Already ten years ago it was noticed that the longer the delay between the subsequent laser pulses, the more efficient the pulses are in inducing photoinhibition (Keren et al. 1997, 2000). These results supported the low-light hypothesis because the time constant of the laser pulse interval effect was similar to the time constant of the charge recombination reactions between the quinone acceptors Q_A/Q_B and the S-states of OEC (Fig. 2 and Keren et al. 1997).

Pumpkin thylakoids were illuminated with 4-5 ns laser pulses of 532 nm or 664 nm and the higher the pulse energy was, the more photoinhibition was induced, although all laser pulses were saturating for PSII electron transfer (Paper V). The increasing photoinhibitory efficiency of pulses of higher intensity shows that the rate of photoinhibition is mainly determined by the intensity of the pulses – also under laser pulse illumination. To further study the role of electron transfer reactions in laser-pulse-induced photoinhibition, 532 nm laser pulses were fired to the thylakoid sample in doublets in which the two pulses of each doublet were separated by only 4 ns. It was found that the second laser pulse that is unable to induce charge separation causes as much photoinhibition as the first laser pulse that causes an electron transfer reaction. This result strongly supports the suggestion that photons absorbed by the Chl antenna are not important in laser-pulse-induced photoinhibition. Although the charge separation reactions do not participate in laser-pulse-induced photoinhibition, the charge recombination reactions seem to do that. Pulses separated by 30 s induce three times more photoinhibition than pulses separated by 0.1 s. The amplitude of this pulse interval effect is proportional to the photoinhibitory efficiency of the pulse, which is in contradiction with the low-light photoinhibition hypothesis, because increasing the energy of saturating pulses does not increase the number of back reactions. The pulse interval effect implies that the concentration of the photosensitive state increases during the dark interval. We suggest that the S_1-state of OEC is the photosensitive state, as in the dark the concentration of the S_1-state increases.

Another piece of evidence for the marginal influence of the Chl-absorbed photons in laser-pulse-induced photoinhibition is that excitation annihilation does not seem to protect against photoinhibition (Fig. 7). Excitation annihilation is a special type of excitation quenching. If two excitations are found in one and the same PSII antenna complex, annihilation means that one of the excited molecules looses its excitation and returns to the ground state, while the other one gets the available energy and is excited to a higher excited electron state from where it decays to the ground state via internal conversion and the excess energy is released as heat (van Amerongen et al. 2000). The annihilation of singlet excitations was monitored by measuring the intensity of Chl fluorescence induced by a laser pulse in the presence of DCMU. In the absence of quenching phenomena, the fluorescence

yield would be proportional to the energy of the laser pulse, but the quenching lowers the fluorescence yield. Like other quenching mechanisms, annihilation of singlet excitations would compete with all photochemical reactions mediated by singlet excitons in PSII, and would therefore protect against Chl-mediated photoinhibition. However, it was seen that the rate constant of laser-pulse-induced photoinhibition increases in proportion to the square of the pulse intensity (Paper V) even though exciton annihilation strongly reduces the number of singlet excitons available for Chl-mediated photochemical reactions (Fig. 7).

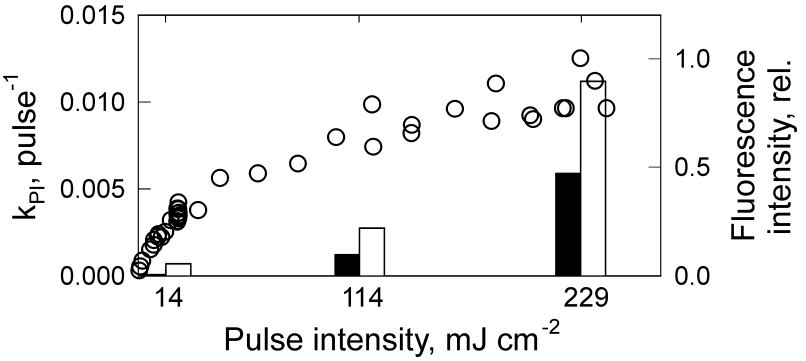

Fig. 7. The increase of the rate constant of photoinhibition at increasing pulse intensity and the quenching of fluorescence by excitation annihilation. The k_{PI} measured at high firing rate (0.1 s between the pulses, black bars) and at low firing rate (10 s between the pulses, open bars) is shown at three different pulse intensities. The fluorescence intensity is shown as open circles.

The effect of anaerobicity on laser-pulse-induced photoinhibition is not fully understood. Under continuous light, anaerobicity obtained with the glucose – glucose oxidase method exacerbated photoinhibition. However, similar anaerobic condition protected against the laser-pulse-induced photoinhibition by 10 to 40% (Fig. 8). The protection was found to be highest in samples illuminated with laser pulses separated with a long dark delay. In the experiments of Keren et al. (1997) pulses spaced by 40 s caused up to 4 times less photoinhibition in nitrogen flushed anaerobic thylakoid samples than in the presence of oxygen. In our system the protection was smaller (Fig. 8), possibly because the laser-illumination cuvette was not sealed to assure the absence of oxygen, although no measurable amount of oxygen was produced in the sample during illumination. The protection by anaerobic conditions against laser-pulse-induced photoinhibition suggests that ROS is somehow involved in laser-pulse-induced photoinhibition, at earlier or later state of the damaging process.

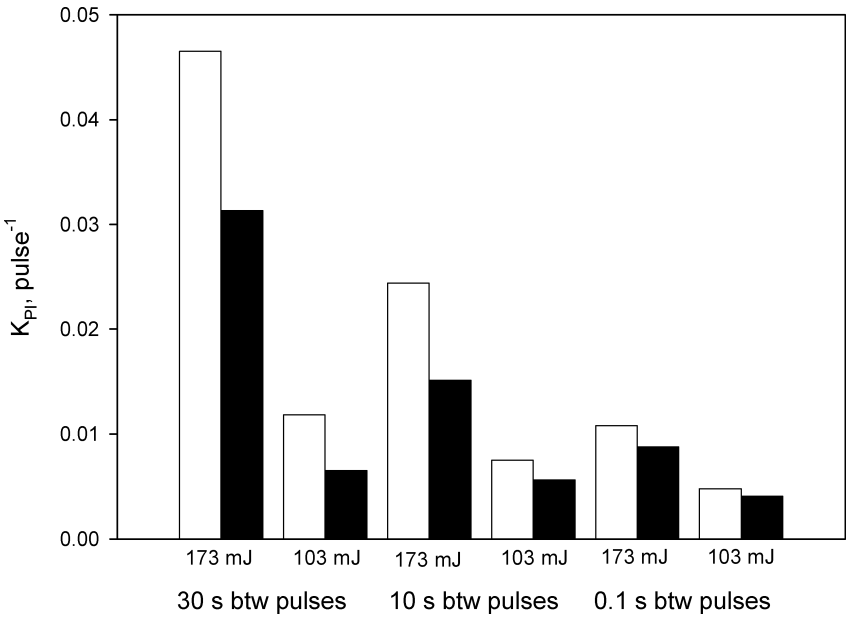

Fig. 8. Laser-pulse-induced photoinhibition under aerobic (open bars) and anaerobic (closed bars) conditions. Photoinhibition was induced with three different frequencies (30 s, 10 s and 0.1 s between the pulses) using two different pulse energies (173 mJ and 103 mJ). Anaerobic conditions were achieved by adding glucose, glucose oxidize and catalase that together remove oxygen from the photoinhibition medium.

4.8. Effect of the Redox-State of the Medium on Photoinhibition of Thylakoids

It has been shown earlier that the rate of photoinhibition is greatly influenced by changes in the redox-potential of the reaction medium (Nedbal et al. 1992; Poulson et al. 1995). Visible light, in the absence of oxygen, was found to cause up to ten times more photoinhibition under reducing than under oxidizing conditions. This was interpreted to lend support to the acceptor-side hypothesis, as oxidizing conditions would alleviate the over-reduction of the PSII acceptor-side and protect against photoinhibition. Nedbal et al. (1992) performed redox titrations of photoinhibition and found that the threshold for the drastic change in the rate of photoinhibition occurs around -20 mV, which is the midpoint potential of the low potential (LP) form of Cytochrome b_{559} (Cyt b_{559}/Cyt b_{559}^{-}). It was shown that in light the Cyt b_{559}LP is first reduced and then PSII is photoinhibited (Poulson et al. 1995). This finding led to the suggestion that the oxidized Cyt b_{559}LP decreases the formation of double reduced Q_A by accepting electrons from over-reduced PSII acceptor side. Gadjieva et al. (2000) found that the electron transfer in OEC-less thylakoids between P_{680} and Q_A is inhibited faster when the ambient midpoint potential is below +350 mV but the electron transfer from Tyr_Z to P_{680} is inhibited more above +350 mV, both experiments done under anaerobic conditions.

We illuminated spinach thylakoids under three different redox conditions: anaerobically in the presence of oxidizing (1,2-naphtoquinone and potassium

ferricyanide) or reducing (2-hydroxy-1,4-naphtoquinone and dithionite) redox poises and aerobically without any addition. An extensive difference was found between the rate constants of visible-light-induced photoinhibition measured under oxidizing and reducing conditions in the absence of oxygen (Fig. 9), in agreement with earlier results (Nedbad et al. 1992). In aerobic conditions, photoinhibition was found to proceed slightly faster than in anaerobic oxidizing conditions. Still, even in the presence of oxygen, photoinhibition was much slower than in anaerobic reducing conditions. It may be that the prevailing redox state near OEC in the sample plays a role in the redox dependency of the rate of photoinhibition. It is known that Mn^{3+} and Mn^{4+} are rapidly reduced to Mn^{2+} in solution. In the photoactivation process, the oxidation of Mn^{2+} into Mn^{3+} is needed to rebind the Mn ion and I suggest that reducing conditions have a negative effect on OEC by making the Mn ions to be more readily released or less easily rebound to OEC. When UV light was used as photoinhibitory illumination, oxidizing conditions did not provide any protection compared to reducing conditions (Fig. 9). Because the redox responses were different under UV and visible light, it may be that different mechanisms of photoinhibition function under UV and visible light. Under UV light, interestingly, photoinhibition proceeded faster in the presence of oxygen compared to anaerobic illumination both under reducing and oxidizing conditions (Fig. 9). The same was noticed under laser-pulse illumination: the presence of oxygen exacerbated photoinhibition (Fig. 8).

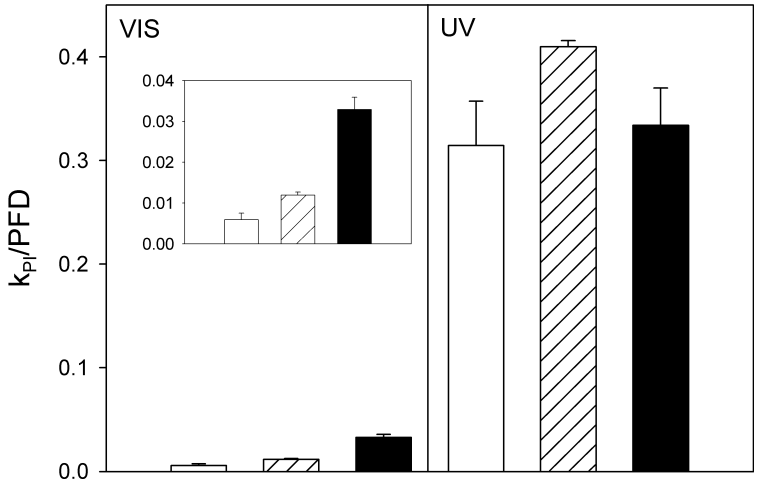

Fig. 9. The dependency of the rate constant of photoinhibition on the midpoint redox potential of the sample medium. Thylakoids were illuminated either with visible light of 2500 µmol photons $m^{-2}s^{-1}$ (VIS) or with UV light (230-410 nm) of 110 µmol photons $m^{-2}s^{-1}$ (UV) under different redox conditions. The bars refer to K_{PI} values of anaerobic photoinhibition in the presence of oxidized quinone and ferricyanide (open bars), aerobic photoinhibition without any additions (hatched bars) and anaerobic photoinhibition in the presence of reduced quinone and dithionite (black bars). The k_{PI} values are normalized to the light PFD of 400 µmol photons $m^{-2}s^{-1}$. More detailed scale for visible light photoinhibition is shown in insert on the left.

4.9. Model Combining Continuous Light and Laser-Pulse Induced Photoinhibition

Photoinhibition induced with continuous light and pulsed light obviously share some reaction pathways, as they both lead to the degradation of D1 protein (Papers II and V) and both are more efficiently induced by UV than by visible light. However, three differences have been seen between photoinhibition under continuous and pulsed light. First, the light response curve is linear under continuous light (Tyystjärvi and Aro 1996) but under laser pulses the rate constant of photoinhibition is proportional to the square of the pulse intensity (Paper V). Another difference between photoinhibition under continuous and pulsed light is that Mn-depleted thylakoid samples under continuous light are more photosensitive than intact thylakoids (Paper II), but Mn-depleted thylakoids are more tolerant against laser-pulse induced photoinhibition than intact thylakoids (Keren et al. 2000). The third difference is that anaerobicity increases the rate of photoinhibition under continuous light while the rate of laser-pulse-induced photoinhibition is slower in the absence than in the presence of oxygen (Fig 8).

In paper V we suggest a model that contains a mechanism for both continuous light and laser-pulse-induced photoinhibition. The model is composed of a Mn-mediated primary reaction that is shared by both laser-pulse-induced and continuous-light-induced photoinhibition. This primary reaction is proposed to be the result of excitation of Mn ion(s) of OEC. We suggest that this excitation leads to a short lived state of OEC, which we call the S_1* state. This state decays independently of light to the state of OEC where one Mn ion is poorly bound and thus OEC is still present but electron donation from OEC to P_{680}^+ does not function. Under continuous light, the absence of electron donation from OEC leads to a P_{680}^+ dependent damaging reaction. The highly oxidizing P_{680}^+ will oxidize the surrounding molecules in a harmful way leading to the irreversible damage of PSII. It is also possible that the dysfunctional electron donation from OEC leads to increased production of singlet oxygen due to charge recombination reactions between P_{680}^+ and Q_A^-/Q_B^-. Under laser-pulse illumination the short-lived S_1* may be excited a second time during the same high-intensity laser pulse. The second Mn-mediated excitation of OEC by a laser-pulse leads to irreversibly damaged OEC and PSII. This two-photon reaction under laser-pulse illumination explains the quadratic relationship between the energy of the laser pulses and their photoinhibitory efficiency.

The Mn model of photoinhibition under continuous light also contains a minor but important factor of reactivation of OEC forming the balance between the active and reversibly inactive OEC. The reversibly inactive OEC has two alternative destinies: it either becomes fully functional PSII by rebinding of a Mn ion, or, while OEC is not functional, a secondary Chl-mediated oxidation by P_{680}^+ or by singlet oxygen production due to charge recombination results in an irreversible damaged PSII. In the first case, we suggest that there is a balance of release and rebinding of Mn ions from OEC. The rebinding probably resembles the photoactivation of PSII. The redox state of the surrounding medium certainly has an influence on the ability of

Mn^{2+} to be oxidized and rebound to OEC, possibly explaining why photoinhibition proceeds remarkably more slowly under oxidizing conditions (Nedbal et al. 1992). The redox midpoint potential of the photoinhibition medium did not affect photoinhibition under UV light (Fig. 8). This suggests that the rebinding reaction does not play an important role under UV light possibly due to the great effect of UV light in Mn-mediated reaction and thus the secondary Chl-mediated damage is minor under illumination with UV light. Under visible light, on the other hand, the rebinding protects against the secondary Chl-mediated damage when OEC is dysfunctional.

To test the model, we illuminated pumpkin thylakoids separately with continuous light, with laser pulses and with a combination of continuous light and laser pulses. We found that simultaneous application of continuous light and laser pulses has essentially the same photoinhibitory efficiency as the sum of those two illumination types separately. It should be pointed out that according to the low-light hypothesis, the pulses on top of the continuous light would not cause any photoinhibition because the rare charge separations induced with laser pulses would not have any effect in the middle of successive charge separations induced by continuous light (Paper V).

4.10. Mn of OEC during Photoinhibition

We found that the activity of OEC is lost before the loss of electron transfer from P_{680} to Q_A during photoinhibition under UV and strong visible light (Paper II). We propose that the primary reaction during photoinhibition is excitation of Mn of OEC, which leads to conversion of the S_1-state to a transient S1*-state. The physico-chemical characteristics of the S1*-state need further examination. The quadratic light response of laser-pulse-induced photoinhibition suggests that the S_1* state is stable enough to undergo another photoreaction during one pulse. An excited state of Mn would probably be too short-lived to be identified as S_1*. Thus, the S_1*-state must be some kind of conformational change occurring before any Mn ion is released to the lumen. Illumination with near-infra-red light causes Mn-centered photochemical reactions in OEC (Ioannidis and Petrouleas 2000) indicating that in spite of the low absorbance of manganese in near-infra-red, photochemistry can occur. It was found that Mn ion(s) are released from OEC during photoinhibition both under ultraviolet and visible light (Virgin et al. 1988; Renger et al. 1989; Paper II; Ohnishi et al. 2007). The Mn release was earlier interpreted to be a consequence of D1 degradation (Virgin et al. 1988). Ohnishi et al. (2005) published results further supporting a scheme of primary Mn-mediated damage and secondary Chl-mediated damage. A scheme of my new proposed hypothesis is presented in Fig. 10 and it covers photoinhibition of PSII under continuous light, pulsed light and the combination of these two illumination modes simultaneously.

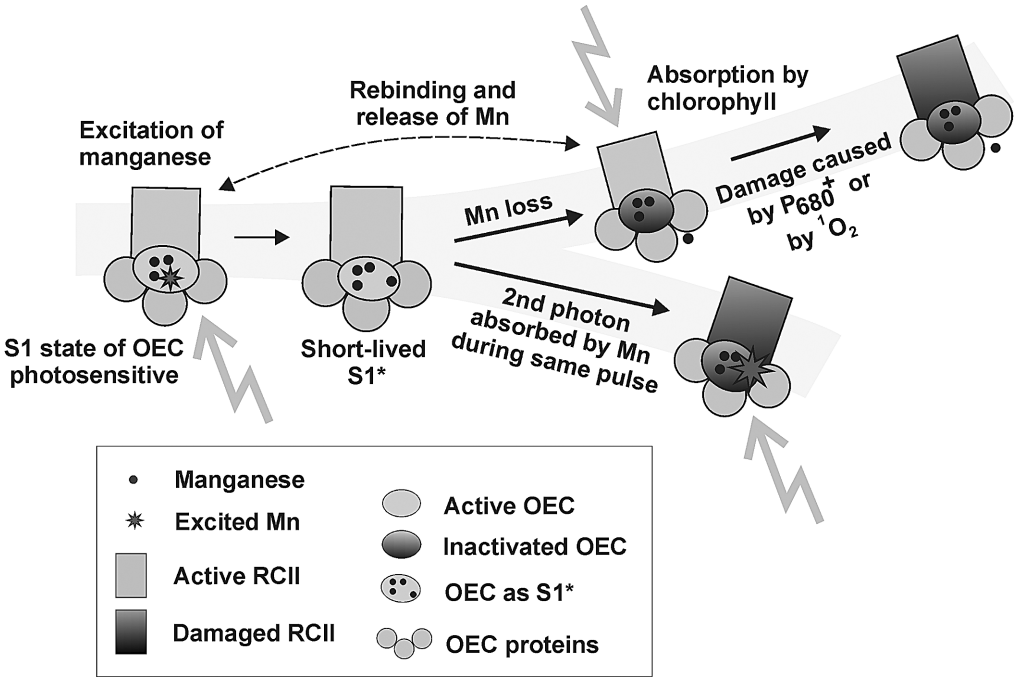

Fig 10. Absorption of light by manganese converts S1 to a short-lived S_1* state that decays to an inactive Mn-less state. Mn-less PSII is susceptible to P_{680}^+ mediated damage or to singlet oxygen produced due to charge recombination reactions, and this pathway dominates under continuous light. Under illumination with laser pulses, PSII becomes irreversibly inhibited mainly by a second photoreaction in which the short-lived S_1* state is the photoreceptor. This Mn mechanism of photoinhibition explains the independency of continuous light induced photoinhibition from electron transfer reactions, the quadratic light intensity dependence and the pulse interval effect of the laser-pulse-induced photoinhibition and photoinhibition induced by laser pulses on top of continuous light.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Based on the experiments in my thesis work related to photoinhibition of PSII I can say that the previous photoinhibition hypotheses are not able explain many of the experimental results. In the Mn mechanism of photoinhibition I suggest that at least two components are required to explain photoinhibition: Mn and Chl mediated components. The roles of these components under different conditions will have to be defined in future studies. Furthermore, the physical characteristics of the S_1*-state should be resolved by EPR studies and the further description of the influence of the secondary reaction under different conditions. When planning biotechnology for improvement of the natural photosynthesis machinery, it is important to consider the function and protection of OEC.

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Turussa syyskuussa 2007

Maija

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