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PHOTOINHIBITION OF PHOTOSYSTEM II

Kinetics, Photoprotection and Mechanism

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.

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II **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.

III **Sarvikas, P., Tyystjärvi T. and Tyystjärvi E.** (2010)
Kinetics of Prolonged Photoinhibition Revisited: Photoinhibited Photosystem II Centres Do Not Protect the Active Ones against Loss of Oxygen Evolution. Photosynthesis Research 103:7-17.

IV **Sarvikas, P., Hakala, M., Dönmez, S. and Tyystjärvi, E.**
Short Flashes and Continuous Light Have Similar Photoinhibitory Efficiency in Intact Leaves. Manuscript.

ABBREVIATIONS

Chl, Chl*, ^{3}Chl	Chlorophyll, excited singlet state of Chl and triplet state of Chl, respectively
$Cytb_{559}$	Cytohrome b_{559}
$Cytb_6/f$	Cytochrome b_6/f complex
CP29, CP26, CP24	Minor Chl *a/b* binding proteins of LHCII
CP43, CP47	Chl *a* binding proteins of PSII RC
D1 and D2	Reaction centre proteins of PSII
DCBQ	2,6-dichlorobenzoquinone
DMBQ	2,6-dimethylbenzoquinone
F_0, F'_0	Minimum fluorescence in the dark-adapted state and in the light-adapted state, respectively
F_M, F'_M	Maximum fluorescence in the dark-adapted state and in the light-adapted state, respectively
F_V/F_M	Ratio of variable to maximal fluorescence
Fd	Ferredoxin
FNR	Ferredoxin-NADP reductase
k_d	Rate constant of dark inactivation
k_{PI}	Rate constant of photoinhibition
LHCII	Light harvesting complex II
NO	Non-regulated non-photochemical quenching
NPQ	Non-photochemical quenching
OEC	Oxygen evolving complex
P_{680}, P_{680}*	Reaction centre Chl of PSII, excited singlet state of reaction centre Chl of PSII
PC	Plastocyanin
Pheo	Pheophytin
PFD	Photon flux density
PPFD	Photosynthetic photon flux density
PSI	Photosystem I complex
PSII	Photosystem II complex
q_E	Energy-dependent quenching of Chl *a* fluorescence
q_I	Quenching of Chl *a* fluorescence related to photoinhibition
q_P	Quenching of Chl *a* fluorescence related to photochemistry
q_T	Quenching of Chl *a* fluorescence related to state-transition
Q_A and Q_B	Primary and secondary plastoquinone electron acceptor of PSII
RC	Reaction centre
ROS	Reactive oxygen species
S_0-S_4	S-states of the oxygen evolving complex
Tyr_Z	Tyrosine Z
UV	Ultraviolet

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ABSTRACT

Photosystem II (PSII) is susceptible to light-induced damage defined as photoinhibition. In natural conditions, plants are capable of repairing the photoinhibited PSII by on-going degradation and re-synthesis of the D1 reaction centre protein of PSII. Photoinhibition is induced by both visible and ultraviolet light and photoinhibition occurs under all light intensities with the same efficiency per photon. In my thesis work, I studied the reaction kinetics and mechanism of photoinhibition of PSII, as well as photoprotection in leaves of higher plants.

Action spectroscopy was used to identify photoreceptors of photoinhibition. I found that the action spectrum of photoinhibition *in vivo* shows resemblance to the absorption spectra of manganese model compounds of the oxygen evolving complex (OEC) suggesting a role for manganese as a photoreceptor of photoinhibition under UV and visible light. In order to study the protective effect of non-photochemical quenching, the action spectrum was measured from leaves of wild type *Arabidopsis thaliana* and two mutants impaired in non-photochemical quenching of chlorophyll *a* excitations. The findings of action spectroscopy and simulations of chlorophyll-based photoinhibition mechanisms suggested that quenching of antenna excitations protects less efficiently than would be expected if antenna chlorophylls were the only photoreceptors of photoinhibition.

The reaction kinetics of prolonged photoinhibition was studied in leaves of *Cucurbita maxima* and *Capsicum annuum*. The results indicated that photoinhibitory decrease in both the oxygen evolution activity and ratio of variable to maximum fluorescence follows first-order kinetics *in vivo*. The persistence of first-order kinetics suggests that already photoinhibited reaction centres do not protect against photoinhibition and that the mechanism of photoinhibition does not have a reversible intermediate. When *Cucurbita maxima* leaves were photoinhibited with saturating single-turnover flashes and continuous light, the light response curve of photoinhibition was found to be essentially a straight line with both types of illumination, suggesting that similar photoinhibition mechanisms might function during illumination with continuous light and during illumination with short flashes.

Omistettu äidille ja isälle

1. INTRODUCTION

1.1. Photosynthesis

Photosynthetic organisms utilize light energy, carbon dioxide and water to synthesize carbohydrates. In plants, photosynthetic reactions take place inside specific organelles, chloroplasts. Inside chloroplasts, a continuum of thylakoid membranes forms appressed grana stacks and non-appressed stroma membranes (Fig. 1A). The soluble space inside thylakoid membranes is called lumen, whereas stroma is the outer aqueous space between the thylakoid membranes and the inner envelope of the chloroplast. Light is absorbed by photosynthetic pigments, most of which are bound to proteins embedded in the thylakoid membranes. In higher plants, these pigments include chlorophylls (Chl *a* and Chl *b*) absorbing blue and red light and carotenoids that absorb blue and green light (for review, see Ke, 2001). The energy of the absorbed photons drives photosynthetic light reactions in the thylakoid membrane and finally the physical energy of sunlight is stored in chemical form as NADPH and ATP. Four thylakoid-membrane-bound protein complexes are involved in the process: photosystem II (PSII), cytochrome b_6/f complex ($Cytb_6$/f), photosystem I (PSI) and ATP synthase (Fig. 1B). The majority of PSII centres are in the grana stacks, whereas most PSI centres and ATP synthases can be found in the stroma-exposed thylakoid membranes and there is no strict stoichiometry between PSII and PSI (Anderson and Andersson 1982; Chow et al. 1991; Albertsson 2001; Danielsson et al. 2004; Chow et al. 2005). When the photosynthetic electron transport from PSII to PSI functions, a proton gradient is formed across the thylakoid membrane and used as the driving force for ATP synthesis. In the stroma, NADPH and ATP are further utilized in carbon assimilation reactions of the Calvin-Benson cycle.

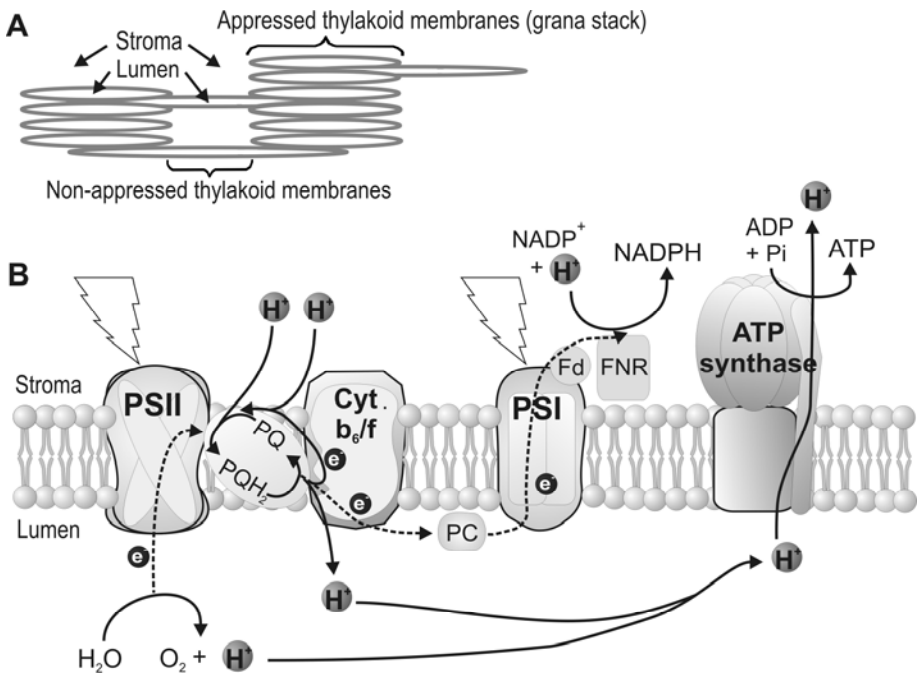

Fig. 1. (A) Simplified scheme of the internal thylakoid membrane structure of higher plant chloroplasts. Thylakoid membranes are organized as a continuum of appressed grana stacks and more spaciously arranged non-appressed stroma thylakoids. The soluble space surrounding the thylakoid membranes is called stroma and the inner soluble space of the thylakoid membranes is called lumen. Thylakoid membranes and stroma are enclosed by inner and outer membrane layers of chloroplast envelope (not shown). **(B)** Simplified scheme of the four protein complexes of the thylakoid membrane: photosystem II (PSII), cytochrome b_6/f complex ($Cytb_6$/f), photosystem I (PSI) and ATP synthase, and the linear electron transport chain of photosynthetic light reactions (dashed line). On the donor-side of PSII, water is split by the oxygen evolving complex (OEC). On the acceptor-side of PSII, plastoquinol (PQH_2) is formed and released from PSII to reduce the $Cytb_6$/f complex. An oxidized plastoquinone (PQ) is released to the plastoquinone pool and binds to PSII. The $Cytb_6$/f complex uses one of the two electrons from PQH_2 to reduce plastocyanin (PC) but the other electron reduces PQ in two reactions back to PQH_2 in the Q-cycle of the $Cytb_6$/f, which leads to transfer of additional protons to the lumen. PC donates an electron to P_{700}^+, the oxidized primary donor of PSI. 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 the transmembrane complex F_0 and the protruding F_1 complex on the stromal side of the thylakoid membrane. The stoichiometries of protons and electrons are not shown.

1.2. Photosystem II

PSII is a pigment-protein complex located in the thylakoid membranes of higher plants, cyanobacteria and algae (for review, see Melis 1999). PSII is the site where water splitting, oxygen release and reduction and protonation of plastoquinone occur (Fig. 2). The structure of PSII has been studied in detail by X-ray crystallography (Zouni et al. 2001; Ferreira et al. 2004; Iwata and Barber 2004; Loll et al. 2005) and it has been estimated that the core of the monomeric PSII complex contains about 20 protein subunits, 36 Chl molecules and 11 carotenoid molecules (Ferreira et al. 2004; Loll et al. 2005; Mulo et al. 2008). Monomeric PSII complexes form dimers on the thylakoid membrane and each monomer is associated with both the minor light-harvesting proteins (CP24, CP25 and CP29) and the major light-harvesting complex (LHCII) (Barros and Kühlbrandt 2009). It has been estimated that each PSII, with the light-harvesting antenna included, contains approximately 200-300 Chl molecules, depending on environmental conditions (for a review, see Hankamer et al. 1997; Melis et al. 1999; Dekker and Boekema 2005).

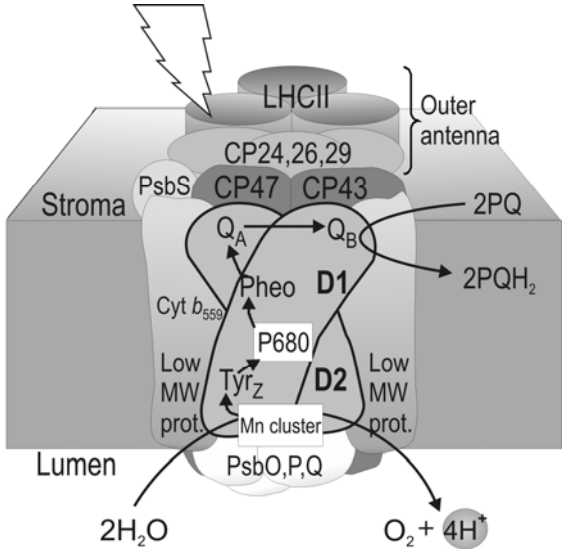

Fig. 2. Simplified scheme of the PSII monomer with associated electron carriers and the light harvesting antenna. The arrows show the direction of electron flow from water to Q_B, which then becomes double reduced and protonated to plastoquinol (PQH_2). Release of plastoquinol from PSII allows a new plastoquinone to bind to the D1 protein. The electron transfer components of PSII are Tyr_Z, P_{680}, pheophytin (Pheo) and plastoquinones Q_A and Q_B. The core of the PSII reaction centre consists of the D1, D2, cyt b_{559}, CP43 and CP47 proteins and is surrounded by several low molecular weight proteins of PSII. The outer antenna of PSII is composed of the major light-harvesting antenna (LHCII trimers) and the minor light-harvesting antenna (CP24, CP26 and CP29). On the lumenal side, water is split by the manganese cluster of the oxygen evolving complex (OEC). The Mn cluster is embedded within the three extrinsic proteins of PSII (PsbO, PsbP and PsbQ).

1.2.1. Linear electron transfer

Photosynthetically active light is absorbed by pigments Chl *a*, Chl *b* and carotenoids associated with the light harvesting antenna systems and the reaction centres of the photosystems. From the PSII antenna systems the excitation energy is transferred to a reaction centre Chl, P_{680}. P_{680} is actually a group of four Chls (P_{D1}/P_{D2} and Chl_{D1}/Chl_{D2}) (Fromme et al. 2006). The excitation energy of P_{680}* (the asterisk denotes excited state of the pigment molecule) drives electron transfer from P_{680} to pheophytin (Pheo), the primary electron acceptor of PSII (Klevanik et al. 1977). After charge separation, the highly oxidizing primary donor P_{680}^+ attracts an electron from the Mn cluster of the oxygen evolving complex via one of the tyrosine residues of the D1 protein (Tyr_Z) (Debus et al. 1988; Metz et al. 1989). $Pheo^-$ reduces the primary quinone acceptor Q_A, which, in turn, reduces the secondary electron acceptor Q_B. After receiving another electron, the double reduced Q_B is protonated to plastoquinol, which is released from PSII and binds to the $Cytb_6/f$ complex at Q_0 site close to the lumen. $Cytb_6/f$ catalyzes the reduction of the soluble plactocyanin electron carriers (PC) (Joliot & Joliot 1992). In addition, there is a cyclic electron transfer route around $Cytb_6/f$, known as the Q-cycle, where plastoquinone at the Q_i site is reduced and protonated. The Q-cycle leads to transport of additional protons to the lumen. Finally PC donates electrons to P_{700}^+, the oxidized primary donor of PSI. The components of PSI

electron transfer are Chl *a* (A_0), phylloquinone (A_1) and three iron-sulfur (4Fe-4S) clusters. PSI reduces ferredoxin that reduces $NADP^+$ to NADPH via ferredoxin-NADP reductase (FNR).

1.2.2. Charge recombination reactions in PSII

Charge recombination reactions, i.e. the reversal of linear electron transfer reactions, are of importance for photoinhibition research mainly because these reactions may lead to formation of the harmful singlet oxygen. In general, reactive oxygen species like singlet oxygen cause oxidative stress in the chloroplast (for review, see Foyer et al. 1994). In PSII, the repair of photoinhibition damage is particularly sensitive to reactive oxygen species (Nishiyama et al. 2006).

1.2.2.1. Charge recombination of the primary radical pair

During illumination of PSII, the primary radical pair (P_{680}^+/$Pheo^-$) is not always successful in producing Q_A^- and charges of P_{680}^+ and $Pheo^-$ may recombine. If the spin configuration of the primary radical pair is equivalent with a triplet state $^3(P_{680}^+/Pheo^-)$, then charge recombination may produce the triplet state of the primary donor, $^3P_{680}$*. The yield of $^3(P_{680}^+/Pheo^-)$ formation is influenced by the competition between (i) P_{680}^+/$Pheo^-$ dissipation via spin conversion to the triplet state, (ii) direct recombination of P_{680}^+/$Pheo^-$ to the ground state (P_{680}/Pheo) and (iii) the equilibrium between the P_{680}^+/$Pheo^-$, P_{680}* and excited antenna Chl (Schatz et al. 1988). The time constant of direct recombination of the primary radical pair to the ground state has been estimated to be about 2.5 ns (Moser et al. 2005) and it is comparable to the few nanosecond time constant of spin change between the ground state and triplet state of the primary radical pair (Hoff 1981). Typically the lifetime of the charge separated primary radical pair is too short (0.3-0.5 ns, Renger and Holzwarth 2005) to markedly yield the triplet intermediate state $^3(P_{680}^+/Pheo^-)$. However, under e.g. anaerobiosis or at cryogenic temperatures, double reduction and protonation of Q_A have been shown to increase the lifetime of the primary radical pair, as well as the probability of $^3(P_{680}^+/Pheo^-)$ formation, which, in turn, leads to increased production of triplet Chl ($^3P_{680}$) (Hideg et al. 1998; Durrant et al. 1990; Vass et al. 1992; van Mieghem et al. 1995; Telfer et al. 1999). It has been suggested that formation of $^3(P_{680}^+/Pheo^-)$ could also take place under physiological conditions when Q_A is in the stably reduced form or even in the presence of unstabilized Q_A^- (Vass et al. 1992; Vass and Aro 2008). However, in the presence of Q_A^-, the lifetime of $^3P_{680}$ is 150-500 fold shorter than in the presence of double reduced Q_A (van Mieghem et al. 1995).

1.2.2.2. Charge recombination of further charge separated states

Charge recombination may also occur between further charge separated states but with much slower time constants than recombination of the primary radical pair. Charge recombination between P_{680}^+ and Q_A^- is typically caused by stochastic failure of electron donation by OEC. In the Mn complex of OEC, electrons derived from manganese ions during advancement of the S-states reduce P_{680}^+ via Tyr_Z. A miss is a failure in the advancement of an S-state after production of the charge separated state $P_{680}^+Q_A^-$ (Kok et al. 1970). In this case, P_{680}^+ is reduced by charge recombination with Q_A^-. In the absence of electron donation from the OEC, the lifetime of P_{680}^+ is 100-200 µs (Renger and Holzwarth 2005), whereas in the presence of functional OEC, the lifetime of P_{680}^+ has been estimated

to be in the range of 23-260 ns before reduction by OEC via Tyr_Z (Diner and Babcock 1996).

Occasionally charge recombination takes place between electron deprived S_2 or S_3 state of OEC on the donor side of PSII and the reduced Q_A or Q_B on the acceptor side of PSII. Charge recombination between the S-states of the OEC and the plastoquinone electron acceptors is very slow and can compete with forward electron transfer only if PSII excitations occur rarely, e.g. under illumination with short flashes of light separated by long dark intervals. Charge recombination of S_2/Q_A^- to S_1/Q_A may take place if electron transfer from Q_A to Q_B is blocked (Ohad et al. 1990; Vass et al. 1992; van Mieghem 1995). Thermoluminescence studies have suggested that the time constant for charge recombination of S_2/Q_B^- to S_1/Q_B is 30 s (Rutherford et al. 1984; Keren et al. 1997; Hakala M, Sarvikas P, Paturi P, Tyystjärvi E, manuscript in preparation). Fluorescence relaxation experiments have suggested somewhat shorter timescales (Vass et al. 1999; Cser and Vass 2007; Antal et al. 2009b).

1.2.2.3. Charge recombination and singlet oxygen

Charge recombination reactions in PSII have been associated with an increased chance of formation of singlet oxygen via triplet Chl (Keren et al. 1997; Szilard et al. 2005; Vass and Cser 2009). Rapid recombination of the primary radical pair does not usually lead to triplet Chl because the radical pair is formed from the singlet ground state of P_{680}, and the spins of the electrons persist in singlet configuration for a short time. An increase in the lifetime of the primary radical pair, e.g. due to stabilization of Q_A^- by protonation, enhances the probability of triplet Chl production (Durrant et al. 1990; Vass et al. 1992; Hideg et al. 1998; Telfer et al. 1999) because spin conversion in the radical pair needs time (Vass et al. 1992; van Mieghem et al. 1995). The very slow charge recombination reactions (e.g. S_2/Q_A^- to S_1/Q_A), in turn, may easily produce triplet chlorophyll because the spin of the electron of the donor molecule does not originally have any specific orientation with regard to the spin of the electron in the acceptor.

Once triplet Chl is formed in PSII by charge recombination reactions, it may react with oxygen and produce singlet oxygen. Singlet oxygen is a highly reactive oxygen species with potential to damage the D1 protein *in vivo* (Telfer et al. 1999). In support, production of singlet oxygen has been observed during photoinhibition of PSII under aerobic conditions (Hideg et al. 1994, 1998, 2001). However, experimental evidence does not support the dependence of photoinhibition on reduced Q_A (Hakala et al. 2005), and Chl triplets produced in the presence of reduced Q_A are short-lived (van Mieghem et al. 1995).

1.3. Oxygen evolving complex

1.3.1. Structure and function of OEC

OEC is the water-splitting apparatus on the lumenal side of PSII. The structure of OEC has recently been studied by X-ray spectroscopy and electron paramagnetic resonance spectroscopy (Loll et al. 2005). The catalytic site of OEC appears to consist of four Mn ions and a Ca^{2+} ion, which is apparently required for structural purposes and proper function of

the OEC (Lee et al. 2007). In addition, a Cl^- ion seems to be required for some of the S-state transitions of OEC (Wincencjusz et al. 1997). It has been suggested that the four Mn ions form a tetramer and that the distances between the four Mn ions are about 3 Å (Loll et al. 2005). In plant thylakoid membranes, the Mn cluster of the OEC is embedded inside three extrinsic proteins of PSII: PsbO, PsbP and PsbQ, which stabilize the function of OEC (Mayes et al. 1991; Debus 1992; Yi et al. 2006) and optimize the levels of ion cofactors in OEC (Debus 1992; Ifuku et al. 2005).

The Mn ions of the OEC cycle through five oxidation states (S_0, S_1, S_2, S_3, S_4) (Kok et al. 1970; Haumann et al. 2005). The cycle of S-states is fuelled by the light-induced generation of P_{680}^+. Oxygen is released after four successive steps, during the conversion of S_3 to first the transient S_4 and then to S_0, the most reduced state of OEC (Dau and Haumann 2006). Operation of OEC has been studied by illuminating thylakoids with short single-turnover flashes, which advance the S-states gradually (Kok et al. 1970). The time constants for the advancements of the S-states are in the order of 0.03-1 ms, the advancement from S_3 to S_0 via S_4 being the slowest step. When leaves are under constant illumination, one fourth of OEC is in each state S_0, S_1, S_2 or S_3. In darkness, states S_2 and S_3 relax so that approximately 75 % of the OEC end up in S_1-state and the rest in S_0-state (Kok et al. 1970; Antal et al. 2009b).

When illumination is provided by single-turnover flashes, the four step oscillating cycle of OEC becomes disturbed by misses and double hits (Kok et al. 1970; Shinkarev 2005). Occasionally OEC is unable to reduce P_{680}^+ due to a miss in the advancement of the S-state cycle (Kok et al. 1970) and P_{680}^+ is reduced either via charge recombination with Q_A^- or by an alternative electron source within PSII. It has been estimated that every tenth charge separation fails to advance S-states (Naber et al. 1993; Antal et al. 2009b). Short flashes of light may sometimes cause also double hits, where two advancements in S-state cycle are brought about by a single flash. For microsecond-length flashes from a Xenon lamp, the probability of a double hit has been estimated to be about 5 %.

1.4. Photoinhibition

1.4.1. Photoinhibitory damage and the repair of damaged PSII

Light is essential for photosynthesis, but light also damages the photosynthetic apparatus. In this thesis, I use the term photoinhibition for the reaction that damages photosystem II in the light, however, the concepts of photodamage and photoinactivation of PSII are essentially similar to photoinhibition (Nishiyama et al. 2006; Chow and Aro 2005). Photoinhibition occurs under all light intensities of visible (400-700 nm) and ultraviolet light (220-400 nm) but UV light is more efficient in inducing photoinhibition than visible light (Jones and Kok 1966; Renger et al. 1989; Jung and Kim 1990). In natural conditions, light stress becomes obvious if the rate of photoinhibition exceeds the rate of repair (for reviews of photoinhibition, see Aro et al. 1993a; Melis 1999; Andersson and Aro 2001; Tyystjärvi 2008; Vass and Aro 2008). After photoinhibition, the damaged PSII migrates to stroma thylakoids where the D1 reaction centre protein is degraded and replaced by a new copy in the repair process of PSII. The reassembled PSII migrates back to grana membranes where other components of PSII are reassembled, and finally a functional PSII dimer is

formed. At high light intensities, where the rate of the damage is fast, also fast rate of repair is required to maintain PSII activity at high level (Ohad et al. 1984).

1.4.2. Reaction kinetics of photoinhibition

Elucidation of the kinetic pattern is important in revealing the mechanism of a photochemical reaction, such as photoinhibition. Analysis of reaction kinetics enables the calculation of the rate constant which, in turn, enables the quantification of positive and negative effects on the rate of the reaction. The rate constant of photoinhibition (k_{PI}) describes the initial rate of loss of PSII activity. Calculation of k_{PI} is necessary for quantitative comparison of the rate of the damaging reaction of photoinhibition under different conditions. In order to determine the k_{PI} *in vivo*, the effect of the repair process of PSII has to be excluded. This can be done by measuring photoinhibition in the presence of an inhibitor of chloroplast protein synthesis (Ohad et al. 1984; Samuelsson et al. 1985; Greer et al. 1986; Tyystjärvi et al. 1994), e.g. lincomycin (Mulo et al. 2003). Alternatively, thylakoids can be isolated from photosynthetic organisms and used directly for photoinhibition measurements, as the repair machinery is not functional *in vitro* (Nedbal et al. 1986; Tyystjärvi et al. 1994).

The k_{PI} value has been shown to be directly proportional to light intensity under both visible and UV light (Jones and Kok 1966; Tyystjärvi and Aro 1996; Santabarbara et al. 2002; Nishiyama et al. 2005). These results suggest that the quantum yield of photoinhibition is the same, irrespective of the light intensity or the wavelength. Some studies have suggested that photons of low light have higher photoinhibitory efficiency than photons of high light (Keren et al. 1995, 1997), however opposite results have also been presented, suggesting that the quantum yield of photoinhibition is greater under high light than at low light (Oguchi et al. 2009). There is general agreement that photoinhibition follows first-order kinetics during the initial phases of typical photoinhibition experiments in which a substantial fraction of PSII centres remains active at the end of the illumination treatment (Jones and Kok 1966; Aro et al. 1993b; Tyystjärvi et al. 1994; Tyystjärvi and Aro 1996; Lee et al. 1999). In contradiction, a clear deviation from first-order kinetics was suggested by photoinhibition experiments with lincomycin-treated *Capsicum annuum* leaves under prolonged illumination (Lee et al. 2001, Matsubara and Chow 2004). This deviation was implied to indicate protection by already photoinhibited PSII centres against photoinhibition of the remaining active PSII centres (Sun et al. 2006). Such a deviation from first-order kinetics might also be caused by the presence of a reversible phase in photoinhibition. I have approached this question in Paper III.

1.4.3. Action spectrum of photoinhibition

Once the kinetic pattern of a photochemical reaction is known, it is possible to quantify the response to different wavelengths of incident light, that is, to measure the action spectrum of the reaction. The general form of the action spectrum resembles the absorption spectra of the photoreceptors of the reaction (Haxo and Blinks 1950), and for this reason, action spectroscopy is used to identify possible photoreceptors of photochemical reactions. Several action spectra of photoinhibition have been measured from thylakoids of higher plants (Jones and Kok 1966; Renger et al. 1989; Jung and Kim 1990; Santabarbara et al. 2001a; Hakala et al. 2005; Ohnishi et al. 2005). In addition, measurements have been made of the action spectrum of general sensitivity of phycoplankton cells to UV light

(Boucher and Prezelin 1996), the action spectrum of D1 protein degradation from *Spirodela oligorrhiza* (Greenberg et al. 1989) and the action spectrum of photoinhibition in the cyanobacterium *Synechocystis* sp. PCC 6803 (Tyystjärvi et al. 2002). However, until Paper II, action spectrum of photoinhibition had not been measured from higher plants *in vivo*.

1.4.4. Hypotheses about the mechanism of photoinhibition

The mechanism of photoinhibition has been under debate for over two decades. No conclusive evidence has been presented to support a single or combined mechanism for photoinhibition *in vivo*. Yet, several hypotheses on the molecular mechanism of the primary photoinhibition damage have been put forward. In the following, I will focus on summarizing the most popular hypotheses that have been presented to explain the mechanism of photoinhibition. Most of the suggested photoinhibition mechanisms deal with the effect of visible light only.

1.4.4.1. Acceptor-side photoinhibition hypothesis

The acceptor-side hypothesis relates photoinhibition to strong light which causes double reduction and dissociation of the Q_A electron acceptor from PSII (Vass et al. 1992). The reaction occurs in PSII centres that contain functional P_{680}, Pheo and D1 protein but in which electron transfer from Q_A^- to Q_B is hampered because the plastoquinone pool is reduced (Vass et al. 1992). The lifetime of the primary radical pair is prolonged by double reduction and when charge recombination of the primary pair finally occurs, the probability of production of ^{3}Chl is high. The experiments leading to the acceptor-side hypothesis were done under anaerobic conditions. Double reduction of Q_A has not been seen in natural, aerobic conditions (Vass et al. 1993) but in aerobic conditions PSII may become inhibited if Q_A^- is stabilized by protonation (Vass et al. 1992). Both double reduction and stabilization of Q_A^- prolong the lifetime of the primary radical pair, which leads to enhanced production of singlet oxygen.

It has also been suggested that singlet oxygen produced in the presence of unstabilized Q_A^- (Vass and Aro 2008) might be the actual damaging agent in photoinhibition. The amount of triplet Chl and singlet oxygen produced are expected to increase with increasing light intensity when the photosynthetic electron transfer is light-saturated (Vass and Cser 2009).

1.4.4.2. Donor-side photoinhibition hypothesis

According to the donor-side hypothesis, PSII becomes damaged because electron donation from OEC is disrupted and the highly oxidized, long lived P_{680}^+ (and/or Tyr_Z^+) oxidizes a wrong component of the reaction centre (Callahan et al. 1986; Chen et al. 1992; Anderson et al. 1998). Electron donation from OEC could be disrupted after partial release of Ca^{2+} from OEC, for example under low lumen pH (Krieger and Weis 1993). Donor-side photoinhibition has been directly observed after OEC has been chemically removed or destroyed in an active PSII unit (Callahan et al. 1986; Eckert et al. 1991; Chen et al. 1992). Accordingly, experiments with an OEC-less mutant of *Scenedesmus* have shown high sensitivity to light (Keren et al. 1995). Furthermore, even a functional OEC is occasionally unable to reduce P_{680}^+ due to a miss in the S-state cycle of the OEC (Kok et al. 1970). After a miss, P_{680}^+ decays slowly (up to 200 µs) by recombination with Q_A^- (Renger and Holzwarth, 2005), and it is possible that some other component of PSII than Q_A^- reduces P_{680}^+, which may lead into donor-side inactivation of PSII (Anderson et al. 1998). In the

donor-side photoinhibition hypothesis, light is not assumed to exert a direct effect on the functionality of OEC itself.

1.4.4.3. Low-light photoinhibition hypothesis

Photoinhibition experiments are typically done with continuous light, but strong, short flashes of light also cause photoinhibition (Keren et al. 1995, 1997). Flashes within the timescale of few microseconds or less in length are called single-turnover flashes because one flash causes only one PSII turnover (S_nQ_A to $S_{n+1}Q_A^-$). Even though single-turnover flashes do not occur in nature, the low-light hypothesis (Keren et al. 1995, 1997, 2000; Szilard et al. 2005) was designed to explain photoinhibition under dim continuous light, and short pulses of light were applied to model dim light conditions. When nanosecond-length laser pulses were separated by long intervals, more photoinhibition was caused per pulse than with shorter intervals and this finding was related to the time constant of charge recombination (Keren et al. 1997). Thus, the low-light photoinhibition hypothesis is based on slow charge recombination reactions of PSII (e.g. $S_2Q_B^-$ to S_1Q_B) which take place if Q_B^- is not immediately double reduced by a subsequent PSII turnover. As the quantum yield of the slow charge recombination of PSII increases, so does the probability of triplet Chl formation and singlet oxygen production. Photoinhibition caused by the low-light mechanism would not depend on the intensity of the flashes, as long as the flashes are saturating and short enough to be considered as single-turnover flashes.

1.4.4.4. Singlet oxygen hypotheses

It has also been suggested that photoinhibition is caused directly by singlet oxygen produced by illumination of iron-sulfur centres or cytochromes (Jung and Kim 1990) or Chls which are weakly coupled or uncoupled from the light harvesting antenna (Santabarbara et al. 2001a, 2002). The involvement of uncoupled Chls was suggested by the findings that (i) quenching of antenna Chl excitations offers only little protection against photoinhibition (Tyystjärvi et al. 1999; Santabarbara 2001b), (ii) Chl triplets are produced by weakly coupled Chl in PSII (Santabarbara et al. 2002) and (iii) that the action spectrum of photoinhibition is blue-shifted in the red region when compared to the action spectrum of photosynthesis (Santabarbara et al. 2001a; Santabarbara et al. 2002). It has been estimated that the size of the uncoupled Chl pool is about 1-3 Chl per PSII reaction centre (Santabarbara and Jennings 2005), however, the uncoupled Chls in the PSII antenna have not been identified. The singlet oxygen hypotheses explain the independence of the quantum yield of photoinhibition of PPFD, but they do not explain the fact that photoinhibition also occurs in anaerobic conditions.

1.4.4.5. Manganese hypothesis

According to the manganese hypothesis, photoinhibition begins when light absorption by Mn ions of the OEC triggers the disintegration of the manganese complex (Hakala et al. 2005; Ohnishi et al. 2005). At the time, it was considered that the disintegration of the manganese complex might be reversible and that a subsequent Chl-dependent step could be responsible for the final, irreversible inhibition of PSII (Hakala et al. 2005). Prior to the development of the manganese hypothesis of photoinhibition, the manganese complex of OEC had been suggested to be the primary target of damage caused by UV light only (Renger et al. 1989; Barbato et al. 1995; Larkum et al. 2001; Vass et al. 2002). However, the forms of the absorption spectra of Mn^{3+} and Mn^{4+} model compounds of OEC (Bodini et

al. 1979; Baffert et al. 2002; Hakala et al. 2005) suggest that if manganese is important for photoinhibition in the UV range, it also contributes to photoinhibition under visible light. Furthermore, the primary photoinhibitory damage has been localized to OEC under both UV and visible light (Hakala et al. 2005; Ohnishi et al. 2005) and Mn ions have been found to dissociate from the OEC during photoinactivation (Virgin et al. 1988; Renger et al. 1989; Hakala et al. 2005; Zsiros et al. 2006; Ohnishi et al. 2007).

1.5. Photoprotection

1.5.1. Mechanical and biochemical photoprotection

Plants have evolved different mechanisms to either protect themselves from the harmful effects of light or to avoid being exposed to strong light (for review, see Demmig-Adams and Adams 1992; Niyogi et al. 1998; Walters 2005). Physical avoidance mechanisms include alteration of leaf blade orientation with respect to direction of light (McMillen and McClendon 1979; Öquist and Huner 1991; Jiang et al. 2006), cuticle compounds and leaf surface waxes that function as sunscreeners (Robinson et al. 1993; Kolb et al. 2001; Markstädter et al. 2001) or morphological adaptations such as leaf hairing, compact growth pattern and leaf size and form. Inside plants, chloroplast movements take place within minutes as light induces the movement of chloroplasts towards the anticlinal cell walls (Chow et al. 1988; Park et al. 1996; Briggs and Christie 2002, Wada et al. 2003; Kagawa and Wada 2004), and mutant plants lacking this avoidance response are more susceptible to photoinhibition than wild-type plants (Kasahara et al. 2002). Accumulation of screening compounds such as anthocyanins and rhodoxanthin can also be a light-induced protection mechanism (Weger et al. 1993; Chalker-Scott 1999; Smillie and Hetherington 1999; Havaux et al. 2000; Steyn et al. 2002). In addition, accumulation of antioxidant molecules, such as tocopherols, tocotrienols and vitamin b6, may be enhanced under strong light to protect plants from photodamage and photo-oxidative stress (Havaux et al. 2000, 2005, 2009; Trebst et al. 2002; Krieger-Liszkay and Trebst 2006; Krieger-Liszkay et al. 2008; Matringe et al. 2008).

1.5.2. Photochemical quenching

Inside thylakoid membranes, light is absorbed by protein-bound pigments and the physical energy is transferred from the light harvesting antenna to the reaction centres to be utilized in photochemical reactions. The excitation energy of a chlorophyll molecule can decay via three routes: it can be utilized in photosynthetic electron transfer (photochemical quenching), emitted as a quantum of light (fluorescence) or dissipated as heat (non-photochemical quenching). As an adaptation to environment, plants are capable of adjusting the electron flow directed to photochemistry. In low light conditions, most of the excitation energy is used to drive photosynthesis and only a few percent of energy are emitted as fluorescence (for review, see Govindjee 2005; Gruszecki et al. 2006; Lazar 2006). However, as the light intensity increases, a bigger portion of the excitation energy is quenched non-photochemically and the yield of photochemical quenching decreases (Demmig-Adams et al. 1996). The distribution of energy between the three routes is flexible and changes constantly due to environmental conditions (Genty et al. 1989; Krause and Weis 1991; Külheim et al. 2002; Kramer et al. 2004).

In spite of different mechanisms described above, plants tend to receive more excitation energy under natural conditions than they can quench photochemically via photosynthetic electron transfer. This is because under strong, saturating light, the yield of photochemistry can be limited by the capacity of the Calvin-Benson cycle. Photorespiratory pathway, by which plant consumes oxygen and releases carbon dioxide during photosynthesis (for review, see Foyer et al. 2009), and alternative electron acceptors, such as oxygen (for a review, see Foyer and Noctor 1999), may alleviate the excitation pressure of PSII (Kozaki and Takeba 1996; Jiang et al. 2006; Kanervo et al. 2005) and even mitochondrial electron transport may decrease the excitation pressure in photosynthetic electron chain (Yoshida et al. 2006). In addition, plants are typically capable of adjusting the size of the light harvesting antenna during adaptation to different environmental conditions (Park et al. 1996). However, with regard to photoinhibition, the effect of excitation pressure can even at best be very small, as it has been shown that blocking photosynthesis protects against photoinhibition under strong illumination (Hakala et al. 2005).

1.5.3. Non-photochemical quenching

Non-photochemical quenching (NPQ) dissipates the excitation energy of Chl *a* molecules safely as heat (for review, see Niyogi et al. 1998; Szabó et al. 2005). Both photochemical and non-photochemical quenching of Chl excitations can be easily and non-invasively monitored by measuring fluorescence emission of intact leaves. The three components of regulated non-photochemical quenching include energy-dependent quenching (q_E), photoinhibitory quenching (q_I) and state-transition induced quenching (q_T) (Horton and Hague 1988; Quick and Stitt 1989). NPQ has been observed from plant leaves, green algae and diatoms and, fairly recently, involvement of an orange carotenoid protein in the mechanism of light-regulated thermal dissipation of antenna excitations in cyanobacteria has been elucidated (Wilson et al. 2006, 2008; Punginelli et al. 2009). Also the IsiA protein, accumulating in iron-deficient cyanobacteria, seems to cause quenching of excitation energy (Sandström et al. 2001; Ihalainen et al. 2005). In addition to light-regulated NPQ, non-radiative quenching occurs via constitutive non-photochemical quenching (NO), which is not regulated actively in leaves (Kramer et al. 2004). NO occurs in the antenna chlorophylls and NO is also associated with direct recombination of the primary radical pair to the ground state. In the literature, the word NPQ sometimes refers to the sum of all components of non-photochemical quenching (NO, q_E, q_I and q_T) and at other times only to the light-regulated component of non-photochemical quenching. In this thesis, I will use the abbreviation NPQ for non-photochemical quenching which includes q_E, q_I, q_T and NO.

1.5.3.1. Energy-dependent quenching

The energy-dependent quenching decreases the flow of energy to the reaction centre of PSII in high light conditions (Niyogi et al. 1998). q_E is induced in the thylakoid membranes within seconds after transfer from dark to light and relaxes within minutes (Horton and Hague 1988, Quick and Stitt 1989). Details of the quenching mechanism and the pigment molecules involved are still unclear (Holt et al. 2004, Cogdell 2006, Barros and Kühlbrandt 2009). However, formation of proton gradient across the thylakoid membrane is required for the induction of q_E (Briantais et al. 1979, Horton et al. 2005, Szabó et al. 2005). Low pH activates the violaxanthin de-epoxidase that converts violaxanthin into zeaxanthin (Demmig et al. 1987) and causes protonation of the PsbS protein of PSII (Li et al. 2000, Li et al. 2004), both of which are important for efficient q_E in plants. Recently, it has been shown

that q_E is associated with PsbS protein-dependent reorganization of the PSII-LHCII protein domains and that these changes are reversible when light intensity changes (Kiss et al. 2008; Betterle 2009; Kereiche 2010). I have studied the photoprotective role of qE in Papers I and II.

1.5.3.2. Photoinhibitory quenching

Photoinhibitory quenching is induced in tens of minutes after transfer of a plant to bright light and the relaxation of qI is relatively slow (Horton and Hague 1988; Krause and Jahns 2004; Stroch et al. 2004). The quenching mechanism of q_I is still unclear, but it has been associated with accumulation of photoinhibited reaction centres. In support, a short-lifetime Chl fluorescence component has been associated with the accumulation of inactive PSII centres (Chow et al. 2002; Sun et al. 2006). The research group of W. S. Chow has proposed that under prolonged photoinhibition already photoinhibited PSII units protect the residual active ones by dissipating the absorbed photon energy (Chow et al. 2002; Sun et al. 2006). I have studied this question in Paper III.

1.5.3.3. State-transition-induced quenching

In addition to q_E and q_I, some light-regulated non-photochemical quenching may be associated with state transition that balances excitation energy between PSII and PSI (for review, see Dekker and Boekema 2005). In darkness, plant thylakoids are in State I. When the plastoquinone pool is reduced in the light, a conformation change in the $Cytb_6/f$ complex activates the LHC kinase and State II is formed either as the phosphorylated LHCII units disassociate from PSII and move to PSI (for review, see Bennett 1983; Aro and Ohad 2003; Kanervo et al. 2005; Dekker and Boekema 2005) or as the PSI-LCHII complexes move closer to PSII-LCHII in grana margins, as recently suggested by Tikkanen et al. (2006). As a result of state transition, the yield of Chl *a* fluorescence is slightly lowered. When the $Cytb_6/f$ becomes oxidized, the kinase is deactivated and transition from State II to State I may take place. Under continuous light, the Arabidopsis *stn7*-mutant, which is deficient in q_T, grows normally (Tikkanen et al. 2006). However, under fluctuating light conditions the growth of q_T-deficient mutants is typically slower and they have a more reduced plastoquinone pool (Bellafiore et al. 2005). It has also been suggested that q_T might be associated with transition from linear to cyclic electron transfer when the electron transfer chain is highly reduced and CO_2 is limiting (Finazzi et al. 1999).

2. AIM OF THE STUDY

In this thesis, I have done photoinhibition experiments mainly with intact plant leaves in an attempt to clarify the mechanism of photoinhibition *in vivo*.

- I have modeled photoinhibition and measured the first action spectrum of photoinhibition *in vivo*, in order to identify the photoreceptor of photoinhibition and the photoprotective effect of q_E type of NPQ.

- I have analyzed the reaction kinetics of photoinhibition under prolonged illumination to find signs of reversibility in the mechanism of photoinhibition or protection by already photoinhibited PSII reaction centres.

- I have studied the mechanism of photoinhibition by comparing the photoinhibitory efficiency of continuous light and short flashes of light under physiological conditions.

3. METHODOLOGY

3.1. Plant material

Arabidopsis thaliana plants were grown under 100 µmol photons m^{-2} s^{-1} in 8/16 h day/night rhythm (Papers I and II) and pumpkin (*Cucurbita maxima*) (Papers III and IV) and pepper (*Capsicum annuum*) (Paper III) plants were grown under 150 µmol photons m^{-2} s^{-1}, in 12/12 h day/night rhythm. The growth conditions are known to affect the sensitivity of plants to photoinhibition via e.g. thermal dissipation and production of light-induced protective substances, such as UV-screening compounds. Generally, similar growth light conditions as I have used in Papers I-IV, are used to grow these plants for research purposes.

Photoinhibition experiments were done on lincomycin (2.3 mM) treated leaves of Arabidopsis (Papers I and II), pumpkin (Papers III and IV) or pepper (Paper III). At this concentration, lincomycin fully inhibits the repair cycle without having other harmful effects on PSII (Mulo et al. 2003). The *in vivo* action spectra in Paper II were measured by using Arabidopsis leaves because two NPQ-deficient Arabidopsis 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 for *in vivo* photoinhibition experiments because pumpkin leaves take up lincomycin easily and light reactions are equally active in all parts of the leaves. For Paper III, pepper was chosen for comparison, as we needed to compare our results with those of Lee et al. (2001) and Matsubara and Chow (2004).

3.2. Photoinhibitory light and measurement of photon flux density

Photoinhibition was induced by illuminating leaves, isolated thylakoids or PSII membranes by either different wavelength regions of light (Papers II and III), white continuous light (Papers I, III and IV), UV light (Papers II and III), sunlight (Paper II) or short single-turnover flashes (Paper IV). For continuous-light experiments, either a 300 W high-pressure ozone-free Xenon lamp equipped with a Schott GG400 UV-blocking filter (Papers I, II, III and IV) or a HPI-T greenhouse lamp (Paper IV) were used as sources of photoinhibitory illumination. The HPI-T greenhouse lamp, which was used in Paper IV for illumination with low continuous light, has several narrow spectral peaks but no discontinuity in the PPFD dependence of k_{PI} was detected between the PPFD values of 500 and 930 µmol m^{-2} s^{-1} where the switch between the two continuous-light lamps was done, indicating that the two continuous-light lamps used in Paper IV are similar with regard to photoinhibitory efficiency. Different visible light wavelength regions were obtained by using pairs of low-pass and high-pass filters (Corion, USA) to define 50 nm wavelength bands (Paper III). 10 nm filters (Corion, USA) were used to define even narrower wavelength bands (Paper II). An ENF-280C lamp (Spectronics, USA) was used as a light source for continuous UV-C and UV-A light illuminations (Papers II and III). Microsecond-length, single-turnover flashes were obtained from FX-200 Xenon flash lamp (EG&G, USA) through a Schott GG400 UV-blocking filter (Paper IV). The FX-200 flash lamp and the continuous-light high-pressure Xenon lamp have fairly similar, continuous spectra, further justifying the comparison of these two light sources at the PPFD level (Paper IV).

The photosynthetic photon flux density (PPFD) of the experiments in continuous visible light was measured with a quantum sensor (LiCor, USA). Prior to measuring the action spectra of photoinhibition (Paper II), the quantum sensor was calibrated for its spectral response. The photon flux density (PFD) of the continuous UV light (Paper II) and short flashes of light (Paper IV) was measured with actinometric methods by measuring the number of photons that hit the sample. A UV-light-sensitive chemical, $K_3[Fe(C_2O_4)_3]$, was used as it undergoes a photochemical reduction from Fe^{3+} to Fe^{2+} upon absorption by UV light (Hatchard and Parker 1956). The Fe^{2+} is released to the medium and the concentration of the free ferro ions can be measured by adding o-phenanthroline which forms a colored complex with Fe^{2+}. To measure the PPFD of the short flashes of light, *meso*-diphenylhelianthrene (Brauer et al. 1983) was used because the number of photons absorbed by the compound is proportional to the increase in absorbance at 429 nm. *Meso*-diphenylhelianthrene is sensitive to wavelengths of 475-610 nm, so the result was corrected with the emission spectrum of the lamp to obtain emission in the 400-700 nm range.

3.3. Measurement of oxygen evolution activity of PSII

In papers II-IV, 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). Measurement of light-saturated activity of oxygen evolution is a commonly used method in photoinhibition studies and it gives a good estimate of the number of active PSII reaction centres in the whole leaf segment. For measurements of unilluminated and illuminated leaves, thylakoids were quickly isolated as described in Paper II, and used for measurement of the oxygen evolving activity of PSII. A slide projector was used as a source of saturating light and either 2,6-dimethylbenzoquinone (DMBQ) (Papers I and II) or 2,6-dichlorobenzoquinone (DCBQ) (Papers III and IV) were used as artificial electron acceptor. Both DMBQ and DCBQ are used as electron acceptors in measurements of oxygen evolution activity of PSII, however, it has been shown that DCBQ oxidizes plastoquinol pool more efficiently than DMBQ (Srivastava et al. 1995).

The rate constant of photoinhibition (k_{PI}) was used as a measure of photoinhibition. The k_{PI} was calculated by fitting the photoinhibitory loss of PSII activity to a first-order reaction equation (Jones and Kok 1966; Ögren et al. 1984, Cleland and Melis 1987, Chow et al. 1989, Setlik et al. 1990, Wünschmann and Brand 1992, Aro et al. 1993a, Tyystjärvi et al. 1994, Tyystjärvi and Aro 1996, Lee et al. 1999). As slight dark inactivation of the samples was detected under prolonged photoinhibition, dark-inactivation experiments were done in the absence of light in the same temperature-controlled cuvette as photoinhibition experiments. Subsequently, the rate constant of dark inactivation was taken into account by subtracting it from the raw k_{PI}.

3.4. Modeling

Photoinhibitory loss of PSII activity was initially analyzed by fitting to first-order reaction kinetics. In papers I and III, the experimental data were further modeled with ModelMaker software (Cherwell Scientifc, Oxford, UK). ModelMaker is a software in which each reactant

is defined as a compartment. During modeling, the amounts of the reactants vary according to differential equations.

3.5. Measurement of Chl *a* fluorescence

Chl *a* fluorescence was measured with pulse-amplitude-modulated (PAM) fluorometry (Schreiber et al. 1986) and the FIP fluorescence program (Tyystjärvi and Karunen 1990) was used to control the fluorometer. Measurement of Chl *a* fluorescence is a commonly used, non-invasive method in photosynthesis studies. However, when fluorescence is measured from leaves, the fluorescence signal is detected only from the topmost layers of the leaf due to reabsorption. Prior to measurements, unilluminated and illuminated pumpkin leaf discs were dark-incubated for at least 20 min to obtain the initial, dark-adapted level of fluorescence. After dark-incubation, the initial fluorescence level (F_0) was measured under a weak measuring beam, and subsequently the leaf was given a saturating white light pulse (2 s) to induce maximum fluorescence (F_M). For measurements of NPQ, whole Arabidopsis leaves or pumpkin and pepper leaf discs were dark-incubated for 30 min and NPQ was induced by 6 min (Paper II) to 20 min (Papers I, III and IV) illumination with actinic light, applied as continuous light of different wavelengths (Paper II) or as either white continuous light or single-turnover flashes fired at 1 flash/s (Papers I, III and IV). For Paper II, the 6-minute treatment time with actinic light was found to be adequate based on preliminary experiments with different durations of the actinic light illumination. After measurements, the NPQ parameter was calculated from the fluorescence curves as $(F_M–F_M')/F_M'$, as described by Bilger and Björkman (1990). The fluorescence excitation spectrum in Paper II was measured from the leaves of wild-type and *npq1-2* and *npq4-1* mutants of Arabidopsis at 77K. These spectra correspond to the action spectrum of PSII electron transfer reactions.

3.6. Determination of D1 protein

D1 protein degradation during photoinhibition under visible and UV light was determined immunologically (Paper III). Thylakoids (200 µg Chl/ml) were solubilized for 5 min at 65 ºC, and the proteins were separated by 13 % SDS-polyacrylamide gel electrophoresis, as described by Pätsikkä et al. (1998). A Dual colour standard (Bio-Rad) was used to indicate molecular weight of the separated proteins. After electrophoresis, the separated proteins were electroblotted onto Immobilon P membrane (Millipore, Watford, Herts., UK). D1 protein was detected with chemiluminescence kit (CDP-StarTM, Sigma) using a commercial antibody (AS06 124A, Agrisera). Quantification of the D1 protein was carried out by densitometry analysis of the films with FluorChemTM 8000 image analyser (Alpha Innotech Corporation, San Leandro, CA, USA).

4. RESULTS

4.1. Modeling of the light response curve of photoinhibition *in vivo*

4.1.1. Acceptor-side photoinhibition

In Paper I, the light response curves of acceptor and donor-side photoinhibition were calculated and the experimental light response curve of photoinhibition was determined by measuring fluorescence from Arabidopsis leaves. Acceptor-side photoinhibition was modeled as the double reduction of the Q_A electron acceptor (Vass et al. 1992) and according to the model, the double reduction of Q_A would follow charge separation in a closed reaction centre basically in the same way as reduction of Q_A follows charge separation in an open centre. Thus, both the quantum yield of open PSII reaction centres and the yield of double reduction of Q_A depend on NPQ in a similar manner. With these assumptions, the yield of double reduction of Q_A was calculated as the product of the concentration of Q_A and an NPQ-dependent intrinsic factor, and the rate constant of acceptor-side photoinhibition was determined as the quantum yield of double reduction of Q_A multiplied by the quantum flux absorbed by PSII. The results of the modeling show that the quantum yield of acceptor-side photoinhibition is small under low light and that the yield of double reduction of Q_A increases to a constant level at saturation of photosynthesis (Paper I, Fig. 1A). However, under high-intensity continuous light, the theoretical light response curve of acceptor-side photoinhibition does not essentially deviate from the direct proportionality seen *in vivo* (Paper I, Fig. 1).

4.1.2. Statistical donor-side photoinhibition

Donor-side photoinhibition has a high quantum yield if the OEC has been chemically destroyed (Callahan et al.1986; Chen et al. 1992), but it has been suggested (Anderson et al. 1998) that random failure of OEC to reduce P_{680}^+ might explain photoinhibition *in vivo* also in normal PSII. This is called statistical donor-side photoinhibition in Paper I. In Paper I, the competition case was modeled in which the damaging oxidation by P_{680}^+ and the normal oxidation of Tyr_Z compete for all P_{680}^+. The results of Paper I show that the calculated light response curve of donor-side photoinhibition partially saturates at moderate PPFD's, before the saturation of photosynthesis, leading to maximum quantum yield at the PPFD of about 200 µmol m^{-2} s^{-1} in the Arabidopsis plants used (Paper I, Fig. 1B).

4.1.3. Experimental light response curve of photoinhibition and effect of NPQ

The experimentally determined rate constant of photoinhibition was found to be directly proportional to the PPFD of 200-1600 µmol m^{-2} s^{-1} in Arabidopsis *in vivo* (Paper I, Fig. 1C). Furthermore, the experimentally determined rate constant of photoinhibition, measured from fully grown leaves of wild-type Arabidopsis and *npq4-1* at the PPFD of 1600 µmol m^{-2} s^{-1}, was compared to the calculated rate constant of acceptor-side photoinhibition in leaves of *npq4-1*. The results of the calculation showed that approximately one third of photoinhibition, induced by visible light, is independent of non-photochemical quenching of excitation energy of PSII antenna (Paper I, Fig. 1C). In the framework of the acceptor-side model, the difference in k_{PI} between the mutant and the wild type was considerably less than would have been expected, if photoinhibition was caused by the acceptor-side

mechanism (Paper I, Fig. 1C). This result suggests that the acceptor-side mechanism is not alone responsible for photoinhibition even under high light, although a significant contribution of the acceptor-side mechanism cannot be excluded by these data.

4.1.4. Photoinhibition and production of singlet oxygen

Singlet oxygen has been shown to slow down recovery from photoinhibition of PSII (Nishiyama et al. 2004). Furthermore, according to some photoinhibition hypotheses, singlet oxygen may even be the primary damaging agent in photoinhibition (Jung and Kim 1990; Keren et al. 1997; Santabarbara et al. 2001a; Vass and Aro 2008). In the original acceptor-side photoinhibition hypothesis, singlet oxygen is a harmful side product, whereas in donor-side-related photoinhibition hypotheses, singlet oxygen does not have a direct role. There is evidence showing that photoinhibition leads to the production of singlet oxygen (Hideg et al. 1998), but it is not clear whether all singlet oxygen is produced by the reaction centre or whether some other sources also contribute. In Paper I, this question was approached by comparing the kinetics of photoinhibition, singlet oxygen production and D1 protein degradation with the help of a model, which does not assume a particular mechanism for photoinhibition. The kinetic data from Hideg et al. (1998) was analyzed with a simple two-step model, according to which D1 protein is degraded in a first-order reaction after photoinhibition of PSII activity (Tyystjärvi et al. 1994). The model fits nicely the photoinhibition and D1 protein data (Paper I, Fig. 2), and allows the calculation of the relative amount of inactive but D1-containing PSII reaction centres. During illumination of plant leaves in the presence of lincomycin, inactive but D1-containing reaction centres tend to accumulate to some extent in the beginning of a typical photoinhibition experiment because loss of D1 protein lags behind the photoinhibitory loss of PSII activity (Aro et al. 1993a, 1993b). The results of Paper I also suggest that singlet oxygen production by the acceptor-side mechanism would be expected to peak at a relatively early phase of photoinhibition in the presence of lincomycin, and then decline due to further degradation of the D1 protein. However, the pattern of singlet oxygen production observed by Hideg et al. (1998) shows a steady increase during photoinhibition (Paper I, Fig. 2).

4.2. Action spectrum of photoinhibition

4.2.1. Photoinhibition *in vivo* under different wavelengths of light

Paper II was the first study to report an *in vivo* measurement of the action spectrum of photoinhibition from a higher plant. The *in vivo* action spectrum was measured from Arabidopsis by illuminating lincomycin-treated leaves with the PPFD of 1600 μmol m^{-2} s^{-1} of different wavelengths with the help of high quality narrow-band (~10 nm half-band width) filters. In addition, a UV lamp that emitted monochromatic 360 nm and 254 nm light was used. The *in vivo* spectrum shows a steady decrease of photoinhibitory efficiency from UV-C light to blue-green region. In addition, the visible light region is relatively flat in comparison with the UV region (Paper II, Fig 1). During measurements, the quantum yield of photoinhibition was independent of the PFD under both UV and visible light (Paper II, Fig. 2), confirming the validity of the action spectrum measurements.

4.2.2. Photoreceptor of photoinhibition

The existence of a peak in the red light region of the action spectrum of photoinhibition is the main argument favoring the importance of Chl as a photoreceptor of photoinhibition (Jones and Kok 1966; Jung and Kim 1990; Santabarbara et al. 2001a). In Paper II, the measured action spectrum of photoinhibition *in vivo* was compared with the action spectrum of PSII fluorescence *in vivo* and with the absorption spectra of manganese model compounds of the OEC (Bodini et al. 1979; Baffert et al. 2002) (Paper II, Fig. 5). Comparison of the action spectrum of PSII fluorescence with the action spectrum of photoinhibition *in vivo* showed, in accordance with earlier results, that the red peak in the action spectrum of photoinhibition can be superficially fitted with the red peak of the absorbance spectrum of the Chl antenna (Paper II, Fig. 5). However, from red to blue light, the action spectrum of photoinhibition gradually deviated upwards from the excitation spectrum of PSII fluorescence, and finally, below 470 nm, the two spectra could not be fitted together. In turn, comparison of the absorption spectra of manganese model compounds with the action spectrum of photoinhibition *in vivo* showed general similarity with a long, flat visible-light tail and an increase from blue towards UV light (Paper II, Fig. 5).

4.2.3. Protective effect of NPQ against photoinhibition

In Paper II, the action spectrum of photoinhibition and NPQ were measured from wild-type of Arabidopsis and from two NPQ-deficient mutants, *npq1-2* and *npq4-1*. Under different wavelengths, the action spectra of the two mutants were very similar in shape with that of the wild type (Paper II, Fig 1). However, when compared to the wild type, *npq1-2* was found to be 15 % and *npq4-1* 25 % more sensitive to visible-light-induced photoinhibition, apparently due to the low NPQ capacities of the mutants. The differences in k_{PI} values between the wild type, *npq1-2* and *npq4-1* were relatively small, showing that NPQ may vary significantly without having a great impact on photoinhibition. Because the mutant plants retain some capacity for NPQ, the data in Fig. 3A (Paper II) were extrapolated to zero NPQ in order to obtain an estimate of the full protective capacity of NPQ. The k_{PI} values calculated for such a theoretical NPQ-0 plant were slightly higher than the actual k_{PI} values of the *npq4-1* mutant. In comparison with the theoretical NPQ-0 plant, the maximum protective efficiency of the NPQ mechanism against visible-light-induced photoinhibition seems to be relatively small, only about 10-30 %, depending on wavelength (Paper II, Fig. 3).

4.3. Reaction kinetics of photoinhibition

4.3.1. Photoinhibition *in vivo* follows first-order kinetics even under prolonged illumination

In Paper III, the effect of photoinhibited PSII centres on the rate of photoinhibition was studied by measuring reaction kinetics of photoinhibition. Earlier fluorescence measurements have suggested that during prolonged illumination of lincomycin-treated *Capsicum annuum* leaves the accumulation of inactive PSII centres leads to stabilization of the oxygen evolving activity of PSII to 15–20 % of control level (Lee et al. 2001). This stabilization effect was explained by photoinhibitory quenching by photoinhibited PSII. In Paper III, the kinetic analysis of both oxygen evolution (Paper III, Figs. 1 and S1) and

fluorescence measurements (Paper III, Figs. 2 and 3) showed that photoinhibition is a first-order reaction *in vivo*, practically to the end of the reaction. Furthermore, the results indicated that the first-order kinetics of photoinhibition is not specifically related to the plant species or the growth light conditions used in the study (Paper III, Figs. 2 and 3). To explore the possibility that the protective effect of photoinhibited reaction centres was masked *in vivo*, a set of *in vitro* experiments were performed with different preparations of isolated PSII membranes under different wavelengths and intensities of light (Paper III, Figs. 5, 6, 7 and 8). In these experiments, isolated thylakoids, randomized thylakoids and PSII core complexes were used, as the connectivity between the PSII centres varies between these preparations and lack of connectivity is expected to abolish the possible photoprotective effect of already photoinhibited reaction centres. The *in vitro* data from different PSII preparations confirmed the first-order nature of photoinhibition (Paper III, Figs. 5, 6, 7 and 8).

4.4. Photoinhibition *in vivo* with single-turnover flashes and with continuous light

4.4.1. Flash-induced photoinhibition depends on flash energy but not on time interval between the flashes *in vivo*

In Paper IV, lincomycin-treated pumpkin leaves were illuminated with different PPFD's of either 3-µs flashes of light or continuous light and the photoinhibitory efficiency of both types of illumination was compared to study the dependence of photoinhibition on the mode of delivery of light. The time constant of Q_A^- reoxidation by electron transfer to Q_B is in the range of 20-500 µs (Tyystjärvi and Vass 2004), and therefore the 3-µs flashes qualify as single-turnover flashes, as the vast majority of PSII reaction centres closed by a flash remain closed until the flash has extinguished. Furthermore, both the low efficiency of the flashes in induction of NPQ and the finding that the level of q_P remains very high during flash illumination support the single-turnover nature of the flashes (Paper IV, Fig. 2 and Table S1). Energies of the flashes used in Paper IV were chosen so that the flashes were saturating for oxygen evolution in pumpkin leaves (Paper IV, Fig. 1). It has been shown *in vitro* that the photoinhibitory efficiency of saturating µs-length flashes (Hakala et al. 2005) and saturating ns-length laser pulses (Tyystjärvi et al. 2008) depends on the energy of the flash or pulse. The results of Paper IV show direct proportionality between the photoinhibitory efficiency of the flashes and the flash energy *in vivo* (Paper IV, Fig. 3). The correlation between k_{PI} calculated per flash and flash energy was statistically highly significant (Paper IV, Table 2). Furthermore, no dependence of the photoinhibitory efficiency of short flashes on the time interval (1 s, 9 s, 90 s) between the flashes was observed (Paper IV, Fig. 4) as the results failed to show statistically significant correlations. Only one set of oxygen evolution experiments (13.5 J) showed correlation between k_{PI} and flash interval that was significant at the 5 % level (Paper IV, Table 2).

4.4.2. Photon flux density dependence of flash-induced and continuous-light-induced photoinhibition

In Paper IV, the dependence of the rate constant of photoinhibition on the photoinhibitory PPFD was studied with both common methods of measuring photoinhibition, oxygen evolution and fluorescence (Paper IV, Fig. 5). The photoinhibitory efficiency of short pulses

can be compared with continuous light by measuring the photon content of the flashes and calculating the average PPFD during the flash treatment (Hakala et al. 2005). In Paper IV, the PPFD range was 0.025-1350 μmol m^{-2} s^{-1} for the flash treatments and 0.025-2300 μmol m^{-2} s^{-1} for continuous light. Comparison of k_{PI} values obtained from fluorescence measurements *in vivo* shows that the flashes and continuous light have essentially similar photoinhibitory efficiency in this PPFD range, with possibly an exception at very low PPFD and at the high PPFD of 2300 μmol m^{-2} s^{-1} (Paper IV, Fig. 5A). Furthermore, the same PPFD dependence was seen in oxygen-evolution-based k_{PI} values of continuous-light photoinhibition treatments (Paper IV, Fig. 5B) and in $1/F_0$-$1/F_M$-based k_{PI} values of continuous-light photoinhibition treatments (Paper IV, Fig. 5C). In comparison with these data, the photoinhibitory efficiency of single-turnover flashes was much stronger on oxygen evolution than on F_V/F_M (Paper IV, Fig. 5B), with the $1/F_0$-$1/F_M$ data being intermediate (Paper IV, Fig. 5C). The finding that the flashes have similar photoinhibitory efficiency as continuous light in fluorescence data but much stronger efficiency in oxygen evolution data (Paper IV, Fig. 5) suggests that the flashes might have a particularly strong effect on deep cell layers, partially because continuous light induces more chloroplast movements (Paper IV, Fig. 6).

5. DISCUSSION

5.1. Light response of photoinhibition *in vivo*

In Papers I and IV, direct proportionality was demonstrated between the rate constant of photoinhibition *in vivo* and PPFD, in accordance with earlier data (Jones and Kok 1966; Park et al. 1995; Tyystjärvi and Aro 1996; Allakhverdiev 2004; Hakala et al. 2005). These results have implications with regard to the mechanism of photoinhibition, especially as the photoinhibition hypotheses modeled in Paper I did not imply direct proportionality between k_{PI} and PPFD under continuous light (Paper I, Fig. 1). Modeling of the light response curve of acceptor-side photoinhibition as double reduction of Q_A (Vass et al. 1992) suggests that the classic acceptor-side photoinhibition can only partially account for photoinhibition under non-saturating light intensities (Paper I, Fig. 1). The model in Paper I did not consider acceptor-side photoinhibition involving stabilization of reduced Q_A or damage caused by singlet oxygen produced in the presence of Q_A^- (Vass et al. 1992; Vass and Aro 2008; Vass and Cser 2009). However, in all these hypotheses, inactivation of PSII occurs only when Q_A is reduced.

The calculated light response curve of donor-side photoinhibition deviated clearly from direct proportionality between k_{PI} and PPFD (Paper I, Fig. 1B). In addition, the donor-side photoinhibition model was found to become partially light-saturated at moderate PPFD's, before the saturation of photosynthesis, leading to maximum quantum yield of donor-side photoinhibition at the PPFD of about 200 μmol m^{-2} s^{-1} (Paper I, Fig. 1B). The result is not in agreement with experimental data, suggesting that the contribution of the statistical donor-side mechanism in photoinhibition of intact leaves must be small. As far as the combined effect of the acceptor and donor-side mechanisms is concerned, the fit of the combination model to experimental results would depend on the relative proportions of both mechanisms, although it would be very likely that a deviation from the direct proportionality between k_{PI} and PPFD would be observed. The results of Paper I suggest that acceptor-side and donor-side photoinhibition hypotheses cannot explain photoinhibition under all light intensities. However, contribution from the acceptor-side mechanism to photoinhibition under strong light conditions cannot be excluded by these data (Paper I, Fig. 1A).

The *in vivo* results of Paper IV support the conclusions obtained by mathematical modeling in Paper I. In Paper IV, pumpkin leaves were treated with continuous light or with saturating single-turnover flashes and both types of illumination were found to cause photoinhibition of F_V/F_M with essentially similar quantum efficiency (Paper IV, Fig. 5). These results suggest that photoinhibition caused by flashes may be mechanistically similar to photoinhibition caused by continuous light, although the possibility remains that photoinhibition occurs with two different mechanisms that have the same quantum yield by coincidence. Photoinhibition induced with 3-μs single-turnover flashes did not show statistically significant dependency on the time interval between the flashes (Paper IV, Fig. 4 and Table 2). Furthermore, direct proportionality, supported by a statistically highly significant correlation, was found between the photoinhibitory efficiency of the flashes and the flash energy (Paper IV, Fig. 3 and Table 2). Lack of dependence on flash interval and dependence on flash energy *in vivo* are in agreement with earlier *in vitro* data (Hakala et al.

2005). The finding that the photoinhibitory efficiency of ns-timescale laser pulses increases with pulse interval has earlier been taken as evidence for involvement of the slow charge recombination reactions of PSII (e.g. $S_2Q_B^-$ to S_1Q_B) in the mechanism of photoinhibition (Keren et al. 1997, 2000). However, if this was the case, a similar interval-effect would be seen also with µs-length flashes. The lack of an interval effect with single-turnover flashes *in vivo* (Paper IV, Fig. 4) indicates that the low-light photoinhibition hypothesis (Keren et al., 1997; 2000) is not a comprehensive explanation for flash-induced photoinhibition in leaves of higher plants.

5.2. Singlet oxygen produced by photoinhibited plants does not originate only in recombination reactions

According to the acceptor-side photoinhibition hypothesis, recombination reactions associated with photoinhibition take place in photoinhibited PSII reaction centres that still contain the D1 protein. The kinetic analysis of Paper I revealed that the pattern of singlet oxygen production observed by Hideg et al. (1998) does not fit the accumulation pattern of photoinhibited, D1-containing PSII centres (Paper I, Fig. 2). The discrepancy between the kinetics of singlet oxygen production and the accumulation of photoinhibited but D1-containing PSII centres indicates that singlet oxygen formed during photoinhibition is not produced only by recombination reactions in already acceptor-side photoinhibited reaction centres. In addition to recombination reactions, production of singlet oxygen during photoinhibition can also take place via triplet Chl formed by intersystem crossing in the light harvesting antenna.

5.3. Action spectrum of photoinhibition suggests that both Mn and Chl function as photoreceptor(s) of photoinhibition

The action spectrum of photoinhibition *in vivo* from a higher plant (Paper II, Fig. 1) shows general similarities with the earlier *in vitro* spectra (Jones and Kok 1966; Renger et al. 1989; Jung and Kim 1990; Santabarbara et al. 2001a; Hakala et al. 2005; Ohnishi et al. 2005), suggesting that the same photoinhibition reactions occur in both conditions. Accordingly, in *Synechocystis* sp. PCC 6803 cells the photoinhibitory efficiency of 400-450 nm light is over twice as high as that of 600-650 nm light and intermediate wavelengths (450-600 nm) are less efficient (Tyystjärvi et al. 2002). Furthermore, transcripts of the *psbA* gene that codes for the D1 protein in *Synechocystis* sp. PCC 6803 decrease after transfer of the cells from darkness to orange light and increase when the cells are transferred from darkness to blue-green light, suggesting that also the redox state of the photosynthetic electron transfer chain regulates *psbA* transcription (El Bissati and Kirilovsky 2001). In comparison with the *in vitro* action spectra, the *in vivo* spectrum is more flat in the visible light region and shows higher sensitivity to UV light. This difference between *in vivo* and *in vitro* action spectra may partially be caused by the fact that leaves are optically thicker than thylakoid suspensions used *in vitro*. Furthermore, leaves show a variety of responses to light quality and intensity that are absent in isolated systems (for review, see Demmig-Adams and Adams 1992; Niyogi et al. 1998).

Comparison of the action spectrum of photoinhibition *in vivo* with the absorption spectra of both the PSII antenna and the model compounds of the oxygen-evolving Mn cluster (Paper II, Fig. 5) suggests that photoinhibition, especially under blue or green light, cannot be explained without assuming a strong contribution from a photoreceptor other than Chl. Although no precise match can be demonstrated between the *in vivo* action spectrum of photoinhibition and the absorption spectra of the model compounds of the manganese cluster, the role of Mn as a photoreceptor of photoinhibition is supported by the spectral similarity (Hakala et al. 2005; Paper II) and other evidence under both visible and UV light, such as (i) correlation between photoinhibition and loss of Mn ions form the OEC (Virgin et al. 1988, Renger et al. 1989, Hakala et al. 2005, Zsiros et al. 2006, Ohnishi et al. 2007), (ii) photosensitivity of both manganese enzymes and model compounds of the Mn cluster (Hakala et al. 2006, Antal et al. 2009a) and (iii) the finding that inhibition of OEC activity precedes the inhibition of the rest of the reaction centre (Hakala et al. 2005; Ohnishi et al. 2005).

5.4. Lack of kinetically resolvable reversibility further defines the mechanism of photoinhibition

Reversibility in photoinhibition could be caused by rebinding of manganese ions to the OEC, as suggested by the original version of the manganese hypothesis (Hakala et al. 2005). A reversibly inhibited state might also be formed in PSII in which light-induced damage has occurred in the manganese complex but release of Mn ions from OEC has not yet occurred (Mohanty et al. 2007). A similar possibility is associated with the acceptor-side hypothesis of photoinhibition (Vass et al. 1992), as reversal of double reduction or stabilization of the reduced Q_A electron acceptor would result in restoration of PSII activity without protein synthesis. Reversibility of inactivated states of PSII without D1 protein turnover has been earlier suggested to explain results from low-temperature photoinhibition of *Chlamydomonas reinhardtii* and *Synechocystis sp.* PCC 6803 (Kirilovsky et al. 1990, Allakhverdiev et al. 2003), as well as results from anaerobic photoinhibition in higher plant thylakoids (Hundal et al. 1990, Kirilovsky and Etienne 1991, Vass et al. 1992, Vass and Styring 1993, Kirilovsky et al. 1994). These results may suggest function of specific inactivation mechanisms of PSII in chilling sensitive organisms at low temperature. Anaerobic reversibility can be explained by acceptor-side photoinhibition mechanism, as double reduction of Q_A has been shown to occur under anaerobiosis (Vass et al. 1992, Vass and Styring 1993). No recovery from photoinhibition without protein synthesis has been seen in pumpkin leaves in normal air at room temperature (Vavilin et al. 1995).

If photoinhibition proceeded via a reversible intermediate, deviation from first-order kinetics would become obvious during prolonged photoinhibition. In Paper III, kinetics of prolonged photoinhibition was studied and accordance to first-order kinetics was found both *in vivo* and *in vitro*, indicating lack of reversibility. In the framework of the manganese mechanism, the first-order nature of photoinhibition under prolonged illumination indicates that the light-induced loss of the activity of the manganese cluster is irreversible. Due to the lack of reversibility, the protective effect of NPQ (Papers I and II) cannot be targeted to Chl-dependent step of a Mn-based mechanism, but NPQ must protect against an independent, parallel Chl-dependent photoinhibition mechanism which remains to be identified. Thus, manganese and chlorophyll do not function in series as photoreceptors of photoinhibition as

previously suggested (Hakala et al. 2005, Ohnishi et al. 2005), but different photoinhibition mechanisms function simultaneously (Fig. 3). Similar conclusions have recently been drawn by Oguchi et al. (2009). The kinetics data of Paper III agree with the donor-side and singlet oxygen photoinhibition hypotheses. With regard to the acceptor-side hypothesis, reversibility of the double reduction or stabilization of the Q_A electron acceptor is not supported by the results of Paper III. However, photoinhibition of PSII due to production of singlet oxygen in the presence of unstabilized Q_A^- in the same reaction centre (Vass and Aro 2008) would probably follow first-order reaction kinetics.

In Paper III, the kinetics data representing both oxygen evolution (Paper III, Figs. 1 and S1) and Chl fluorescence measurements (Paper III, Figs. 2 and 3) are not in agreement with the results of Lee et al. (2001). The reason for the contradiction is not clear. Failure in blocking chloroplast protein synthesis in the experiments of Lee et al. (2001) might be an obvious explanation as it causes stabilization of PSII activity to an above-zero level (Greer et al. 1986; Samuelsson et al. 1987; Melis 1999), however, a similar concentration of lincomycin was used in both studies. The fluorescence parameter $1/F_0$-$1/F_M$, which was also used by Lee et al. (2001), has been found to correlate linearly with oxygen yield per saturating single-turnover flash, but the data is rather scattered and the linear correlation has been indicated only in the range of 20-100 % of control (Park et al. 1995; Lee et al. 1999). It is also possible that the induction of NPQ has a role in causing stabilization of $1/F_0$-$1/F_M$ during long photoinhibition treatments, as it may lower the F_0 value. However, in Paper III, both F_V/F_M and $1/F_0$-$1/F_M$ data showed compliance with first-order kinetics, and the F_0 value remained constant during the photoinhibition treatments.

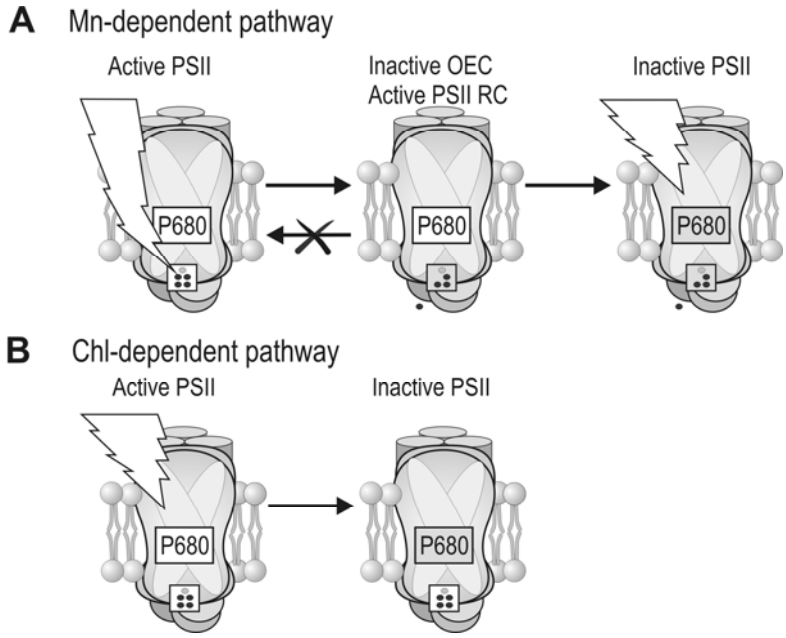

Fig. 3. Simplified scheme of the mechanism of photoinhibition showing Chl-independent and Chl-dependent pathways functioning parallel to each other. **(A)** In Mn-dependent pathway, absorption of light by Mn ions of the OEC (white square showing four Mn ions and one Ca ion)

triggers the disintegration of the Mn cluster. As the OEC is inactivated (grey square), excitation of the PSII reaction centre (white rectangle) leads to inactivation of the PSII reaction centre (grey rectangle) via P_{680}^{+} mediated damage. Light-induced loss of the activity of the OEC is irreversible (crossed arrow) and later reaction steps have no additional effect on the loss of oxygen evolution activity. **(B)** In the Chl-dependent pathway, light is absorbed by active PSII (white rectangle) causing inactivation of PSII (grey rectangle) possibly due to production of singlet oxygen. Mechanisms such as non-photochemical quenching of Chl *a* fluorescence may offer protection to PSII against Chl-dependent damage.

5.5. The protective efficiency of NPQ against photoinhibition is small

5.5.1. Protective role of q_I

In Paper III, the effect of photoinhibited PSII centres on the rate of photoinhibition was studied by kinetic analysis of oxygen evolution and fluorescence data. The first-order kinetics of photoinhibition *in vivo* and *in vitro* supports the conclusion that photoinhibited PSII centres do not significantly protect the remaining active ones, contrary to what was previously suggested by Lee et al. (2001). The finding that the q_I-component of NPQ does not offer kinetically resolvable protection against photoinhibition of the remaining active PSII centres is supported by data showing that under strong light over 90 % of PSII excitations are quenched by reversible NPQ, together with non-regulatory quenching (Kramer et al. 2004). Thus, even under optimal conditions, photoinhibited PSII centres would only marginally increase the efficiency of thermal dissipation of excitation energy.

5.5.2. Protective role of q_E

In Papers I and II, the protective role of NPQ against photoinhibition was studied in wild-type Arabidopsis and *npq4-1* and *npq1-2* mutants defective in the q_E component of NPQ. In Paper I, a calculation based on comparison of photoinhibition in the wild type and *npq4-1* mutant of Arabidopsis indicated that the q_E-component of NPQ lowers the rate constant of photoinhibition by approximately one fourth *in vivo* (Paper I, Fig. 1C). In Paper II, similar results were obtained as the maximum protective efficiency of NPQ against photoinhibition was estimated to be 10-30 % (Paper II, Fig. 3). The small protective effect of NPQ against photoinhibition is in agreement with earlier *in vitro* results showing no protection against photoinhibition with chemicals that quench over two thirds of the excitations of the antenna of PSII (Santabarbara et al. 1999; Tyystjärvi et al. 1999), as well as with qualitative results *in vivo* (Grasses et al. 2002). Recently, the *npq4*-mutant of Arabidopsis was shown to reach wild-type NPQ-levels via a mechanism essentially similar to q_E, but with a time constant of one hour (Johnson and Ruban 2010). In Paper II, the *npq4-1* mutant was found to retain some capacity for NPQ (Paper II, Fig. 3) but the slowly inducible NPQ in the *npq4-1* is not expected to influence significantly the 20-min photoinhibition treatments of Paper II.

The protective efficiency of NPQ tells about the importance of Chl as a photoreceptor of photoinhibition as NPQ can only protect against photoinhibition via the light harvesting antenna of PSII. The results of Papers I and II indicate that NPQ protects against visible-light-induced photoinhibition less efficiently than would be expected if photoinhibition was fully dependent on photons absorbed by PSII antenna. The small protective role of NPQ and the presence of a small peak in the red light region of the action spectrum of

photoinhibition (Jones and Kok 1966; Jung and Kim 1990; Santabarbara et al. 2001a; Paper II) suggest that both Chl-dependent and Chl-independent mechanisms have a role in photoinhibition (Fig. 3).

5.5.3. Photoinhibition and NPQ outdoors

NPQ can only protect against Chl-dependent photoinhibition and NPQ does not protect against UV-light-induced photoinhibition. Comparison of the action spectrum of photoinhibition in Arabidopsis with the emission spectrum of sunlight indicated that the UV part of sunlight is responsible for the major part of photoinhibition under natural conditions (Paper II, Fig. 6). However, the photoinhibitory efficiency of UV light depends greatly on the growth conditions and the age of the plant material. Whereas in phytotron-grown, young leaves of *Arabidopsis* over 80 % of photoinhibition is caused by UV-part of sunlight (Paper II, Fig. 6), in phytotron-grown mature leaves of pumpkin, the proportion of UV-light-induced photoinhibition is only 35 % in sunlight (unpublished results: Marja Hakala-Yatkin, Mika Mäntysaari and Esa Tyystjärvi). Thus, the very high photoinhibitory efficiency of UV light in the action spectrum of photoinhibition *in vivo* (Paper II, Fig. 1) may be connected to the relatively young age and growth conditions of the *Arabidopsis* leaves. Furthermore, UV-absorbing compounds, especially flavonoids and hydroxycinnamic acids, are synthesized by plants grown under natural sunlight (Cockell and Knowland 1999; Day and Neale 2002). These compounds are expected to lower the photoinhibitory efficiency of the UV-part of sunlight in comparison to plants grown under artificial illumination.

The differences in k_{PI} values between the wild type and the *npq1-2* and *npq4-1* mutants were relatively small with regard to differences in the levels of NPQ in mature leaves of Arabidopsis (Paper II, Fig. 3A). These results indicate that NPQ may vary significantly without having a great impact on photoinhibition *in vivo*. The small magnitude of the protective effect of NPQ also explains why NPQ-deficient plants do not show symptoms of chronic photoinhibition when grown under high light in the laboratory (Niyoqi et al. 1998; Havaux and Niyogi 1999; Li et al. 2000; Niyogi et al. 2001). Even though these results indicate that protection by NPQ against photoinhibition is a relatively moderate effect, NPQ-deficient plants consume more resources to repair PSII after photoinhibition and NPQ also protects against other damaging reactions, e.g. against oxidative damage (Demmig-Adams and Adams 1992; Horton et al. 1996; Havaux and Niyogi 1999; Niyogi 1999; Müller et al. 2001). These additional effects may in part explain why the fitness of NPQ-deficient mutant plants is lower than the fitness of wild-type plants of Arabidopsis in field conditions (Külheim et al. 2002).

6. CONCLUDING REMARKS

My thesis work on photoinhibition of PSII *in vivo* suggests that both Chl-dependent and Chl-independent pathways are required to explain the features of photoinhibition *in vivo.* Furthermore, these pathways function parallel to each other under both continuous-light-induced and flash-induced photoinhibition. The Chl-independent pathway apparently begins with light-induced inactivation of the Mn cluster of the OEC and the secondary Chl-mediated step in the pathway has no additional effect on the loss of oxygen evolution activity. On the other hand, mechanisms such as NPQ may offer protection by slowing down the damage to PSII via the Chl-dependent pathway.

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