Journal of Experimental Botany, Vol. 61, No. 15, pp. 4239–4247, 2010
doi:10.1093/jxb/erq224 Advance Access publication 19 July, 2010
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Short flashes and continuous light have similar photoinhibitory efficiency in intact leaves

Päivi Sarvikas, Marja Hakala-Yatkin, Sirin Dönmez* and Esa Tyystjärvi†

Molecular Plant Biology, Department of Biochemistry and Food Chemistry, University of Turku, FI-20014 Turku, Finland

* Permanent address: Plant Material and Cultivation, Department of Landscape Architecture, University of Bartin, 74100 Bartin, Turkey.
† To whom correspondence should be addressed. E-mail: esatyy@utu.fi

Received 19 April 2010; Revised 3 June 2010; Accepted 1 July 2010

Abstract

Lincomycin-treated pumpkin leaves were illuminated with either continuous light or saturating single-turnover xenon flashes to study the dependence of photoinactivation of photosystem II (PSII) on the mode of delivery of light. The flash energy and the time interval between the flashes were varied between the experiments, and photoinactivation was measured with oxygen evolution and the ratio of variable to maximum fluorescence (F_v/F_m). The photoinhibitory efficiency of saturating xenon flashes was found to be directly proportional to flash energy and independent of the time interval between the flashes. These findings indicate that a low-light-specific mechanism, based on charge recombination between PSII electron acceptors and the oxygen-evolving complex, is not the main cause of photoinactivation caused by short flashes *in vivo*. Furthermore, the relationship between the rate constant of photoinactivation and photon flux density was similar for flashes and continuous light when F_v/F_m was used to quantify photoinactivation, suggesting that continuous-light photoinactivation has a mechanism in which the quantum yield does not depend on the mode of delivery of light. A similar quantum yield of photoinhibition for flashes and continuous light is compatible with the manganese-based photoinhibition mechanism and with mechanisms in which singlet oxygen, produced via a direct photosensitization reaction, is the agent of damage. However, the classical acceptor-side and donor-side mechanisms do not predict a similar quantum yield for flashes and continuous light.

Key words: *Cucurbita maxima*, light response curve, photoinhibition, photosystem II, single-turnover flashes, xenon flashes.

Introduction

Light is needed for photosynthesis, but light also damages the photosynthetic machinery. Light-induced inhibition of the activity of photosystem II (PSII) has been termed photoinhibition (for reviews, see Aro *et al.*, 1993; Melis, 1999; Tyystjärvi, 2008; Vass and Aro, 2008). A characteristic feature of photoinhibition is that degradation and *de novo* synthesis of the D1 protein are needed for restoration of the activity of PSII. All light intensities cause inactivation of PSII with a rate constant directly proportional to the light intensity (Tyystjärvi and Aro, 1996), and therefore plants need to repair the inactive PSII units continuously. The rate of inactivation of PSII can be measured by illuminating leaves in the presence of an inhibitor such as lincomycin that prevents the concurrent synthesis of the D1 protein.

Photoinhibition is usually caused by continuous light, but strong, short single-turnover flashes also inactivate PSII (Keren *et al.*, 1995, 1997). Single-turnover flashes do not occur in nature, but short flashes are valuable tools in studies of the mechanism of photoinactivation of PSII (see, for example, Keren *et al.*, 1995, 1997, 2000; Szilard *et al.*, 2005; Tyystjärvi *et al.*, 2008).

Photoinactivation of PSII has been explained by several hypothetical mechanisms. In the acceptor-side hypothesis (Vass *et al.*, 1992), PSII is inactivated when the primary

Abbreviations: Chl, chlorophyll; cytD, cytochalasin D; F_m, maximum fluorescence of a dark-adapted leaf; F_0, minimum fluorescence of a dark-adapted leaf; fps, flashes per second; F_v, variable fluorescence (F_m–F_0); k_{PI}, reaction rate constant of photoinhibition; NPQ, non-photochemical quenching of chlorophyll excitation energy; P_{680}, P_{680}^+, $^3P_{680}^*$, the ground state, oxidized state and excited triplet state of the primary donor of PSII; PPFD, photosynthetic photon flux density; PSII, photosystem II; Q_A and Q_B, the primary and secondary quinone electron acceptor of PSII, respectively.

quinone electron acceptor of PSII, already in state Q_A^-, becomes double reduced to Q_A^{2-} or stabilized by protonation. Simulations imply that double reduction would not occur under low light (Tyystjärvi *et al.*, 2005) or during illumination with single-turnover flashes. However, if Q_A remains reduced from flash to flash, then double reduction might occur even during illumination with single-turnover flashes. It has also been suggested that photoinactivation is caused by chlorophyll (Chl) triplets produced in the presence of unstabilized Q_A^- (Vass and Aro, 2008).

In the 'statistical' donor-side hypothesis (Anderson *et al.*, 1998), PSII is inactivated when the oxygen-evolving complex is occasionally unable to donate an electron, that is a miss occurs in the oxygen-evolving complex (Kok, 1970; Antal *et al.*, 2009). After a miss, the oxidized primary donor P_{680}^+ lives for 100–200 μs and then recombines with Q_A^- (Renger and Wolff, 1976; Renger and Holzwarth, 2005) or damages PSII by oxidizing an inappropriate target. Saturating single-turnover flashes would saturate the formation of long-lived P_{680}^+, and therefore donor-side photoinhibition would be saturated at the same flash energy that saturates PSII electron transfer.

Generation of singlet oxygen by uncoupled Chls (Santabarbara *et al.*, 2001) or by iron–sulphur centres and cytochromes (Jung and Kim, 1990) only requires the formation of the triplet state of the photoreceptor and would therefore have the same quantum yield in continuous light and short flashes. Also manganese-dependent inactivation of PSII (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005) would have the same quantum yield in continuous and flash light because manganese ions have low absorbance in the visible range, and therefore xenon flashes would not saturate absorption.

The low-light photoinhibition hypothesis (Keren *et al.*, 1995, 1997, 2000; Szilard *et al.*, 2005) applies to inactivation of PSII with short pulses of light or with low light. According to this hypothesis, generation of the triplet excited state of the primary donor ($^3P_{680}$*) by recombination reactions (e.g. $S_2Q_B^- \rightarrow S_1Q_B$) triggers the inactivation of PSII. The hypothesis is supported by the finding that nanosecond laser pulses fired with long dark intervals are more photoinhibitory than pulses fired with short intervals (Keren *et al.*, 1997, 2000). This flash interval dependence has been explained by the fact that the flash yield of charge recombination increases with the length of the dark interval between the flashes. The low-light hypothesis is also supported by data showing that in the green alga *Chlamydomonas*, less degradation of the D1 protein per flash is caused by pairs of microsecond xenon flashes than by single flashes (Keren *et al.*, 1995). *In vitro* experiments of inactivation of PSII with xenon flashes have produced contrasting results. The measurements of Szilard *et al.* (2005) suggest that the photoinhibitory efficiency of xenon flashes depends on the length of the interval between flashes, but the data of Hakala *et al.* (2005) did not show any flash interval dependence. The low-light hypothesis predicts that flash-induced inactivation of PSII becomes saturated at the same flash energy that saturates PSII electron transport.

The aim of the present study was to probe the mechanism of photoinactivation of PSII by comparing the effects of short flashes and continuous light in intact leaves. For this, photoinhibition from lincomycin-treated pumpkin (*Cucurbita maxima*) leaves was measured under continuous white light and under illumination with 3 μs single-turnover xenon flashes, varying the energy of the flashes and the time interval between the flashes.

Materials and methods

Plant material and growth conditions

Pumpkin (*C. maxima*) plants were grown in a research greenhouse under the rhythm of 18 h light/6 h dark until leaves were fully expanded. Photosynthetic photon flux density (PPFD) was 150 μmol m^{-2} s^{-1}, relative air humidity was 70%, and temperature was 24 °C.

In vivo *photoinhibition treatments*

Prior to illumination, leaves were incubated overnight with their petioles in 2.3 mM lincomycin. The leaf petioles were also kept in 2.3 mM lincomycin throughout the photoinhibition treatments during which the leaf blades were gently pressed on moist paper. A 400 W Philips HPI-T Plus lamp was used for continuous-light photoinhibition experiments at a PPFD of 0.025, 3, 30, or 50 μmol m^{-2} s^{-1} (duration 45 h) and 300 μmol m^{-2} s^{-1} or 500 μmol m^{-2} s^{-1} (duration 4 h). A 10 cm water filter and a cooling fan were used to remove heat. For photoinhibition experiments at a PPFD of 900, 1500, 1800, or 2300 μmol m^{-2} s^{-1} (duration 6–12 h), a 300 W high-pressure ozone-free xenon lamp (Oriel Instruments, Stratford, CT, USA) equipped with a 9 cm water filter and a Schott GG400 UV-blocking filter was used. Neutral density filters (Lee Filters, Andover, UK) were used to fine-tune the PPFD, which was measured with a LI-189 quantum sensor (LiCor, Lincoln, NE, USA).

Photoinhibition experiments with xenon flashes were done by illuminating lincomycin-treated pumpkin leaves with 3 μs flashes from an FX-200 xenon flash lamp (EG&G, Gaithersburg, MD, USA) through a Schott GG400 filter. The upper surface of the filter was cooled with an air flow. Capacitors were used to adjust the flash energy, and the time interval between the flashes was adjusted with a computer. The durations and flash energies of the flash treatments are listed in Table 1. Each flash treatment was done with 1800 flashes and the duration of the treatment was varied in accordance with the time interval between the flashes, as indicated in Table 1. The average PPFD of each flash treatment was determined as previously described (Hakala *et al.*, 2005).

Prior to photoinhibition treatments, two leaf disks (2.8 cm^2) were always cut off from each leaf and analysed as control samples. In the middle and at the end of each continuous-light illumination treatment, two leaf disks were cut off from the illuminated area and used for measurements of fluorescence and oxygen evolution. One time point was used in the flash photoinhibition treatments. Pearson correlation coefficients and *P*-values reflecting their statistical significance were calculated with SPSS software (SPSS Inc., Chicago, IL, USA).

Flash energy dependence of oxygen evolution in vivo

The dependence of the amount of oxygen evolved per flash on the energy of the xenon flashes was measured from pumpkin leaf disks (9.6 cm^2) with a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) at 23 °C in ~5% carbon dioxide, essentially as in Lee *et al.* (1999). The FX-200 xenon lamp was put in place of the standard lamp of the device, 5.7 cm above the leaf, and the flash energy was adjusted to 0.1–1.35 J. During the measurements,

Table 1. Photoinhibition treatments with xenon flashes

The average PPFD of each flash treatment of lincomycin-treated pumpkin leaves was determined as previously described (Hakala *et al.*, 2005). The flash treatments were done with 1800 flashes by varying the flash energy, the time interval between the flashes, and the duration of the experiment, as indicated.

PPFD (μmol m^{-2} s^{-1})	Flash energy (J)	Flash interval (s)	Duration of the experiment (h)
1.5	1.35	90	45
15	1.35	9	4.5
15	13.5	90	45
40	3.4	9	4.5
130	1.35	1	0.5
150	13.5	9	4.5
340	3.4	1	0.5
530	5.3	1	0.5
930	9.3	1	0.5
1350	13.5	1	0.5

xenon flashes were applied for 4 min, followed by 4 min of darkness. As high a repetition rate of the flashes was used as technically possible in order to obtain a reasonable signal to noise ratio of the oxygen measurements, and therefore flashes of 0.1–0.4 J were applied at 30 flashes per second (fps), flashes of 0.5–0.6 J at 20 fps, and flashes of 0.7–1.35 J at 10 fps. Several cycles of flashing (each with different energy, in random order) and darkness were measured from each leaf disk, and the measurements were repeated with several leaf disks.

Dark inactivation

Experiments were also done to determine the extent of dark inactivation of PSII activity during the maximum time span of the experiments (45 h). These assays were done in the same set-up as was used for photoinhibition treatments, except that the leaves were covered with several layers of aluminium foil.

Fluorescence measurements

Chl *a* fluorescence of leaves was measured with a PAM-101 fluorometer (Heinz Walz GmbH, Effeltrich, Germany) and the FIP fluorescence program (Tyystjärvi and Karunen, 1990) was used to control the fluorometer. For measurements of F_v/F_m, unilluminated and illuminated pumpkin leaf disks were dark-incubated for 20 min. The initial fluorescence level (F_0) was measured under a weak measuring beam, and a saturating white light pulse (2 s; 5800 μmol m^{-2} s^{-1}) was used to induce maximum fluorescence (F_m). For measurements of non-photochemical quenching (NPQ), pumpkin leaf disks were dark-incubated for at least 30 min and NPQ was induced by 20 min actinic illumination, either with continuous light (175, 300, 930, or 1350 μmol m^{-2} s^{-1}) or with xenon flashes of 1.35, 9.3, or 13.5 J fired at 1 fps. The respective PPFD values are 130, 930, and 1350 μmol m^{-2} s^{-1}. NPQ was calculated as $(F_m–F_m')/F_m'$ (Bilger and Björkman, 1990).

The effect of the xenon flashes on the openness of PSII centers was tested with the Walz PAM-2000 fluorometer by flashing pumpkin leaf disks from 1 cm distance through a GG400 filter under the light guide. After 2 min of flashing with a xenon lamp, a saturating pulse of 0.8 s (4500 μmol m^{-2} s^{-1}) was fired to measure F_m', and then flashing was switched off and a far red light-emitting diode (LED) was switched on for 2 s to measure F_0'. These measurements were done using the flash rates of 1, 10, 20, or 30 fps, and the respective flash energies of 13.5, 1.35, 0.6, and 0.1 J.

Determination of Chl concentration and oxygen evolution activity

Thylakoids were isolated from illuminated and unilluminated leaf disks as described by Sarvikas *et al.* (2010). Chl concentration was determined according to Porra *et al.* (1989) and adjusted to 10 μg ml^{-1} with PSII measuring buffer (40 mM HEPES-KOH pH 7.6; 0.33 M sorbitol; 5 mM $MgCl_2$; 5 mM NaCl; 1 M glycine betaine; 1 mM KH_2PO_4; 5 mM NH_2Cl). Oxygen evolution activity was measured with an oxygen electrode (Hansatech, King's Lynn, UK) in saturating red light with 0.125 mM 2,6-dichlorobenzoquinone as electron acceptor. Chl concentrations of pumpkin leaves were determined in *N*,*N*-dimethylformamide (Inskeep and Bloom, 1985) before and after the illumination treatments.

Measurements of transmittance

Pumpkin leaves were first kept at a PPFD of 20 μmol m^{-2} s^{-1} for 30 min. Leaf petioles were then immersed either in control solution containing water and 2.5% dimethylsulphoxide (DMSO) or in solution containing 5 μM cytochalasin D (cytD), in 2.5% (v/v) DMSO, and the leaves were kept at a PPFD of 20 μmol m^{-2} s^{-1} until the transmittance was measured the following morning. Leaf disks (4.5 cm^2) were then placed on wet paper on a temperature-controlled surface and illuminated from the adaxial side at a PPFD of 1350 μmol m^{-2} s^{-1} with either a 300 W high-pressure ozone-free xenon lamp (continuous light) or an FX-200 flash lamp (xenon flashes, 13.5 J, 1 fps). Before the illumination treatment and every 10 min after the beginning of illumination, the transmittance of the leaf was measured in a separate set-up in which the leaf was illuminated from the adaxial side with continuous light (PPFD 1350 μmol m^{-2} s^{-1}) and transmission was measured from the abaxial side with an LI-189 quantum sensor. The positions of the lamp and the quantum sensor were fixed and the leaf was always placed in exactly the same position in the transmission set-up. Measurement of transmission of light through a leaf is a standard method for measuring chloroplast movements (see, for example, Berg *et al.*, 2006).

Confocal microscopy

Laser scanning confocal microscopy was performed with an inverted confocal laser scanning microscope (Zeiss LSM510 Meta) with a ×20/0.50 water objective. Chloroplasts were imaged by Chl autofluorescence excited at 488 nm with an argon diode laser and detected through a 650 nm emission filter. Maximal projections of the sequential confocal images were created with Zeiss LSM Image Browser.

Results

Xenon flash-induced photoinhibition in vivo depends on flash energy but not on the time interval between flashes

Properties of the flashes. In the photoinhibition experiments, flash energies above the saturation of PSII electron transfer were used. Such flashes cause one turnover of PSII irrespective of the flash energy. To ensure saturation, the flash energy response of PSII oxygen evolution was measured. In these measurements, the lamp to leaf distance was five times longer than in the photoinhibition set-up, and therefore the flash energy response of oxygen evolution provides an upper limit for the flash energy required to saturate PSII

electron transport in the photoinhibition set-up. The results indicate that saturation of oxygen evolution of intact leaves occurred at 0.7–0.9 J (Fig. 1). Pumpkin leaves contain ~600 μmol Chl m^{-2}, and therefore the maximum rate of oxygen evolution (1.2 μmol O_2 per four flashes) corresponded to 88% of reaction centres, assuming one PSII per 440 Chl molecules. Separate fluorescence measurements showed that PSII reaction centres remain open between the flashes when pumpkin leaves are flashed at 10–30 fps as in the flash energy response measurements (Supplementary Table S1 available at *JXB* online).

For comparison of flashes and continuous light, average PPFD values for flash treatments were calculated, using the known relationship between the flash energy and flash photon content of this lamp (Hakala *et al.*, 2005). In the original flash photon content measurements, the number of visible-light photons per flash was first measured at a narrow wavelength range (475–610 nm) by illuminating *meso*-diphenylhelianthrene, a chemical actinometer substance (Brauer *et al.*, 1983), and the flash photon content in the 400–700 nm range was obtained by correcting these data with the emission spectrum of the flash lamp (Supplementary Fig. S2 at *JXB* online). Average PPFD values, used to compare flashes with continuous light, were obtained by dividing the flash photon content by the target area and multiplying by the number of flashes in unit time.

Experiments were also carried out to measure how efficiently the flashes induce NPQ, as compared with continuous light. For this, pumpkin leaves were illuminated for 20 min either with continuous light of different intensities up to a PPFD of 1350 μmol m^{-2} s^{-1} or with xenon flashes of 1.35, 9.3, or 13.5 J fired at 1 fps, producing an average PPFD range similar to continuous light (Table 1). Measurements of NPQ at the end of the illumination showed a linear increase of NPQ with PPFD of continuous light (Fig. 2). Illumination with xenon flashes at 1 fps at the same PPFD range caused much less NPQ than continuous light, and flashes of 9.3 J and 13.5 J caused the same NPQ response (Fig. 2). Flashes fired at 90 s intervals did not cause NPQ, and the NPQ response caused by flashes fired at 9 s intervals was not statistically significant (data not shown). The coefficient of photochemical quenching q_P remained at 0.98 during flashing of pumpkin leaf disks at 1 fps with the flash energy of 13.5 J (Supplementary Table S1 at *JXB* online).

Dependence of photoinactivation of PSII on flash energy. To induce photoinhibition with flashes, a lincomycin-treated pumpkin leaf was placed under the xenon flash lamp and flashing was repeated until 1800 flashes had been fired. Two alternative methods were used to measure photoinhibition: light-induced decrease in F_v/F_m, measured from leaves, and light-induced decrease in the light-saturated rate of PSII oxygen evolution, measured from thylakoids isolated from treated leaves. The flash number-based rate constant of photoinactivation (k_{PI}) was calculated by fitting the original data to the first-order reaction equation using the formula $k_{PI}=(1/1800)\ \text{flash}^{-1}\times\ln(100/Act)$, where *Act* is the percentage of PSII activity (F_v/F_m or oxygen evolution, as indicated) left after treatment with 1800 flashes. Slow loss of PSII activity in the dark was measured separately and taken into account by subtracting the rate constant of dark inactivation from the raw k_{PI}. The Chl content of the leaves remained at 80–100% of the control for 45 h.

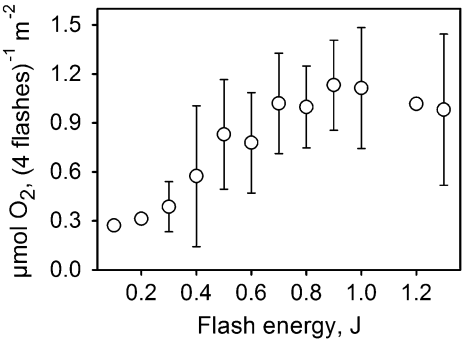

Fig. 1. Rate of oxygen evolution during illumination of pumpkin leaf disks with xenon flashes of 0.1–1.35 J. The error bars show the SD of 2–5 experiments.

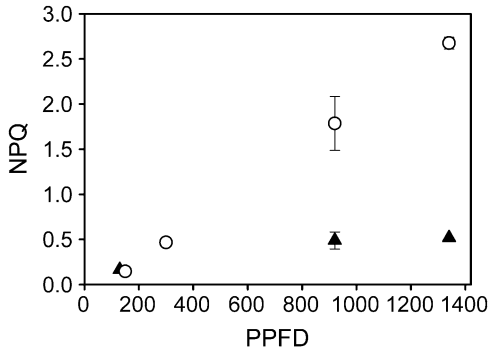

Fig. 2. NPQ induced by 20 min illumination at different PPFDs of continuous light (circles) and with xenon flashes (triangles). The flash to flash interval was 1 s and the flash energies were 1.35, 9.3, and 13.5 J. Prior to the experiments, pumpkin leaves were dark-adapted for at least 30 min. Each symbol represents an average of 3–5 individual experiments, and the error bars, drawn if larger than the symbol, show the SD.

Comparison of photoinhibition induced with saturating xenon flashes of different energies indicated that the k_{PI} of flash photoinactivation was directly proportional to the flash energy, regardless of whether k_{PI} was determined from oxygen evolution or from F_v/F_m (Fig. 3). The correlation between k_{PI} and flash energy was statistically highly significant whether the correlation was calculated from all data or from subsets with the same flash interval (Supplementary Table S2 at *JXB* online). No saturation of photoinactivation could be detected. Larger k_{PI} values were obtained from oxygen evolution measurements than from fluorescence measurements (Fig. 3).

Dependence of photoinactivation on flash interval. To measure the effect of the flash to flash interval on inactivation of PSII *in vivo*, xenon flashes with 1, 9, and 90 s intervals were applied on pumpkin leaves. The photoinhibitory efficiency of the flashes did not show a consistent dependence on the flash interval when F_v/F_m was used to

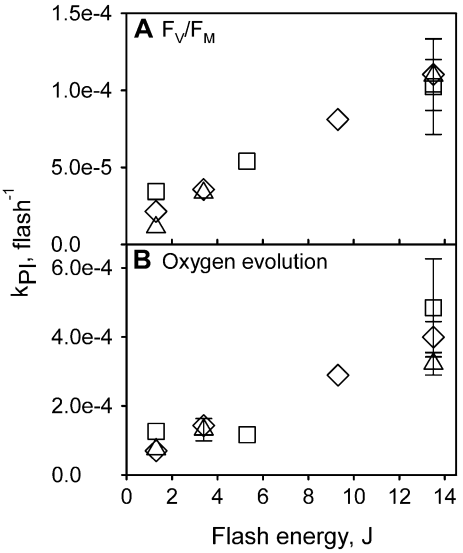

Fig. 3. Flash energy dependence of the rate constant of photoinhibition (k_{PI}) calculated per xenon flash. The k_{PI} values were determined from both the photoinhibitory loss of F_v/F_m (A) and the decrease in the light-saturated rate of oxygen evolution (B). The photoinhibition treatments were done by illuminating lincomycin-treated pumpkin leaves with xenon flashes of the indicated energies. F_v/F_m was measured from the leaf surface, and oxygen evolution was measured from thylakoids isolated from treated leaves. In thylakoids isolated from untreated leaves, the rate of oxygen evolution was 335±45 μmol O_2 mg Chl^{-1} h^{-1}. The time interval between xenon flashes was either 1 s (diamonds), 9 s (triangles), or 90 s (squares). Each data point represents 4–9 independent experiments, and the error bars, drawn if larger than the symbol, show the SE.

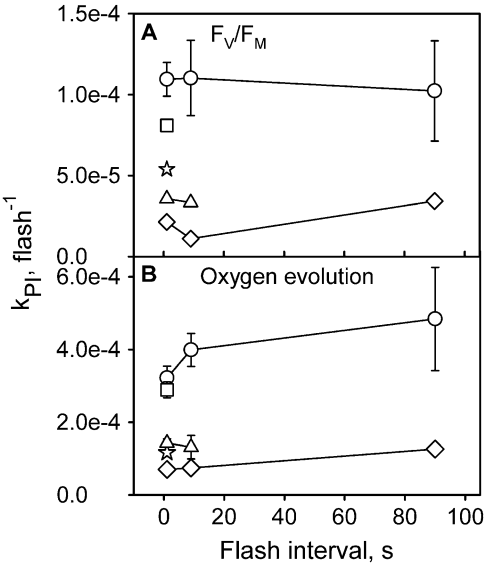

Fig. 4. Dependence of k_{PI}, calculated per flash, on the time interval between xenon flashes. The k_{PI} values were determined from the photoinhibitory loss of F_v/F_m (A) or from the decrease in the light-saturated rate of oxygen evolution (B). Fluorescence was measured from leaves, and oxygen evolution was measured from thylakoids isolated from the treated leaves. The energies of the xenon flashes were 13.5 J (circles), 9.3 J (squares), 5.3 J (stars), 3.4 J (triangles), or 1.35 J (diamonds). Each data point represents 4–9 individual experiments, and the error bars, drawn if larger than the symbol, show the SE.

measure PSII activity, and independence or a slight increase of photoinhibitory efficiency with flash interval was seen in the oxygen evolution data (Fig. 4). However, no statistically significant correlations between k_{PI} values and flash interval could be found (Supplementary Table S2 at *JXB* online). Thus, when k_{PI} values are plotted as a function of flash energy, all experiments were found to fall on the same line independently of the flash interval (Fig. 3).

Photon flux density dependence of flash-induced photoinhibition

The k_{PI} values used to compare continuous-light-induced and flash-induced photoinactivation were obtained by fitting the data to the first-order reaction equation $Act=100\times\exp(-k_{PI}\times t)$, where Act is the percentage activity remaining at time t. The k_{PI} values of the flash and continuous-light treatments showed an essentially direct proportionality to PPFD irrespective of the measurement method (F_v/F_m or oxygen evolution) (Fig. 5), although some deviation from direct proportionality could be found both in very low light and at the high PPFD of 2300 μmol m^{-2} s^{-1}. The slope of the plot describing dependence of k_{PI} on PPFD was the same for flash and continuous-light treatments when F_v/F_m was used to measure photoinhibition (Fig. 5A). Essentially the same slope was also obtained for continuous-light-induced photoinhibition measured with oxygen evolution (Fig. 5B, circles), whereas single-turnover flashes had a stronger inhibitory effect on oxygen evolution (Fig. 5B).

Chloroplast movements under short xenon flashes and continuous light

The oxygen evolution measurements represent all cell layers, whereas Chl fluorescence is emitted by the cell layers close to the leaf surface. Therefore, the difference between F_v/F_m and oxygen evolution in flash-photoinhibited leaves (Fig. 5) prompted measurement of chloroplast movements, as the avoidance response in which chloroplasts stack on each other can obviously not protect the topmost chloroplasts of the leaf. During 30 min illumination with continuous light, PPFD 1350 μmol m^{-2} s^{-1}, the transmittance of pumpkin leaves increased from 7.5% to 10.4% (Fig. 6A). Transmission of blue light increased more than transmission of red light, and that of green light was intermediate (Fig. 6B).

The increase in transmission was apparently caused by chloroplast movements, as 5 μM cytD, an actin-depolymerizing agent, partially inhibited the increase. For comparison, changes in the transmittance of *Arabidopsis* leaves were also studied, and it was found that 30 min illumination induced an increase in transmission from 13% to 25%, twice as much as in pumpkin, and confocal microscopy showed light-induced movement of chloroplasts

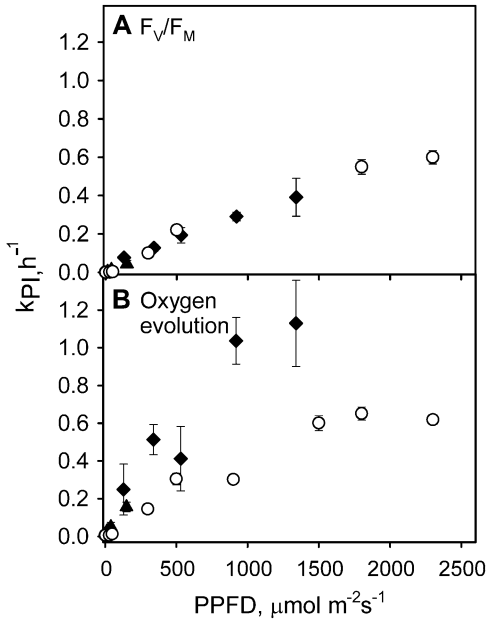

Fig. 5. Dependence of k_{PI} on PPFD in lincomycin-treated pumpkin leaves. The k_{PI} value was calculated per hour from the photoinhibitory decrease in F_v/F_m (A) or from the photoinhibitory loss of oxygen evolution (B). The leaves were illuminated with continuous light (open circles) or with xenon flashes (filled symbols). The flash to flash intervals of the flash photoinhibition treatments were 1 s (diamonds), 9 s (triangles), or 90 s (squares). The flash energies of the xenon flash treatments were adjusted between 1.35 J and 13.5 J, and the mean PPFD values of the xenon flash treatments were calculated by multiplying the photon content of one flash by the number of flashes in unit time and dividing by the area of the illuminated spot. Each data point represents 3–9 independent experiments, and the error bars, drawn if larger than the symbol, show the SD.

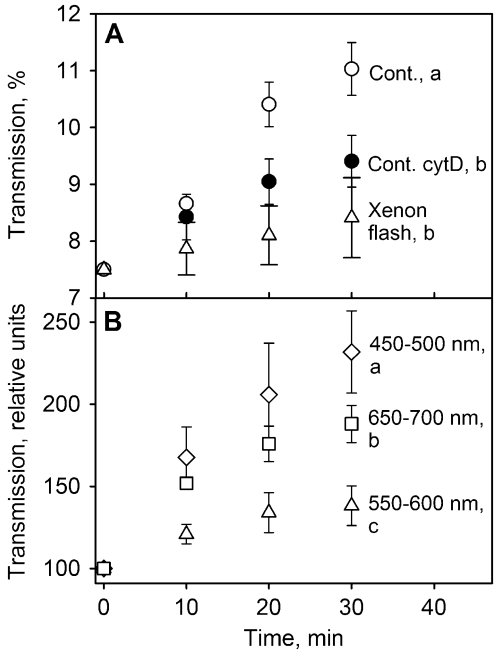

Fig. 6. Light-induced increase in transmission, measured from the adaxial to the abaxial side of pumpkin leaves with a Li-189 quantum sensor. (A) Increase in transmission measured during illumination with continuous light (PPFD 1350 μmol m^{-2} s^{-1}, circles) in the absence (open symbols) or presence (filled circles) of cytochalasin D (cytD), or with xenon flashes (13.5 J, 1 fps, triangles). (B) Relative changes in transmission at the wavelength ranges of 450–500 nm (diamonds), 550–600 nm (triangles), and 650–700 nm (squares). The wavelength-specific values were measured by using a pair of low-pass and high-pass filters between the leaf and the quantum sensor. Each data point represents an average of 3–5 experiments, and the error bars, drawn if larger than the symbol, show the SD. Different letters (a, b, c) indicate that the 30 min treated values differ significantly from each other according to the *t*-test (P <0.05).

to the anticlinal cell walls in *Arabidopsis* (Supplementary Fig. S1 at *JXB* online). However, both before and after illumination, pumpkin chloroplasts appeared to stay in the anticlinal position in the mesophyll cells accessible with the confocal microscope (Supplementary Fig. S1).

When pumpkin leaves were illuminated for 30 min with xenon flashes (13.5 J, 1 fps, 1350 μmol m^{-2} s^{-1}), leaf transmission increased only to 8.4% from the initial value of 7.5% (Fig. 6A).

Discussion

The aim of the present study was to gain insight into the mechanism of photoinactivation of PSII by illuminating intact lincomycin-treated pumpkin leaves with saturating 3 μs single-turnover xenon flashes. The single-turnover nature of the flashes was ensured by showing that flashing does not cause accumulation of reduced Q_A (Supplementary Table S1 at JXB online) and that flashing causes very little NPQ even at the highest flash rate of 1 fps (Fig. 2). Measurements of both oxygen evolution and F_v/F_m show that the photoinhibitory efficiency of single-turnover flashes is directly proportional to flash energy (Fig. 3). Furthermore, the fluorescence data showed that the photoinhibitory efficiency of photons of single-turnover flashes is similar to the photoinhibitory efficiency of photons of continuous light (Fig. 5). Direct proportionality between light intensity and k_{PI} is a key feature of continuous-light photoinhibition (Jones and Kok, 1966; Park *et al.*, 1995; Tyystjärvi and Aro, 1996; Allakhverdiev and Murata, 2004). Thus, the finding that the same proportionality constant applies to photoinhibition under flashing light and under continuous light (Fig. 5) suggests that the mechanism of photoinactivation is the same in both conditions.

Similar photoinhibitory efficiency of continuous light and flash light can be used as a criterion for evaluation of photoinhibition hypotheses. Full opening of PSII reaction centres between the flashes (Supplementary Table S1 at *JXB* online) allows the possibility that the acceptor-side mechanism (Vass *et al.*, 1992) causes flash photoinhibition to be excluded, as neither double reduction of Q_A nor production of singlet oxygen by charge recombination due to stabilized

Q_A^- can happen unless Q_A is singly reduced when a single-turnover flash is fired. Proportionality between k_{PI} and the energy of the saturating flashes also indicates that the 'statistical' donor-side mechanism (Anderson *et al.*, 1998) cannot be responsible for flash photoinhibition, as the amount of long-lived P_{680}^+ cannot depend on how over-saturating a flash is.

The *in vivo* photoinhibitory efficiency of the flashes did not consistently depend on the time interval between the flashes (Fig. 4). This finding is in agreement with earlier *in vitro* data (Hakala *et al.*, 2005). The flash interval range of 1–90 s was chosen on the basis of the 35 s half-time of the charge recombination reaction $S_2Q_B^- \rightarrow S_1Q_B$, measured with thermoluminescence (Keren *et al.*, 1997). At 90 s intervals, 83% of Q_B^- produced by a flash recombines with the S_2 state of the oxygen-evolving complex before the next flash comes, while at 1 s intervals only 2% recombines and most Q_B^- is converted to Q_B^{2-} and replaced by oxidized plastoquinone. Thus, flashes fired at 90 s intervals cause 40 times as many recombination reactions as flashes fired at 1 s intervals. Lack of dependence on flash interval (Fig. 4) indicates that the low-light photoinhibition hypothesis which assumes that photoinactivation starts with charge recombination (Keren *et al.*, 1997, 2000) does not explain flash photoinhibition *in vivo*. Furthermore, the low-light hypothesis cannot explain why the photoinhibitory efficiency of saturating flashes depends on flash energy, as the same number of recombination reactions is induced with all saturating flashes. The results of the present study are in agreement with the finding that the quantum yield of photoinactivation of PSII is the same from 6 μmol m^{-2} s^{-1} to 2000 μmol m^{-2} s^{-1} of continuous light (Tyystjärvi and Aro, 1996). Thus, a specific mechanism for photoinactivation of PSII under low light is improbable.

The manganese mechanism (Hakala *et al.*, 2005) and generation of singlet oxygen by uncoupled Chls (Santabarbara *et al.*, 2001) or by iron–sulphur centres (Jung and Kim, 1990) would have the same quantum yield for continuous light and single-turnover flash illumination, and therefore these mechanisms qualify as explanations for the present *in vivo* photoinhibition data. The manganese mechanism functions in parallel with photoinactivation mediated by the PSII antenna (Sarvikas *et al.*, 2010). The results of the present study do not define the Chl-dependent mechanism, but generation of singlet oxygen may have a role in it.

In the present study, three flash intervals and five flash energies were tested. The possibility that use of subsaturating flashes or a larger number of flash intervals might provide more insight cannot be excluded.

A similar photoinhibitory efficiency of continuous light in F_v/F_m and oxygen evolution measurements (Fig. 5) is in agreement with earlier data (Krause *et al.*, 1992; Schnettger *et al.*, 1994). Flash-induced and continuous-light-induced photoinactivation had the same quantum yield in F_v/F_m measurements (Fig. 5A), but flashes were more efficient at causing photoinhibition of oxygen evolution (Figs 3, 5). This finding can be explained by assuming that the photoinhibitory efficiency of flashes and continuous light is similar but chloroplasts of deep cell layers are more efficiently illuminated by flashes than by continuous light. The chloroplast avoidance response (Wada *et al.*, 2003) would not protect the top layer of chloroplasts, and the finding that flashes cause very little chloroplast movements may partially explain why the flashes penetrate better in the leaf. The chloroplast avoidance response affects mostly transmission of blue light (Fig. 7), which is the most effective visible-light wavelength range in photoinhibition *in vivo* (Sarvikas *et al.*, 2006). Chloroplast movements have little effect on leaf reflectance (Park *et al.*, 1996) and therefore transmission measurements estimate changes in leaf absorptance.

The data presented in this paper lead to two important conclusions about the mechanism of photoinhibition *in vivo*. First, the finding that the photoinhibitory efficiency of saturating flashes depends on flash energy but is independent of flash to flash interval indicates that charge recombination between PSII electron acceptors and the oxygen-evolving complex cannot be the trigger of photoinhibition caused by short flashes *in vivo*. Secondly, photons of continuous light and photons of short flashes were found to have similar photoinhibitory efficiency, suggesting that continuous-light photoinhibition has a mechanism in which the quantum yield does not depend on the mode of delivery of light.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Confocal microscope images from pumpkin and *Arabidopsis* leaves.

Figure S2. Emission spectra of the xenon flash lamp and the continuous-light lamps used in this study.

Table S1. Effect of illumination with xenon flashes on photochemical quenching in pumpkin leaves.

Table S2. Pearson correlation coefficients between k_{PI} and flash energy, and between k_{PI} and flash interval.

Acknowledgements

This work was financially supported by the Academy of Finland (grant number 110409), the Turku University Foundation, the Finnish Cultural Foundation, and the Emil Aaltonen Foundation.

References

Allakhverdiev SI, Murata N. 2004. Environmental stress inhibits the synthesis *de novo* of proteins involved in the photodamage–repair cycle of photosystem II in *Synechocystis* sp. PCC 6803. *Biochimica et Biophysica Acta* **1657,** 23–28.

Anderson JM, Park Y-I, Chow WS. 1998. Unifying model for the photoinactivation of photosystem II *in vivo*: a hypothesis. *Photosynthesis Research* **56,** 1–13.

Antal TK, Sarvikas P, Tyystjärvi E. 2009. Two-electron reactions $S_2Q_B \rightarrow S_0Q_B$ and $S_3Q_B \rightarrow S_1Q_B$ are involved in deactivation of higher S states of photosystem II. *Biophysical Journal* **96,** 4672–4680.

Aro E-M, Virgin I, Andersson B. 1993. Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochimica et Biophysica Acta* **1143,** 113–134.

Berg R, Königer M, Schjeide B-M, Dikmak G, Kohler S, Harris GC. 2006. A simple low-cost microcontroller-based photometric instrument for monitoring chloroplast movement. *Photosynthesis Research* **87,** 303–311.

Bilger W, Björkman O. 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of Hedera canariensis. *Photosynthesis Research* **25,** 173–185.

Brauer H-D, Schmidt R, Gauglitz G, Hubig S. 1983. Chemical actinometry in the visible (475–610 nm) by meso-diphenylhelianthrene. *Photochemistry and Photobiology* **37,** 595–598.

Hakala M, Tuominen I, Keränen M, Tyystjärvi T, Tyystjärvi E. 2005. Evidence for the role of the oxygen-evolving manganese complex in photoinhibition of photosystem II. *Biochimica et Biophysica Acta* **1706,** 68–80.

Inskeep W, Bloom PR. 1985. Extinction coefficients of chlorophyll a and b in N, N-dimethylformamide and 80% acetone. *Plant Physiology* **77,** 483–485.

Jones LW, Kok B. 1966. Photoinhibition of chloroplast reactions. I. Kinetics and action spectra. *Plant Physiology* **41,** 1037–1043.

Jung J, Kim H- S. 1990. The chromophores as endogenous sensitizers involved in the photogeneration of singlet oxygen in spinach thylakoids. *Photochemistry and Photobiology* **52,** 1003–1009.

Keren N, Berg A, van Kan PJM, Levanon H, Ohad I. 1997. Mechanism of photosystem II photoinactivation and DI protein degradation at low light: the role of back electron flow. *Proceedings of the National Academy of Sciences, USA* **94,** 1579–1584.

Keren N, Gong H, Ohad I. 1995. Oscillations of reaction center II-D1 protein degradation *in vivo* induced by repetitive light-flashes. Correlation between the level of RCII-Q_B^- and protein degradation in low light. *Journal of Biological Chemistry* **270,** 806–814.

Keren N, Ohad I, Rutherford AW, Drepper F, Krieger-Liszkay A. 2000. Inhibition of photosystem II activity by saturating single turnover flashes in calcium-depleted and active photosystem II. *Photosynthesis Research* **63,** 209–216.

Kok B, Forbush B, McGloin M. 1970. Cooperation of charges in photosynthetic O_2 evolution. *Photochemistry and Photobiology* **11,** 457–475.

Krause GH, Somersalo S, Zumbusch E, Weyers B, Laasch H. 1992. On the mechanism of photoinhibition in chloroplasts. Relationship between changes in fluorescence and activity of photosystem II. *Journal of Plant Physiology* **136,** 472–479.

Lee H-Y, Chow WS, Hong Y-N. 1999. Photoinactivation of photosystem II in leaves of *Capsicum annuum*. *Physiologia Plantarum* **105,** 377–384.

Melis A. 1999. Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage *in vivo? Trends in Plant Science* **4,** 130–135.

Ohnishi N, Allakhverdiev SI, Takahashi S, Higashi S, Watanabe M, Nishiyama Y, Murata N. 2005. Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center. *Biochemistry* **44,** 8494–8499.

Park Y-I, Chow WS, Anderson JM. 1995. Light inactivation of functional photosystem-II in leaves of peas grown in moderate light depends on photon exposure. *Planta* **196,** 401–411.

Park Y-I, Chow WS, Anderson JM. 1996. Chloroplast movement in the shade plant *Tradescantia albiflora* helps protect photosystem II against light stress. *Plant Physiology* **111,** 876–875.

Porra RJ, Thompson WA, Kriedemann PE. 1980. Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-a and chlorophyll-b extracted with 4 different solvents—verification of chlorophyll standards by atomic-absorption spectroscopy. *Biochimica et Biophysica Acta* **975,** 384–394.

Renger G, Holzwarth AR. 2005. Primary electron transfer. In: Wydrzynski T, Satoh K, eds. *Photosystem II: the light-driven water:plastoquinone oxidoreductase*. Dordrecht, The Netherlands: Springer, 139–175.

Renger G, Wolff C. 1976. The existence of a high photochemical turnover rate at the reaction centers of system II in Tris-washed chloroplasts. *Biochimica et Biophysica Acta* **423,** 610–614.

Santabarbara S, Neverov KV, Garlaschi FM, Zucchelli G, Jennings RC. 2001. Involvement of uncoupled antenna chlorophylls in photoinhibition in thylakoids. *FEBS Letters* **491,** 109–113.

Sarvikas P, Hakala M, Pätsikkä E, Tyystjärvi T, 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.

Sarvikas P, Tyystjärvi T, 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.

Schnettger B, Critchley C, Santore UJ, Graf M, Krause GH. 1994. Relationship between photoinhibition of photosynthesis, D1 protein turnover and chloroplast structure: effects of protein synthesis inhibitors. *Plant, Cell and Environment* **17,** 55–64.

Szilard A, Sass L, Hideg E, Vass I. 2005. Photoinactivation of photosystem II by flashing light. *Photosynthesis Research* **84,** 15–20.

Tyystjärvi E. 2008. Photoinhibition of photosystem II and photodamage of the oxygen evolving manganese cluster. *Coordination Chemistry Reviews* **252,** 361–376.

Tyystjärvi E, Aro E-M. 1996. The rate constant of photoinhibition, measured in lincomycin-treated leaves, is directly proportional to light intensity. *Proceedings of the National Academy of Sciences, USA* **93,** 2213–2218.

Tyystjärvi E, Hakala M, Sarvikas P. 2005. Mathematical modelling of the light response curve of photoinhibition of photosystem II. *Photosynthesis Research* **84,** 21–27.

Tyystjärvi E, Hakala M, Tyystjärvi T. 2008. Photoinhibitory efficiency of saturating laser pulses depends on pulse energy. In: Allen

JF, Gantt E, Golbeck JH, Osmond B, eds. *Photosynthesis. energy from the sun: 14th International Congress on Photosynthesis*. Springer, 1587–1590.

Tyystjärvi E, Karunen J. 1990. A microcomputer program and fast analog to digital converter card for the analysis of fluorescence induction transients. *Photosynthesis Research* **26,** 127–132.

Vass I, Aro E-M. 2008. Photoinhibition of photosystem II electron transport. In: Renger G, ed. *Primary processes of photosynthesis: basic principles and apparatus. Comprehensive Series in Photochemical and Photobiological Sciences*, Vol. 8. Cambridge: The Royal Society of Chemistry, 393–411.

Vass I, Styring S, Hundal T, Koivuniemi A, Aro E-M, Andersson B. 1992. Reversible and irreversible intermediates during photoinhibition of photosystem II. Stable reduced QA species promote chlorophyll triplet formation. *Proceedings of the National Academy of Sciences, USA* **89,** 1408–1412.

Wada M, Kagawa T, Sato Y. 2003. Chloroplast movement. *Annual Review of Plant Biology* **54,** 455–468.