Photosynthesis Research

Whole-tissue determination of the rate coefficients of photoinactivation and repair of Photosystem II in cotton leaf discs based on flash-induced P700 redox kinetics

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Author Comments:	Dear Editor, We wish to submit a revised version of the paper "Whole-tissue determination of the rate coefficients of photoinactivation and repair of Photosystem II in cotton leaf discs based on flash-induced P700 redox kinetics", as a contribution to the special issue that celebrates the 80th birthdays of Govindjee and Joliot. Thank you for considering our manuscript. Professor Wah Soon (Fred) CHOW
Abstract:	Using radioactively-labelled aminoacids to investigate repair of photoinactivated Photosystem II (PS II) gives only a relative rate of repair, while using chlorophyll fluorescence parameters yields a repair rate coefficient for an undefined, variable location within the leaf tissue. Here we report on a whole-tissue determination of the rate coefficient of photoinactivation ki, and that of repair kr in cotton leaf discs. The method assays functional PS II via a P700 kinetics area associated with PS I, as induced by a single-turnover, saturating flash superimposed on continuous background far-red light. The P700 kinetics area, directly proportional to the oxygen yield per single-turnover, saturating flash, was used to obtain both ki and kr. The value of ki, directly proportional to irradiance, was slightly higher when CO2 diffusion into the abaxial surface (richer in stomata) was blocked by contact with water. The value of kr, sizable in darkness, changed in the light depending on which surface was blocked by contact with water. When the abaxial surface was blocked, kr first peaked at moderate irradiance and then decreased at high irradiance. When the adaxial surface was blocked, kr first increased at low irradiance, then plateaued, before increasing

	markedly at high irradiance. At the highest irradiance, kr differed by an order of magnitude between the two orientations, attributable to different extents of oxidative stress affecting repair (Nishiyama et al. 2001, EMBO J 20: 5587-5594). The method is a whole-tissue, convenient determination of the rate coefficient of photoinactivation ki and that of repair kr.
Response to Reviewers:	Dear Editor, We thank the reviewers for their constructive comments, in response to which we have made the following modifications where needed. Changes in the re-submitted manuscript text are indicated in red. Reviewer #1: It is a very well-written paper and I enjoy reading it. Below are some minor suggestions for the amendments before publication: P6, line 122, it would be better to include the maximal light intensity under natural light inside the greenhouse from April to August 2012. RESPONSE: added as suggested P6, lines 172 to 173, Mexp and Mfitted should be indicated on Fig. 1B or in the Figure Legend of Fig. 1B. RESPONSE: added in the figure legend as suggested P10, lines 218 - 224, "Measurement of carbon assimilation rates": "Temperature and chamber CO2 concentration were kept at 25°C and 400 µmol mol−1, respectively", what was the source of CO2, internal supply or ambient [CO2]? When were the measurement carried out during the day? RESPONSE: added as suggested P10, lines 225 to 231, "Measurement of electron transport rates". It would be better to include some details on how to obtain PSII. and Y(I) RESPONSE: In the interest of brevity, a full description is not added, but a reference to Khughammer and Schreiber (2007) for details of determining Y(I) and phi PS II has been added Fig. 4, the symbols of 913 and 1300 µmol did not match those shown on the curves. RESPONSE: symbols corrected as suggested Discussion -This paper also studied and discussed the effect of different CO2 diffuse (through two orientations in which leaf discs were floated on water) on the rate coefficient of photoinactivation ki and the rate coefficient of repair kr. It is concluded that at the highest irradiance, the repair rate coefficient depends on CO2 diffusion via stomata, probably reflecting whether oxidative stress exceeded the capacity for detoxifying ROS or not. I am also wondering if cyclic electron flow (CEF) would also affect ki and kr as CO2 diffusion may affect the process of CEF. It has been reported that CEF-dependent generation of pH across the thylakoid membrane helps to avoid photoinhibition by different photoprotection mechanisms [Takahashi et al. Plant Physiology 149:1560-1567 (2009)]. These are just my comments. RESPONSE: Thanks for the suggestion. While CEF may well have an effect on ki and kr, the difficulty of quantifying CEF presents a problem. CEF is combined with the linear electron flux and other minor fluxes in the parameter ETR1, the total electron flux through PS I (Fig. 8,), but we are not sure exactly how large the CEF component is. For this reason, we have refrained from bringing the protective effect of CEF into the Discussion. Reviewer #2: General comments: This manuscript presents a new method to determine the rate coefficients for photoinactivation and repair of PSII. The flash-induced P700 kinetics that the authors have developed seems to be solid and convenient to monitor the two processes that occur simultaneously in vivo during photoinhibition of PSII. This method allowed the authors to determine the rate coefficients for photoinactivation and repair of PSII in a whole tissue of cotton and also to find that the repair process is susceptible to inactivation under high light when the availability of CO2 is limited. Based on the findings, they conclude that the repair process is sensitive to oxidative stress. I totally agree with their conclusion.

	Specific comments: 1. In Fig. 6B, profiles of the rate coefficient of repair do not look very consistent. In particular, the rate coefficient in the leaf disc with adaxial side faced water goes up and down. This complicated profile might be due to the results of the repair assay shown in Fig. 5B. The P700 kinetics area at zero time is not the same in all samples so that the repair rate might be changed (see illumination at 30 and 133). If this is true, I would like to suggest the authors to diminish the discussion about the profile. RESPONSE: As this reviewer suggested, we have modified the Discussion to simply say that kr was between 0.55 and 0.75 h1 in the irradiance range 30-611 µmol m-2 s-1. 2. In Fig. 6A, there is a slight difference in the rate coefficient of photoinactivation between the two leaves with different orientation. I am a bit wondering if lincomycin can efficiently penetrate into the cells from the adaxial side. RESPONSE: The slight difference is probably real, and is probably due to a difference in the electron transport rate permitted in the two orientations at a given irradiance, a difference that exists at least above ~300 umol m-2 s-1.

Whole-tissue determination of the rate coefficients of photoinactivation and repair of Photosystem II in cotton leaf discs based on flash-induced P700 redox kinetics

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Abstract

Using radioactively-labelled aminoacids to investigate repair of photoinactivated Photosystem II (PS II) gives only a relative rate of repair, while using chlorophyll fluorescence parameters yields a repair rate coefficient for an undefined, variable location within the leaf tissue. Here we report on a whole-tissue determination of the rate coefficient of photoinactivation k_i, and that of repair k_r in cotton leaf discs. The method assays functional PS II via a P700 kinetics area associated with PS I, as induced by a single-turnover, saturating flash superimposed on continuous background far-red light. The P700 kinetics area, directly proportional to the oxygen yield per single-turnover, saturating flash, was used to obtain both k_i and k_r. The value of k_i, directly proportional to irradiance, was slightly higher when CO_2 diffusion into the abaxial surface (richer in stomata) was blocked by contact with water. The value of k_r, sizable in darkness, changed in the light depending on which surface was blocked by contact with water. When the abaxial surface was blocked, k_r first peaked at moderate irradiance and then decreased at high irradiance. When the adaxial surface was blocked, k_r first increased at low irradiance, then plateaued, before increasing markedly at high irradiance. At the highest irradiance, k_r differed by an order of magnitude between the two orientations, attributable to different extents of oxidative stress affecting repair (Nishiyama et al. 2001, EMBO J 20: 5587-5594). The method is a whole-tissue, convenient determination of the rate coefficient of photoinactivation k_i and that of repair k_r.

Key words Chlorophyll fluorescence • P700 • Photosystem II • Photoinactivation of Photosystem II • Repair of Photosystem II

Abbreviations

Chl	chlorophyll
f	functional fraction of PS II

F_o, F_m	minimum and maximum Chl fluorescence yield of a dark-adapted leaf, respectively
F_v	= ($F_m - F_o$), variable fluorescence
k_i, k_r	rate coefficient of photoinactivation and repair, respectively
P700	special Chl pair in the PSI reaction center
PI	photoinactivation
PS	photosystem
ROS	reactive oxygen species

Introduction

While light is essential for photosynthesis, too much light can lead to dysfunction of the photosynthetic apparatus (Ewart 1896; Powles 1984; Tyystjärvi 2008; Nishiyama et al. 2011; Oguchi et al. 2011a; Ohad et al. 2011; Vass 2011) because oxygenic photosynthesis, i.e. Photosystem II (PS II), is intrinsically suicidal (van Gorkom and Schelvis 1993). After photoinactivation, PS II needs to be repaired (Prásil et al. 1992; Aro et al. 1993; Melis 1999; Chow and Aro 2005). During illumination, both photoinactivation and repair occur simultaneously. If repair cannot keep up with photoinactivation, net loss of PS II function ensues. When repair keeps up with photoinactivation, the whole PS II population may turn over at least once during a sunny day. The underlying mechanism of repair and the associated energy cost (Raven 2011; Miyata et al. 2012) are crucial for understanding how leaves alleviate light-induced decrease of photochemical efficiency of PSII on the one hand and balance their energy budget on the other, including leaves of woody plants grown in the field (Losciale et al. 2010).

Repair of photoinactivated PS II requires *de novo* synthesis of the D1 protein in the PS II reaction centre, as shown, for example, in *Chlamydomonas* (Kyle et al. 1984; Ohad et al. 1984), *Anacystis* (Samuelsson et al. 1985), pea (Ohad et al. 1985) and beans (Greer et al. 1986). Protein synthesis can be

monitored by the incorporation of radioactively-labelled aminoacids into proteins (Fish and Jagendorf 1982; Mattoo et al. 1984; Aro et al. 1992). For example, Sundby et al. (1993) studied the parallel synthesis and degradation of the D1 protein in *Brassica napus* leaves by measuring (1) the *net* incorporation of ^{35}S-methionine as a function of irradiance at a fixed duration of illumination (1 h) and (2) D1 protein degradation as revealed by the exponential loss of radiolabel in a pulse-chase experiment. Chow (2001) used the data of Sundby et al. (1993) to derive the *gross* rate of D1 protein synthesis (in arbitrary units) as a function of irradiance. However, the use of radiolabel is not always convenient, and the results give only relative rates of photoinactivation and repair.

Photoinactivation and repair of PS II are best characterized by their rate coefficients. The rate coefficient of photoinactivation k_i can be obtained from the first-order time course of the loss of functional PS II in the absence of repair (e.g. in the presence of lincomycin, Tyystjärvi and Aro 1996; Kou et al. 2012); once obtained, it can be multiplied by the concentration of functional PS II to give the rate of photoinactivation. The rate coefficient of repair k_r can deduced from the parallel photoinactivation (with k_i separately determined in the absence of repair under otherwise identical conditions) and recovery processes that occur in the presence of repair; once obtained, it can be multiplied by the concentration of non-functional PS II to give the rate of recovery. The *in vivo* PSII functionality could be assessed by using chlorophyll *a* fluorescence or oxygen evolution. However, these two methods have their drawbacks (Chow et al. 2012). For example, one inherent problem of using Chl fluorescence is that the signal is detected from an unspecified depth in the leaf tissue, and that the depth of signal detection may well vary during the course of the experiment: as functional PS II complexes are rendered less fluorescent upon photoinactivation, the contribution to the Chl fluorescence yield from deeper tissue becomes more prominent (Oguchi et al. 2011b). That is, a moving target that represents a sub-population of PS II is monitored during the onset of PS II photoinactivation as well as recovery from photoinactivation. This may have been a reason for the poor curve fitting in some treatments (He and Chow 2003). Another

inherent problem of using Chl fluorescence is that the use of F_v/F_m or $1/F_o - 1/F_m$ (to represent PS II
96 functionality) could be reliably obtained only after darkness of a certain duration, mainly to allow relaxation of energy-dependent quenching. However, if the repair process persists in darkness (see
98 results below), a long dark treatment before measurement will inevitably complicate the actual repair that has occurred in the light.

100 In this study, we used a whole-tissue measure of the functional PS II content that is rapid and convenient, and can be applied as soon as 1 min after the cessation of a light treatment, provided the flash
102 is saturating. The integrated delivery of electrons from PS II to $P700^+$ (the oxidized primary donor in PS I), after a single-turnover saturating flash, is a whole-tissue measure of the functional PS II content, as
104 supported by two findings. First, a simple analysis of the integrated flash-induced delivery of electrons to $P700^+$ gave a single linear correlation with the relative oxygen yield per repetitive flash for various
106 plant species of diverse anatomy (Losciale et al. 2008; for a review of assays of PS II *in vivo*, see Chow et al. 2012). Second, a simple flash-induced P700 redox kinetics area was measured, bounded by (1) the
108 horizontal line corresponding to the steady-state value in background far-red light and (2) the re-reduction of $P700^+$ and the oxidation of P700 following a saturating, single-turnover flash; this P700 kinetics area,
110 measured from either the upper (adaxial) side or the lower (abaxial) side of a leaf, gave essentially the same fraction of functional PS II remaining after photoinhibition (Oguchi et al. 2011b). We took
112 advantage of this simple flash-induced P700 redox kinetics area to assay the relative content of functional PS II in the whole tissue, and to evaluate the rate coefficients of photoinactivation and repair of PS II in
114 cotton leaves under varied irradiance and oxidative stress. The results demonstrate a reliable method for characterizing both photoinactivation of PS II and recovery from photoinactivation in the whole leaf
116 tissue, superior to the use of Chl fluorescence. Further, we observed that the rate coefficient of repair can be highly sensitive to oxidative stress.

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Materials and methods

Growth of plants

Cotton (*Gossypium hirsutum* L. cv. Deltapine) plants were grown in a glasshouse at approximately 28/18ºC (day/night) under natural light (maximum irradiance ~1000 µmol m^{-2} s^{-1}) from April to August 2012. The plants were provided with a nutrient solution of "Aquasol" (Yates Australia, Padstow, NSW).

Photoinhibitory treatment of leaf discs

For photoinactivation of PS II in the presence of lincomycin, cotton leaf discs (1.5 cm^2) were first floated on 1 mM lincomycin solution overnight in darkness to allow uptake of the inhibitor of chloroplast-encoded protein synthesis. Leaf discs that were to be exposed to photoinhibitory light with the abaxial side facing air were floated overnight in darkness with the adaxial side in contact with a lincomycin solution in a clear petri dish; the subsequent light exposure was applied vertically up onto the adaxial side. Leaf discs that were to be exposed to photoinhibitory light with the adaxial side facing air were floated overnight in darkness with the abaxial side in contact with a lincomycin solution; the subsequent light exposure was applied vertically down onto the adaxial side. Illumination was applied for up to 6 h to obtain the first-order time course of photoinactivation of PS II, which yielded the rate coefficient of photoinactivation k_i.

For observing the time course of recovery of PS II after photoinactivation, leaf discs were immersed under water (~15°C) in the absence of lincomycin, and pre-illuminated on the adaxial side with light (1800 µmol photons m^{-2} s^{-1}) from an HMI Universal Spotlight (Model HMI 575 W/GS; Osram) behind a heat-reflecting filter (Schott 115, Tempax) and a piece of soda glass. The duration of pre-illumination needed to decrease the functional PS II content to ~50% was found to be 72 min, after which leaf discs were allowed to recover under varied irradiance applied to the adaxial side, with either the abaxial or adaxial side facing air, while the opposite side was in contact with water. Depending on

142 the selected orientation, illumination was provided vertically up or down, always to the adaxial side. The pre-treatment at a lower temperature and high irradiance speeded up photoinactivation of PS II, while
144 the relatively low [O_2] in the vicinity of the submerged leaf discs during high-light treatment allowed good recovery to be measured, at least at low irradiances.

146 **PSII functionality**

A time-consuming method of quantifying the functional PSII content is based on the flash-induced
148 oxygen evolution in 1% CO_2, using repetitive single-turnover, saturating xenon flashes (full width at half height = 3 μs) and assuming that each functional PSII evolves one O_2 molecule after four flashes (Chow
150 et al. 1989). The O_2 yield flash^{-1} m^{-2} of photoinhibited leaf segments was normalized to the value of the non-photoinhibited control to obtain the functional fraction of PSII remaining. The functional fraction
152 of PS II so obtained was used to check a simple flash-induced P700 redox kinetics area which was determined immediately after the O_2 measurement, as described below.

154 **Measurement of redox kinetics of P700**

Leaf segments were used for measurement of redox changes of P700 with a dual wavelength (810/870
156 nm) unit (ED-P700DW) attached to a phase amplitude modulation fluorometer (PAM 101/102/103, Walz, Effeltrich, Germany) and used in the reflectance mode (Chow and Hope 2004). To obtain redox changes
158 due to a flash superimposed on continuous far-red light, a steady-state was sought by illumination with far-red light (12 μmol photons m^{-2} s^{-1}, peak wavelength 729 nm, 102-FR, Walz, Effeltrich, Germany) for
160 ≥ 1 min. Then a single-turnover, saturating xenon flash (Walz XST 103 xenon flash, full width at half height = 9 μs) was applied to the adaxial side of the leaf disc. When necessary, the transmitted energy of
162 the flashes was lowered in steps by introducing neutral density films (Lee filters, Mediavision, Australia). Timing of the start of data acquisition (time constant = 95 μs), the triggering of the flash, and the
164 repetition rate were controlled by a pulse/delay generator (Model 555, Berkeley Nucleonics Corporation,

USA). The analogue output from the fluorometer was digitized and stored in a computer using a program written by the late A.B. Hope. Flashes were given at 0.2 Hz, and 4 consecutive signals were averaged automatically. The maximum signal immediately after the flash was taken as the total amount of photo-oxidizable P700, and used to normalize the trace (Fig. 1A). The area bounded by the trace and the horizontal line corresponding to steady state in continuous weak far-red light is here termed the P700 kinetics area (the shaded area in Fig. 1A).

Lowering the flash intensity by neutral density films produced a smaller P700 kinetics area. At the maximum flash energy, the measured relative maximum area (M_{exp}) was found to be almost identical to the extrapolated maximum area obtained by curve fitting (M_{fitted}): M_{exp} and M_{fitted} values were 94.5 and 94.1 for control leaf discs; 55.6 and 54.8 for leaf discs photoinhibited in the absence of lincomycin; and 45.7 and 45.4 for leaf discs photoinhibited in the presence of lincomycin, respectively (Fig. 1B).

The P700 kinetics area of a sample after photoinhibition pretreatment was obtained approximately 1 min after the end of high-light pre-treatment. To test the extent to which the P700 kinetics area, obtained with a *saturating*/*near-saturating* flash, is affected by energy-dependent quenching, we measured the P700 kinetics area (obtained using the maximum flash energy) as a function of dark time after cessation of actinic illumination. Control leaf discs were pre-illuminated with white LED actinic light at 1500 µmol photons m^{-2} s^{-1} for 5 min, so as to induce steady-state photosynthesis, with weak background far-red light present throughout. Photoinhibted leaf discs were taken from the photoinhibition light (30 min at 1700 µmol photons m^{-2} s^{-1}) and immediately exposed to white LED actinic light at 1500 µmol photons m^{-2} s^{-1} for only 1 min to maintain steady-state photosynthesis while minimizing further photoinactivation of PS II, weak background far-red light being present. To begin measurements, promptly after the 5-min/1-min pre-illumination, a pulse/delay generator started a new illumination with the same white actinic light for at least 39 s to re-establish steady state. At the instant

that the actinic illumination ceased (at time $t = 0$), a 1-s pulse of strong far-red light (~2000 μmol photons m^{-2} s^{-1}, applied to the abaxial side from below) helped to quickly bring the concentration of $P700^+$ towards the steady state level corresponding to the weak far-red light alone. At a selected time t ($\geq$ 3 s) after cessation of actinic illumination in near darkness (the weak far-red light being on), data acquisition was started by the pulse-delay generator; then at time (t + 0.05) s, the xenon flash was triggered, and the P700 kinetics curve recorded to yield the P700 kinetics area. In this way, the 'dark' time t was varied from 3 s to 60 s, during which energy-dependent quenching was expected to relax gradually. Fig. 1C shows that at t = 60 s, the kinetics area was within ~2% of the maximum that was obtained in the presence of weak far-red light and before any actinic illumination, whether a sample was a control (squares) or one that had been photoinhibited in the presence of lincomycin (triangles).

Determination of rate coefficients of photoinactivation and repair using a simple P700 kinetics area as a measure of the functional PS II population in the whole tissue

The maximum P700 kinetic area of a control sample at flash saturation is taken to represent the fraction f = 1 of functional PS II. The exponential decrease (Kou et al. 2012) of f from the value 1 during the onset of photoinactivation of PS II in the absence of repair (i.e., in the presence of lincomycin) gives the rate coefficient of photoinactivation k_i at a given irradiance. That is, at any time t the rate of photoinactivation PS II is

$$df/dt = -k_i f \qquad (1)$$

During recovery from photoinactivation at a given irradiance, both photoinactivation and repair occur simultaneously. The rate of repair is directly proportional to the fraction of non-functional PS II, $(1 - f)$. During recovery at a given irradiance, the net rate of increase in f is the algebraic sum of the two rates:

$$df/dt = k_r (1 - f) - k_i f \qquad (2)$$

When recovery occurs from time $t = 0$ at $f = f_0$ (typically ~0.5), the solution of the above equation is (He

and Chow 2003):

$$f(t) = \left(f_0 - \frac{k_r}{k_i + k_r} \right) e^{-(k_i + k_r)t} + \frac{k_r}{k_i + k_r} \quad (3)$$

This equation was used to fit the recovery data points using the software Origin 7 (Microcal Software Inc, Northhampton, MA, USA), allowing k_i and k_r to vary from initial estimates until stable values are obtained after a number of iterations.

Measurement of carbon assimilation rates

Gas-exchange measurements were determined in the afternoon with main-stem leaves using a portable open-circuit infra-red gas analyzer (LI6400, LI-COR, Lincoln, USA), and a normal 2 cm × 3 cm chamber with a 6400-02B (LI-COR) light emitting diode light source was used. Leaves attached to cotton plants were first kept at 2000 µmol m^{-2} s^{-1} for at least 30 min; thereafter, the irradiance was decreased in a stepwise manner. Leaf temperature and chamber CO_2 concentration were kept at 25°C and 400 µmol mol^{-1} (supplied from a CO_2 cartridge), respectively.

Measurement of electron transport rates

The total electron transport rate through PS I (ETR1) was obtained as the product Y(I) × irradiance × 0.85 × 0.5, where Y(I) is the photochemical yield of PS I. The rate of linear electron flow through PS II was determined as the product $\phi_{PS\,II}$ × irradiance × 0.85 × 0.5 Both Y(I) and $\phi_{PS\,II}$ were determined using a Dual-PAM (Walz, Effeltrich, Germany), as described by Miyake et al. (2005) and Klughammer and Schreiber (2007), assuming that the leaf absorptance was 0.85 and equal partitioning of absorbed light energy between the two photosystems.

Results

Linear correlation of P700 kinetics area with the O_2 yield per single-turnover flash

PSII functionality was monitored in leaf discs after photoinactivation in the presence of lincomycin, an inhibitor of repair that depends on chloroplast-encoded protein synthesis. The number of functional PSII complexes in leaf segments was quantified by the oxygen yield per flash, followed by measurement of the flash-induced P700 kinetics area. Fig. 2 shows the correlation between P700 kinetics area and the oxygen yield per flash for cotton. The data points are scattered on both sides of a straight line through the origin (0, 0) and the point (100, 100), showing a one-to-one empirical relationship between the two parameters. That there is a one-to-one correlation is not surprising: both parameters are whole-tissue values, one measuring the release of electrons from the splitting of water molecules in PS II, and the other measuring the cumulative delivery of electrons from PS II to $P700^{+}$.

Recovery of PS II in cotton in darkness from photoinactivation is largely inhibited by lincomycin

Given that the P700 kinetics area is a rapid measurement, it offers the possibility of monitoring the functionality of PS II at various times after a photoinactivation treatment. In the absence of lincomycin, the P700 kinetics area in darkness increased gradually from the cessation of photoinactivation light treatment (Fig. 3, open circles). When cotton leaf discs were pre-infiltrated with lincomycin before photoinactivation light treatment, on the other hand, there was only a little increase in P700 kinetics area in darkness after the light treatment (Fig. 3, closed squares). When infiltration with lincomycin was done *after* the photoinactivation light treatment, substantial recovery occurred initially while the inhibitor was not yet fully effective (Fig. 3, closed circles). For all subsequently measurements of the P700 kinetics area, in a compromise between minimizing repair in the dark and minimizing energy-dependent quenching, we chose to make the measurements at $t \approx 1$ min after a photoinactivation treatment.

Photoinactivation of PS II and recovery in the light while the adaxial side of leaf discs faced air

We investigated the time course of photoinactivation of PS II in the absence of repair by floating leaf discs with the abaxial side (richer in stomates) in contact with a lincomycin solution and the adaxial side

facing air, while illuminating the adaxial side at an irradiance of 1300 μmol m^{-2} s^{-1}. This is an orientation that substantially restricts the diffusion of CO_2 into the leaf tissue. Fig. 4A depicts the negative exponential time courses in the presence of lincomycin. That is, the functional PS II content decreased with first-order kinetics (Tyystjärvi and Aro 1996; Kou et al. 2012).

Fig. 4B shows the recovery of PS II from a photoinactivation pre-treatment (in the absence of lincomycin) which had decreased f to approximately 0.5. The recovery took place at 25°C and at various irradiances, including darkness, with the adaxial side of leaf discs facing air and the abaxial side in contact with water. During recovery in the absence of lincomycin, both photoinactivation and repair occur simultaneously, and the time course of changes in the P700 kinetics area represents the net sum of photoinactivation and repair. In darkness, there was clear recovery (closed squares, Fig. 4B). Net recovery was near optimal at low irradiance, e.g. 27 μmol m^{-2} s^{-1}. At high irradiance, however, net loss of P700 kinetics area (functional PS II) was observed during the 'recovery' phase. By fitting curves to the data points according to equation (3) using the k_i values from Fig. 4A, we obtained the fitted curves plotted in Fig. 4B as well as the k_r values (see below).

Photoinactivation of PS II and recovery in the light while the abaxial side of leaf discs faced air

We next investigated the time course of photoinactivation of PS II in the absence of repair by floating leaf discs on a lincomycin solution with the abaxial side facing air, while illuminating the adaxial side at an irradiance of 1300 μmol m^{-2} s^{-1}, with the light directed upwards at the adaxial side. This orientation allowed easier diffusion of CO_2 into the leaf tissue. Fig. 5A depicts the negative exponential time courses of photoinactivation in the presence of lincomycin. That is, the functional PS II content in leaf discs in this orientation also decreased with first-order kinetics, as observed in the previous orientation.

Fig. 5B shows the recovery of PS II from a photoinactivation pre-treatment (in the absence of lincomycin) which had decreased f to approximately 0.5. The recovery took place at 25°C and at various irradiances, including darkness, with the abaxial side of leaf discs facing air and the adaxial side

in contact with water. In darkness, there was again clear recovery in leaf discs in this orientation. Net recovery was near optimal at low irradiance, e.g. 30 μmol m^{-2} s^{-1}. At high irradiance, recovery occurred to a lesser extent, but no net loss of P700 kinetics area was observed. Fitting curves to the data points according to equation (3) using the k_i values from Fig. 5A, we obtained the fitted curves plotted in Fig. 5B as well as the k_r values (see below).

Variation of k_i and k_r with irradiance

The rate coefficient of photoinactivation k_i increased linearly with irradiance. At a given irradiance, k_i was marginally smaller when the adaxial side of leaf discs was in contact with a lincomycin solution, while the abaxial side faced air (Fig. 6A), thereby allowing easier diffusion of CO_2 into leaf tissue.

The variation of k_r with irradiance was more complex. In darkness, k_r was about 0.16 h^{-1} (non-zero) in both orientations of leaf discs (Fig. 6B). When the abaxial side faced air and the adaxial side faced water, k_r increased rapidly at low irradiance; it then remained on a plateau until the irradiance exceeded 600 μmol m^{-2} s^{-1}. Above 600 μmol m^{-2} s^{-1}, it again increased substantially, reaching 1.6 h^{-1}. When the adaxial side faced air and the abaxial side was in contact with water, k_r increased to a peak at about 350 μmol m^{-2} s^{-1} but declined at higher irradiances (Fig. 6B). At the highest irradiance, k_r differed by an order of magnitude between the two orientations.

Response of rates of carbon assimilation and electron transport to irradiance

The net rate of carbon assimilation of cotton leaves was determined as function of irradiance (Fig. 7). The most rapid increase in the observed carbon assimilation rate occurred over the irradiance interval 0-600 μmol m^{-2} s^{-1}. The total rate of electron transport through PS I (ETR1) assayed by the P700 signal, and the rate of linear electron transport through PS II (ETR2) assayed by Chl fluorescence are depicted in Fig. 8. From moderate to high light, each rate was higher when the abaxial side faced air, allowing better diffusion of CO_2 into the leaf tissue.

306 **Discussion**

The P700 kinetics area represents electrons delivered from PS II to $P700^+$ in the whole leaf tissue

308 The P700 kinetics area, indicated as a shaded area in Fig. 1A, decreased during progressive photoinactivation pre-treatment of PS II. Using the Walz single-turnover flash at maximum intensity, we 310 obtained an area that was practically identical to the extrapolated maximum obtained by curve fitting (Fig. 1B); that is, the flash was saturating for leaf discs which were in a 'relaxed' state in the presence of weak 312 far-red light alone. This maximum corresponded to the capacity for flash-induced delivery of electrons from the functional PS II complexes to $P700^+$.

314 However, the possibility of increased energy-dependent quenching persisting after high-light treatment of leaf discs, reducing delivery of excitation to the PS II reaction centre, and making the flash 316 less than saturating, could not be excluded. Indeed, lincomycin treatment of leaves under high light has the potential to lower the quantum efficiency of PS II by slowing the relaxation of non-photochemical 318 quenching (Bachmann et al. 2004). Nevertheless, as our measurements were made approximately 1 min after cessation of light treatment for photoinactivation or recovery, it appears that energy-dependent 320 quenching seemed to have diminished sufficiently for the Walz flash to be saturating (Fig. 1C). The results mean that this technique is usable at 1 min or longer after cessation of a light treatment; to employ 322 a shorter dark time would require a stronger flash to ensure saturation in the presence of strong energy-dependent quenching.

324 The P700 kinetics area was directly proportional to the oxygen yield per single-turnover flash in repetitive-flash illumination of cotton leaf discs (Fig. 2). Spinach and capsicum also followed a single 326 one-to-one relation (Kou et al. 2012). This simple, empirical correlation forms the basis on which a rapid relative assay of functional PS II content is conducted.

That there is a one-to-one linear correlation between the two parameters is not surprising. Both are whole-tissue measurements: the measuring beam at 810 nm that reports the $P700^+$ signal is multiply scattered in the leaf tissue until it is eventually absorbed by $P700^+$, while oxygen is evolved from throughout the tissue on excitation of PS II with single-turnover, saturating flashes. Indeed, the P700 kinetics area of a photoinactivated sample relative to that of a control is similar, whether measured from the adaxial or the abaxial side of photoinhibited leaves (Fig. 3 in Oguchi et al. 2011b). This superior feature of the $P700^+$ signal contrasts with the variably localized detection of the Chl fluorescence signal (Terashima et al. 2009). Further, the depth of Chl fluorescence signal detection may well vary during the time courses of photoinactivation of PS II and recovery as the contribution to the fluorescence intensity from a particular depth varies.

It should be borne in mind, however, that the method presented here requires that the maximum photo-oxidizable P700 signal did not decline after high-light treatment. Under most circumstances at favourable temperatures, this is the case, as PS I is normally well protected against photoinhibitory damage at normal temperatures. Indeed, photoinactivation of PS II in cotton leaf discs in either the presence or absence of lincomycin in the present study did not significantly decrease the maximum photo-oxidizable P700 signal induced by a flash superimposed on background far-red light (data not shown), indicating no photodamage to PS I. Another assumption is that the two photosystems operate in series, such that all electrons originating from water splitting in PS II arrive at $P700^+$. This measurement may be reasonable in leaf tissue under weak far-red light to which a flash is added.

Recovery of PS II in darkness

In this whole-tissue assay of functional PS II in cotton, slow but steady recovery of PS II from photoinactivation was observed in darkness (Figs. 3, 4B and 5B). Perhaps after illumination with strong light, mitochondria supplied the ATP needed for biochemical reactions required to replace photodamaged D1 protein by newly synthesized D1 protein (Mattoo et al. 1984; Taniguchi et al. 1993) or for the uptake

of cytoplasmically synthesized polypeptides into the chloroplast (Grossman et al. 1980). Indeed, recovery could be partially induced by floating photoinhibited leaf segments on a 50 mM solution of ATP in the dark (H.-Y. Lee and W.S. Chow, unpublished).

In any case, recovery of PS II in cotton leaf discs in the dark was largely inhibited by lincomycin, provided sufficient time was allowed for the uptake of lincomycin into the chloroplast (Fig. 3), demonstrating that by far the major part of the recovery was sensitive to an inhibitor of chloroplast-encoded protein synthesis. A small residual recovery in darkness could be seen in cotton in the presence of lincomycin, amounting to about 3% of the total population of PS II, functional or non-functional. In spinach, bean and maize, the lincomycin-insensitive recovery in 5 h darkness was larger than in cotton, being 12%, 20% and 25% of the total PS II population, respectively (data not shown). This residual recovery could represent (1) the reversible inactivation of PS II that is unrelated to D1 protein synthesis (Hong and Xu 1999), (2) a readily-available pool of D1 protein not yet incorporated into PS II in thylakoids (Wettern 1986) and/or (3) the inability of lincomycin to reach all D1 synthesis sites. This lincomycin-insensitive recovery component was reported for low light conditions, using dark-relaxed F_v/F_m to assay photodamage (Aro et al. 1993). Our measurement of the P700 kinetics area was made at approximately 1 min after the cessation of recovery-light treatment, not long enough to allow any substantial recovery in darkness, but long enough to allow energy-dependent quenching to relax to such an extent that the flash was saturating.

The rate coefficient of photoinactivation k_i

The rate coefficient of photoinactivation k_i was directly proportional to irradiance, as previously reported (Tyystjärvi and Aro 1996; Lee et al. 2001; Kato et al. 2003). Its value at a given irradiance was slightly different between the two orientations of floating leaf discs on a lincomycin solution during illumination on the adaxial side (Fig. 6A). Leaf discs floated with their abaxial side facing air had a smaller k_i, consistent with better CO_2 diffusion into leaf tissue and less excess light energy leading to one of the dual

376 mechanisms of photoinactivation (Oguchi et al. 2009, 2011a, 2011b); indeed, leaf discs floated in this orientation exhibited a (24-33%) higher rate of electron transport at saturating irradiance, assayed either
378 as linear electron flow through PS II via Chl fluorescence or as the total electron flux through PS I *via* P700 redox measurement (Fig. 8).

380 **The rate coefficient of repair k_r**

By far, the greatest difference between the two orientations resided in the rate coefficient of recovery k_r
382 (Fig. 6B). In leaf discs floated with their abaxial side facing water, k_r increased from 0.16 h^{-1} in darkness to a peak of about 0.55 h^{-1} at an irradiance of about 350 µmol m^{-2} s^{-1}; thereafter, it declined at
384 high irradiance, reaching at the highest irradiance a value similar to that in darkness. This rise-and-fall behaviour of cotton is qualitatively similar to that observed in *Capsicum annuum* using Chl fluorescence
386 when leaf discs were also floated with their abaxial side in contact with water while the adaxial side faced air (He and Chow 2003). He and Chow (2003) invoked the suggestion of Nishiyama et al. (2001, 2011)
388 to explain the surprisingly low k_r at high irradiance, just when repair of is most needed: the restriction of CO_2 entry into leaf tissue may lead to O_2 playing a more prominent role as an electron acceptor, with the
390 consequent enhancement of oxidative stress which impaired repair (Nishiyama et al. 2001, 2011).

When leaf discs were floated with their abaxial side facing air, k_r was between 0.55 and 0.75
392 h^{-1} in the irradiance range 30-611 µmol m^{-2} s^{-1}. It was only at much higher irradiance that k_r increased again (Fig. 6B). The highest k_r observed at the highest irradiance was 1.6 h^{-1}. Considering gross
394 recovery only, without taking into account the concomitant photoinactivation (i.e., setting k_i = 0), equation (3) becomes:

396 $$f(t) = (f_0 - 1)e^{-k_r t} + 1$$

At time $t_{1/2}$, when half of the non-functional PS II complexes have recovered function,

398 $$\frac{1 - f(t_{1/2})}{1 - f_0} = e^{-k_r t_{1/2}} = 0.5$$

For k_r = 1.6 h^{-1}, we obtain $t_{1/2}$ = 26 min. That is, half of the non-functional PS II complexes would have recovered function in 26 min if concurrent photoinactivation had not occurred. In the other orientation of leaf discs, however, k_r was an order of magnitude smaller at the highest irradiance, and the $t_{1/2}$ correspondingly longer.

It is not clear why k_r, while remaining at moderately high, did not increase over the irradiance interval 30-600 μmol m^{-2} s^{-1} when leaf discs were floated with their adaxial side in contact with water. One possible reason is that much of the increase in carbon assimilation rate in a leaf attached to the plant occurred over this irradiance range (Fig. 7). Further, under conditions of recovery, the electron transport rate was saturated at about 600 μmol m^{-2} s^{-1} or lower, depending on the orientation of leaf discs floating on water (Fig. 8). Perhaps until light saturation, carbon assimilation out-competed repair of PS II for the available ATP.

A relatively small difference was observed between the two orientations of leaf discs in terms of the rate of electron transport, assayed as either the total electron flux through PS I (ETR1, 24%) at the highest irradiance or as linear electron flow through PS II via Chl fluorescence (ETR2, 27%) (Fig. 8). It is consistent with a slightly (27%) lower stomatal density on the adaxial side compared with the abaxial side (determined for another cultivar grown in the field by Hu et al. 2013). For such a small difference in stomatal density, it is surprising that the difference in k_r between the two orientations was an order of magnitude (Fig. 6B). A possible explanation is that when the abaxial side faced air, CO_2 diffusion into the leaf tissue was able to meet photosynthetic demand, and the production of reactive oxygen species (ROS) was still below the capacity of the scavenging enzymes to detoxify the ROS. On the other hand, when the abaxial side was in contact with water, oxidative stress could have exceeded the capacity of the scavenging enzymes, such that during illumination at high irradiance the repair mechanism was impaired by oxidative stress (Nishiyama et al. 2001, 2011).

Conclusions

Using the P700 kinetics area as a convenient and whole-tissue measure of functional PS II, we obtained the rate coefficients of photoinactivation and repair of PS II for cotton leaf segments. In darkness, the repair rate coefficient of cotton was substantial, perhaps due to energy storage during high-light illumination of cotton leaf discs, and was lincomycin-sensitive. At the highest irradiance, the difference in the repair rate coefficient, between two orientations in which leaf discs were floated on water, was an order of magnitude, probably reflecting whether oxidative stress exceeded the capacity for detoxifying ROS or not.

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Figure Legends

Fig. 1. (A) The P700 kinetics area (shaded) used to assay functional PS II. A cotton leaf disc was continuously illuminated by weak far-red light, resulting in the photo-oxidation of almost 90% of the total photo-oxidizable P700. A single-turnover, saturating flash superimposed on the background far-red light photo-oxidized the remainder of the P700, giving the spike (set to 1.0). Subsequent to the flash, electrons arrived from PS II to $P700^+$, but the background far-red light brought the [$P700^+$] back to the steady-state level. The trace is an average of four scans. (B) The P700 kinetics area plotted as a function of relative flash intensity (I) as varied by neutral density filters. Leaf discs were either control, non-photoinactivated samples or leaf discs that had been photoinactivated (PI) for 6 h, in the presence or absence of lincomycin. Each data set was fitted by an equation of the form $y = M_{fitted}\ (1 - e^{-kI})$, yielding both M_{fitted} and k after a number of iterations, where the M_{fitted} values are indicated by the horizontal dashed lines. The experimental maximum, M_{exp}, at 100% of flash intensity was close to the M_{fitted} value in each case. Each point is a mean of 4 replicates ± se. (C) The P700 kinetics area, measured at maximum flash energy, as a function of dark time after cessation of actinic illumination (1500 µmol m^{-2} s^{-1} for 5 min). The control samples (squares) were exposed to actinic light to steady state. The photoinhibited samples (triangles) were obtained by pre-illumination with white light at 1700 µmol m^{-2} s^{-1} for 30 min in the presence of lincomycin;

564 immediately after, they were given actinic illumination (1500 μmol m^{-2} s^{-1}) for 1 min to maintain steady state prior to measurement. The P700 signals were normalized to the maximum
566 photo-oxidizable P700 obtained after a dark time of 60 s. Each point is a mean of 4 replicates ± se. The P700 kinetics areas for samples without actinic illumination are indicated by the horizontal
568 dashed lines.

Fig. 2. The P700 kinetics area is linearly correlated with the O_2 yield per repetitive, single-turnover
570 saturating flash. Functional PS II content was varied by progressive photoinactivation of PS II in leaf discs in the presence of lincomycin. Measurements were made after the samples had been
572 dark-treated for about 30 min following removal from the high light.

Fig. 3. Recovery of the P700 kinetics area in darkness following photoinactivation treatment at 1800
574 μmol m^{-2} s^{-1} for 72 min at 15 °C, which approximately halved the functional PS II complexes. Recovery took place (1) in the absence of lincomycin (open circles), (2) in leaf discs that took up
576 lincomycin overnight prior to photoinactivation treatment (solid squares), or (3) in leaf discs infiltrated with lincomycin *after* the photoinactivation treatment (closed circles). Each point is a
578 mean of 4 replicates ± se.

Fig. 4. Photoinactivation and recovery of PS II while cotton leaf discs were floated with their abaxial
580 side in contact with water and the adaxial side facing air. Illumination was directed at the adaxial side. The irradiance during recovery was varied from zero to 1300 μmol m^{-2} s^{-1} (μE for short). (A)
582 The time course of photoinactivation of PS II in cotton leaf discs in the presence of lincomycin at various irradiances, including darkness. (B) The time course of recovery of PS II from
584 photoinactivation in the absence of lincomycin. The irradiance during recovery was varied from zero to 1300 μmol m^{-2} s^{-1}. Cotton leaf discs had been given a photoinactivation treatment to render
586 about half of the PS II complexes inactive before recovery was allowed to occur. Each point is a

mean of 4 replicates ± se.

588 **Fig. 5.** Photoinactivation and recovery of PS II while cotton leaf discs were floated with their adaxial side in contact with water and the abaxial side facing air. Illumination was directed at the adaxial 590 side. The irradiance during recovery was varied from zero to 1300 μmol m^{-2} s^{-1}. (A) The time course of photoinactivation of PS II in cotton leaf discs in the presence of lincomycin at various 592 irrdiances, including darkness. (B) The time course of recovery of PS II from photoinactivation in the absence of lincomycin. Cotton leaf discs had been given a photoinactivation treatment to render 594 about half of the PS II complexes inactive before recovery was allowed to occur.

Fig. 6. Rate coefficients of photoinactivation (A) and repair (B) as a function of irradiance. Open 596 circles represent cotton leaf discs floated with the adaxial side facing air while the abaxial side was in contact with a 1 mM lincomycin solution in the determination of k_i in (A) or with water in the 598 determination of k_r in (B) during recovery. Closed circles represent cotton leaf discs floated with the abaxial side facing air while the adaxial side was in contact with a 1 mM lincomycin solution in the 600 determination of k_i or with water in the determination of k_r in (B) during recovery. The k_i and k_r values were derived from the curve fitting in Figs. 4 and 5. Each point is a mean of 4 replicates.

602 **Fig. 7.** Response of net carbon assimilation rate (P_n) to irradiance. Each point is a mean of n = 2 replicates ± se.

604 **Fig. 8.** Total electron transport rate through PS I (ETR1) and the linear electron transport rate through PS II (ETR2) as a function of irradiance. ETR1 and ETR2 were determined using the P700 signal 606 and Chl fluorescence, respectively, for two orientations of cotton leaf discs: (1) the abaxial side faced air while the adaxial side was in contact with water; (2) the adaxial side faced air while the abaxial 608 side was in contact with water. Illumination with red LED light was provided on the adaxial side

only, by using an RG9 filter to block the actinic light that normally is supplied along with the 810/870 nm measuring light by the Dual-PAM, leaving only the actinic light that is supplied along with the Chl fluorescence excitation light. Each point is a mean of 4 replicates ± se.

Line figure

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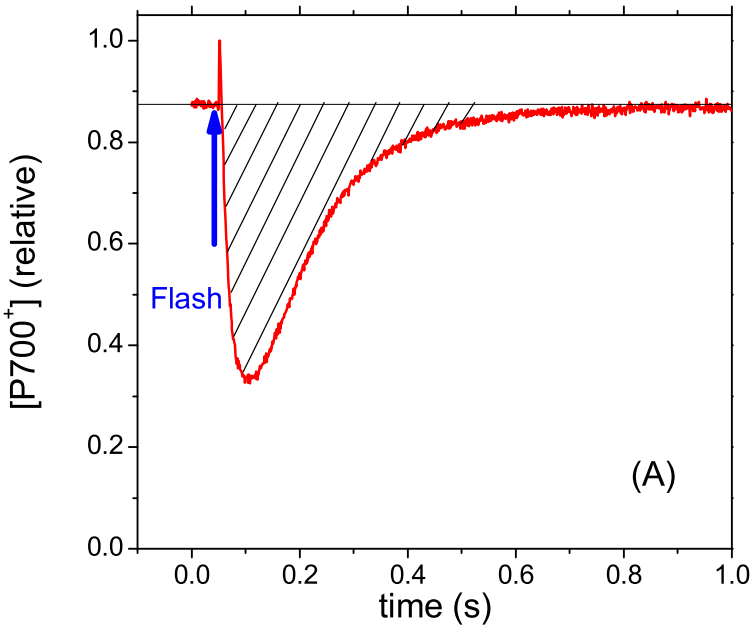

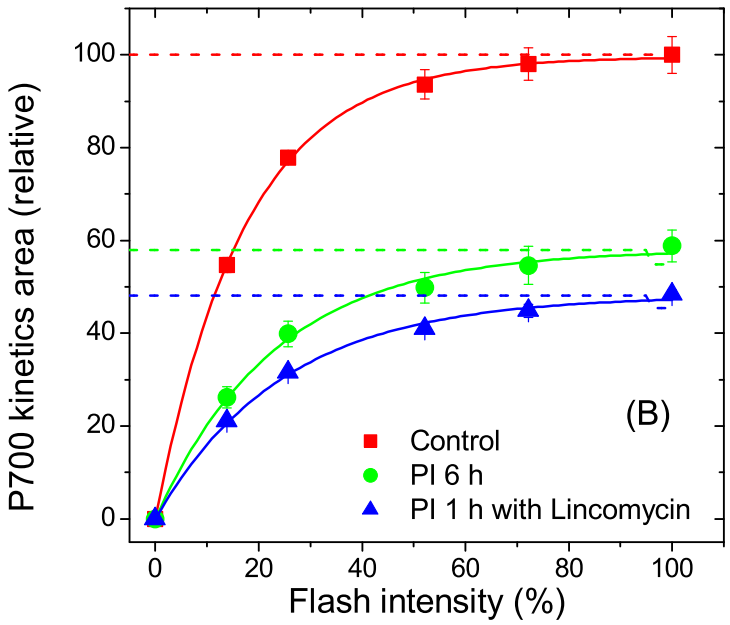

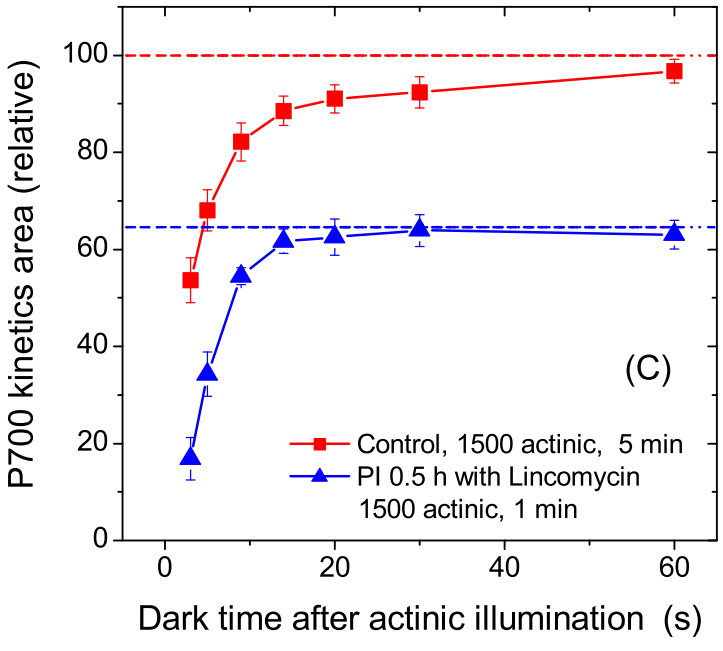

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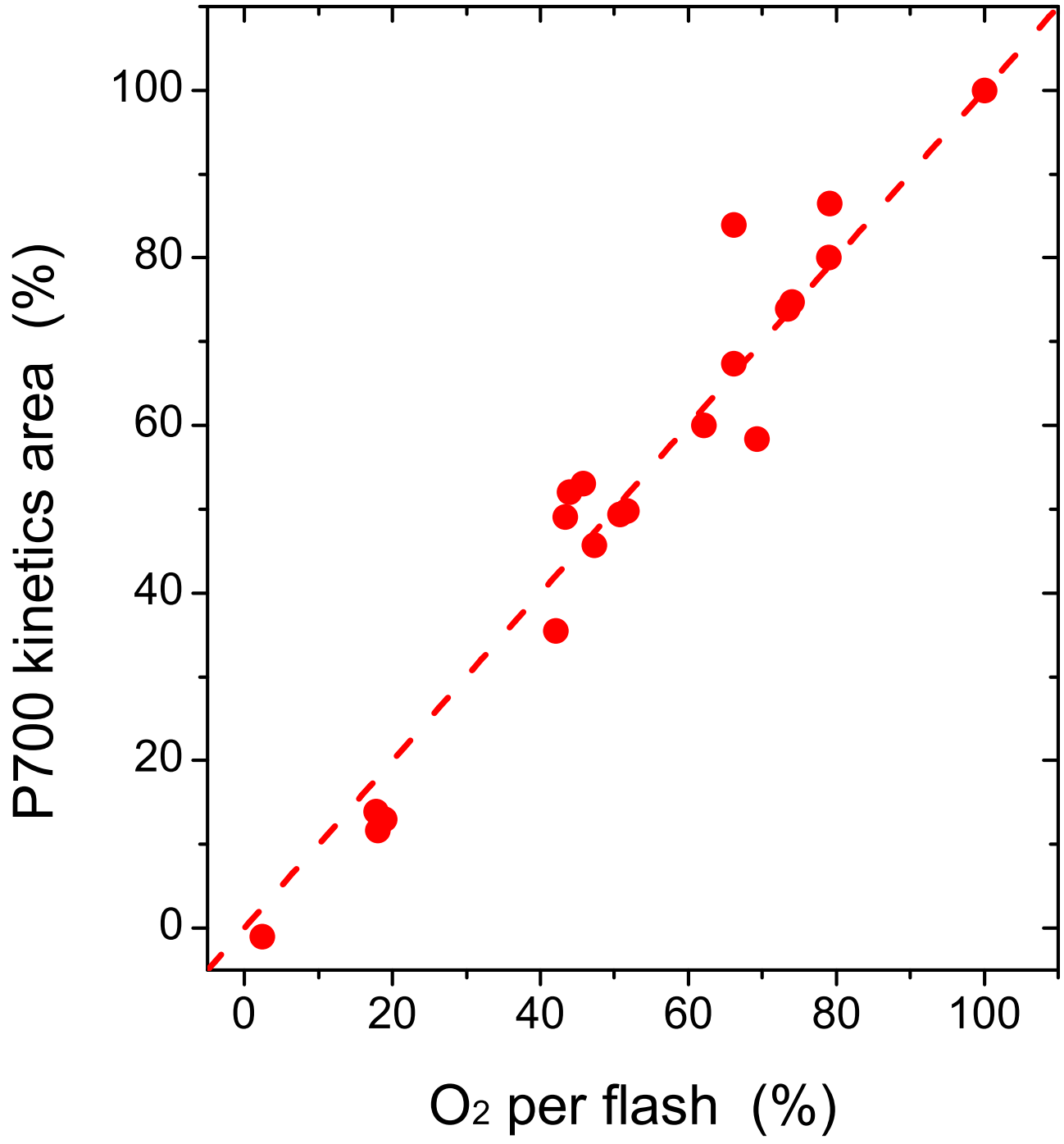

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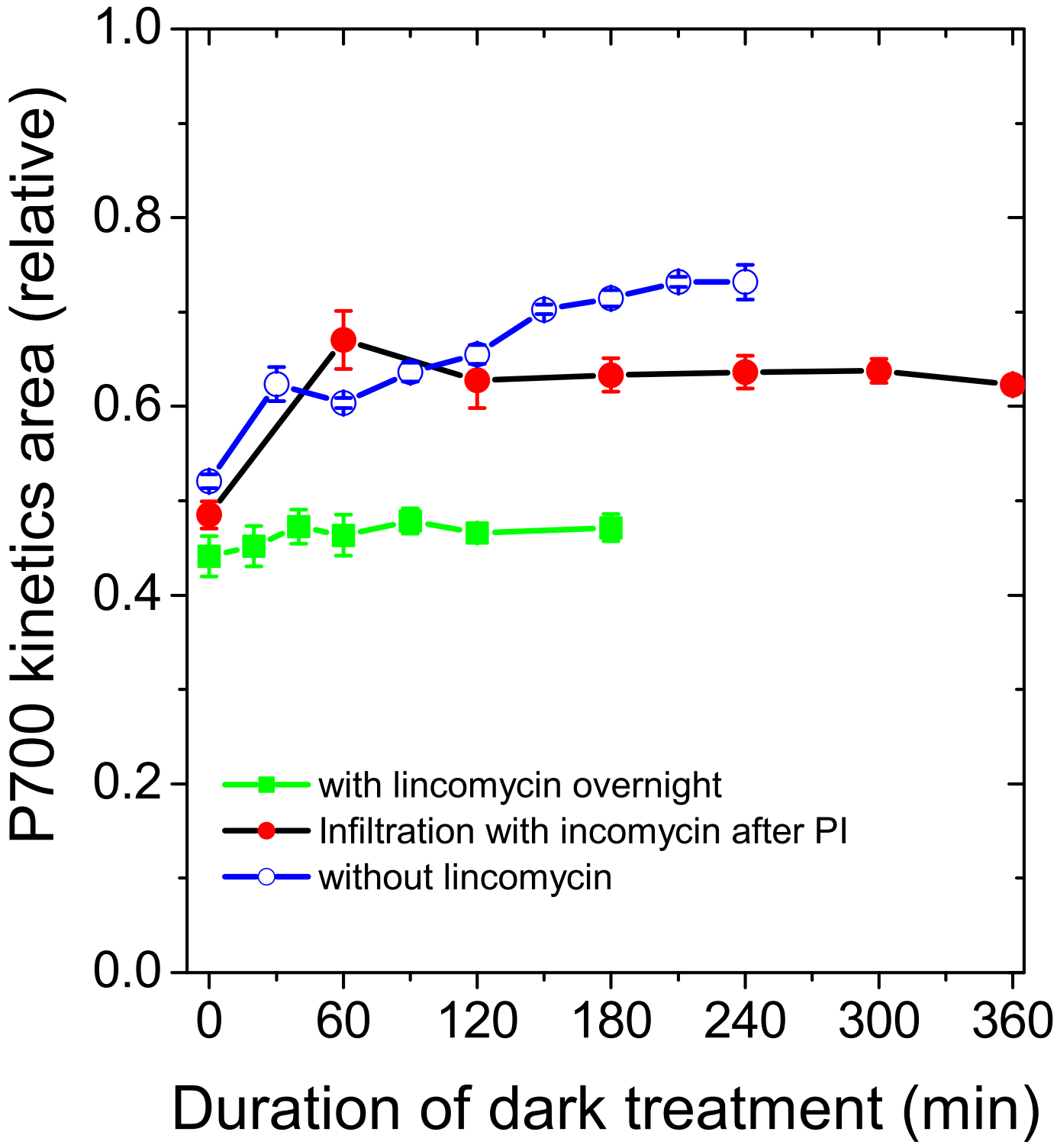

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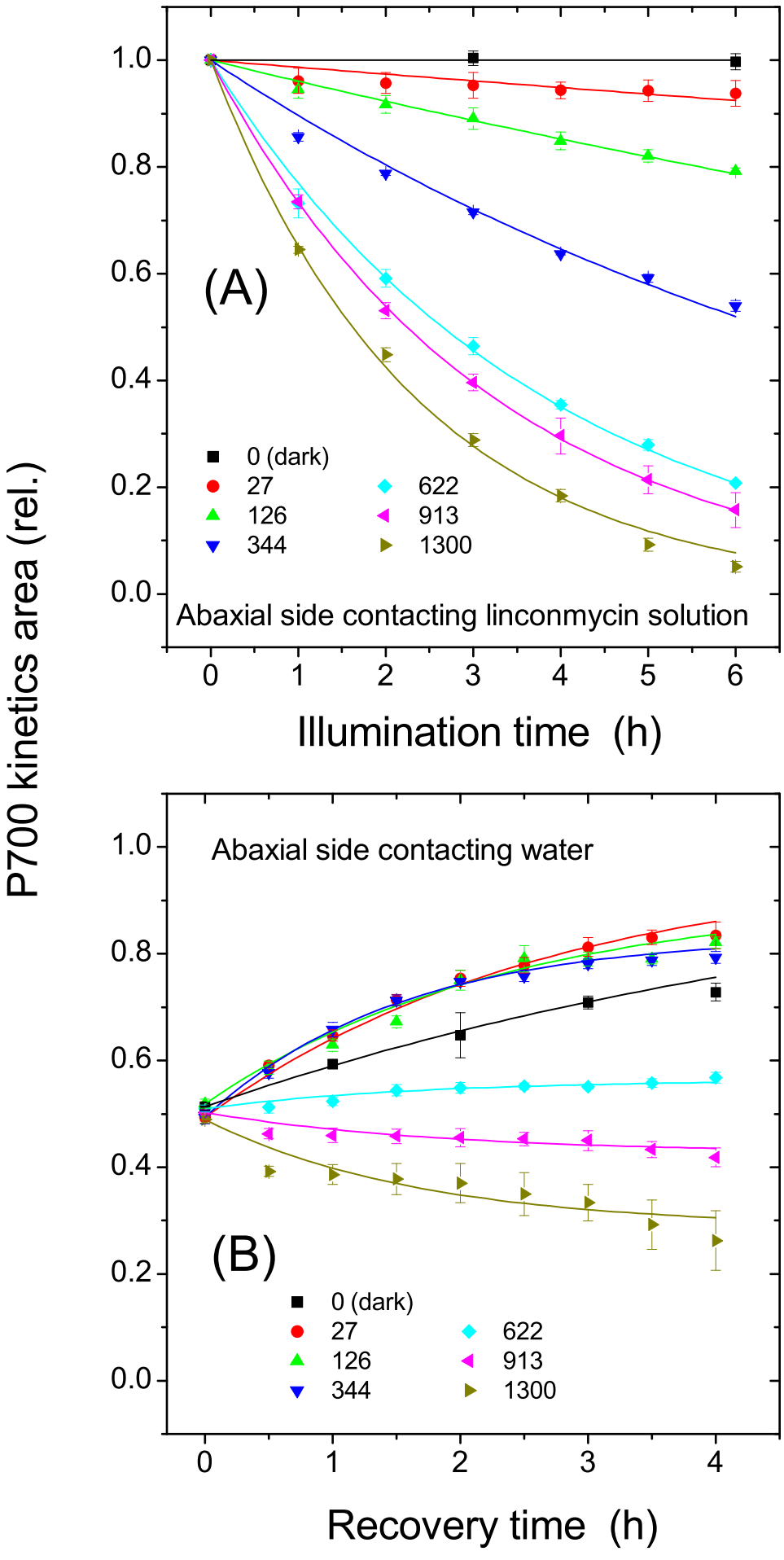

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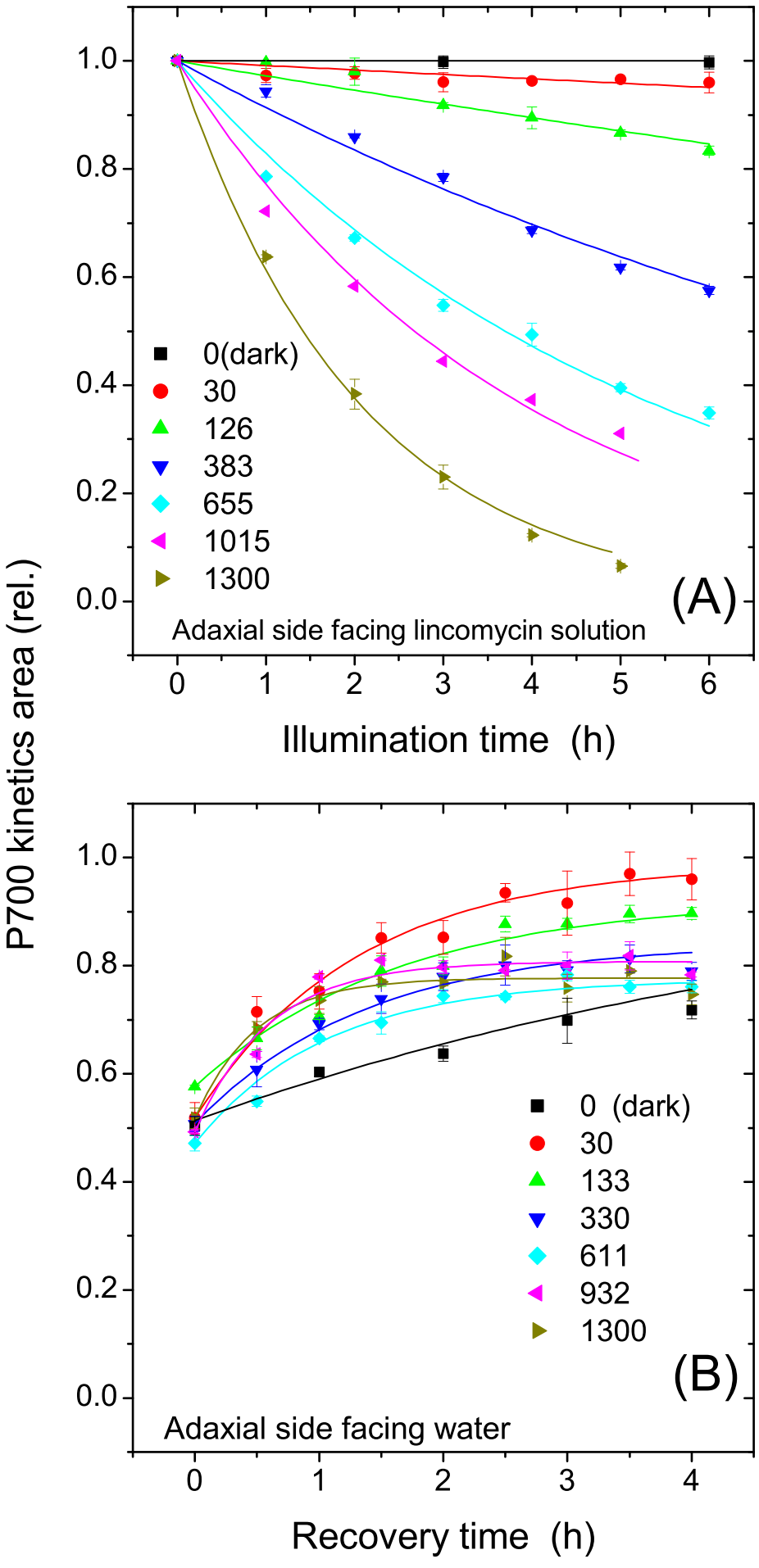

Line figure
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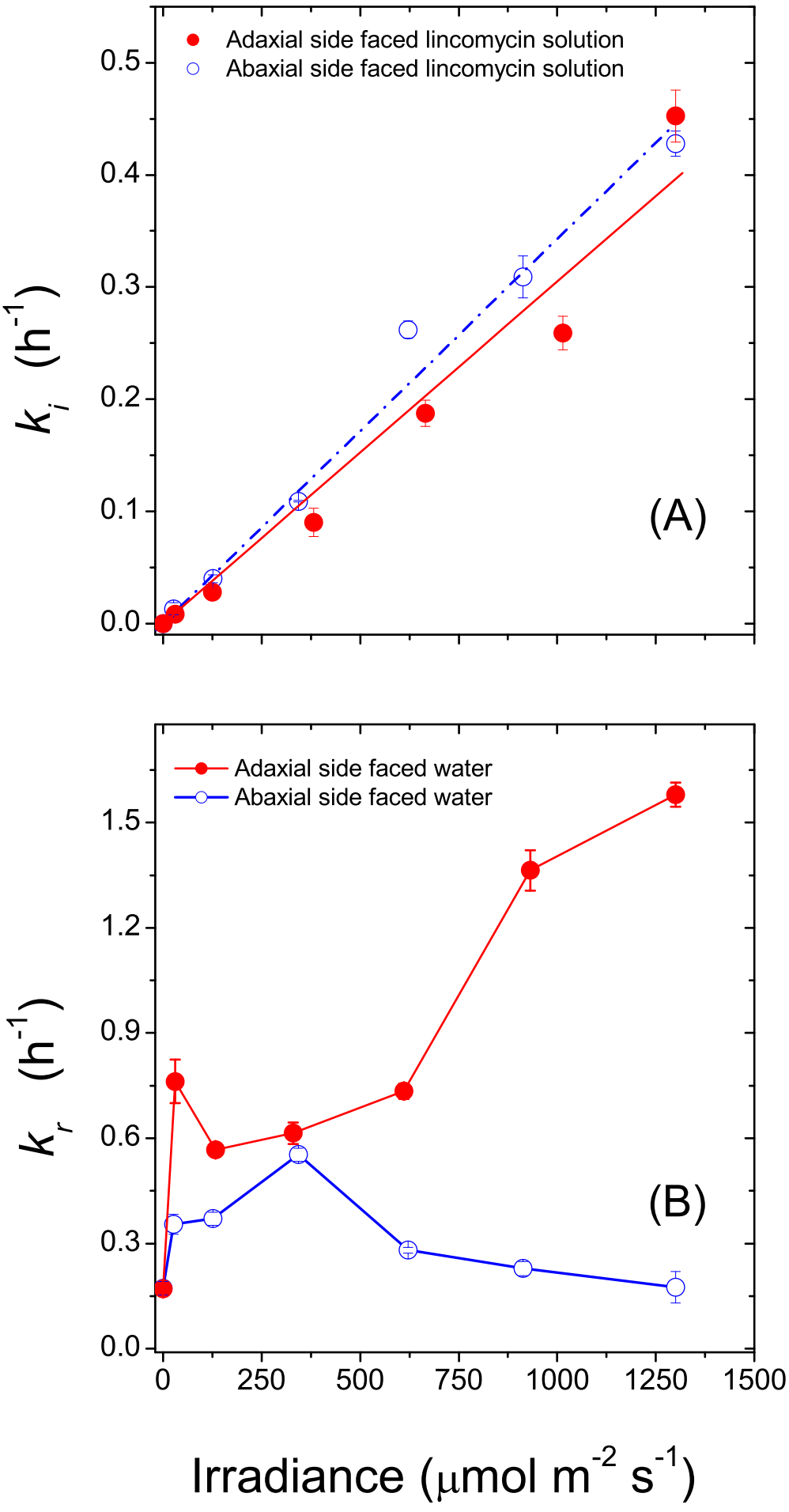

Line figure
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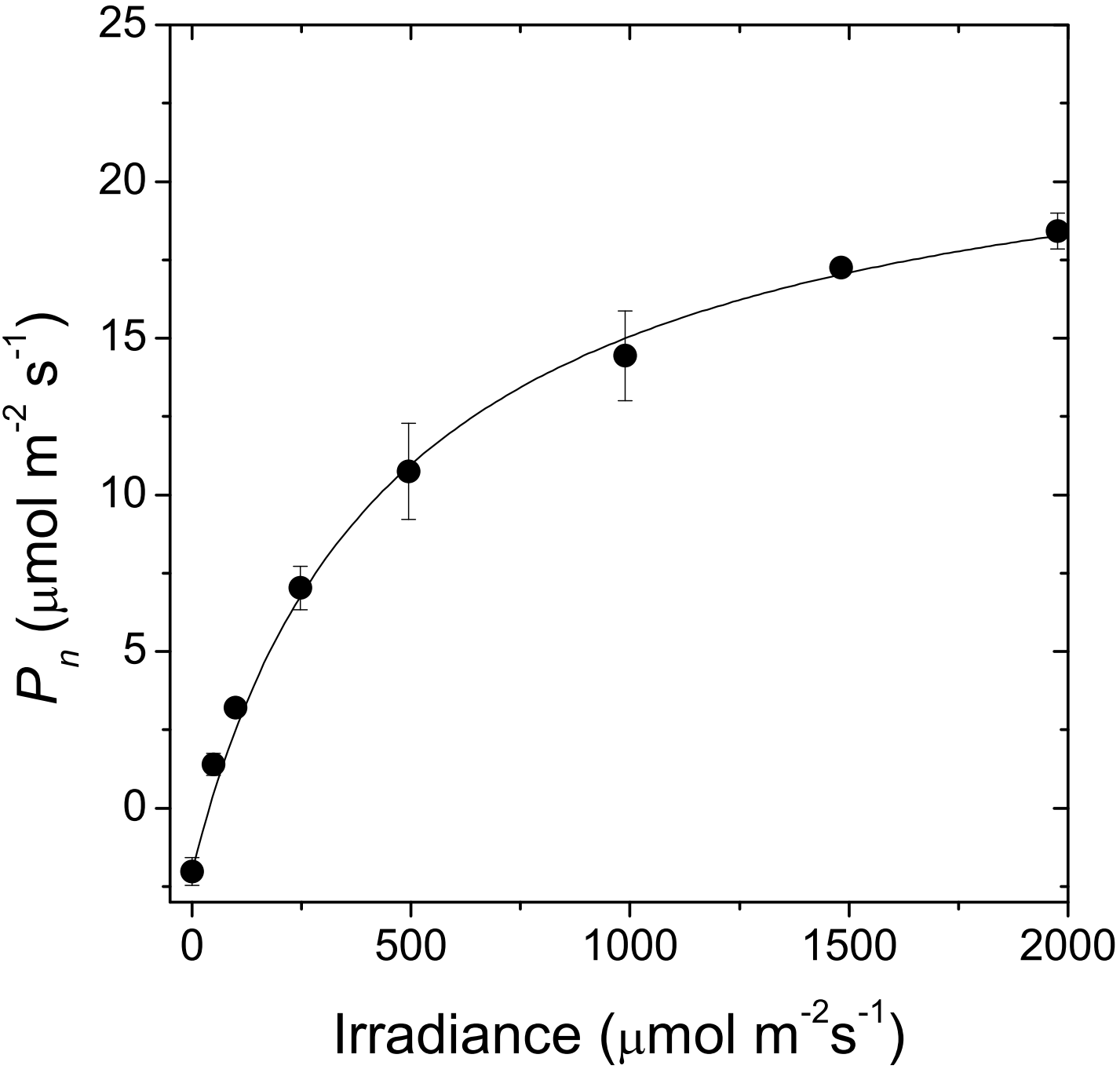

Line figure
Click here to download Line figure: Fig 8.eps