

The energy cost of repairing photoinactivated photosystem II: an experimental determination in cotton leaf discs

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Summary

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- Photosystem II (PSII), which splits water molecules at minimal excess photochemical potential, is inevitably photoinactivated during photosynthesis, resulting in compromised photosynthetic efficiency unless it is repaired. The energy cost of PSII repair is currently uncertain, despite attempts to calculate it.
- We experimentally determined the energy cost of repairing each photoinactivated PSII in cotton (*Gossypium hirsutum*) leaves, which are capable of repairing PSII in darkness.
- As an upper limit, 24 000 adenosine triphosphate (ATP) molecules (including any guanosine triphosphate synthesized at the expense of ATP) were required to repair one entire PSII complex. Further, over a 7-h illumination period at 526–1953 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the ATP requirement for PSII repair was on average up to 4.6% of the ATP required for the gross carbon assimilation.
- Each of these two measures of ATP requirement for PSII repair is two- to three-fold greater than the respective reported calculated value. Possible additional energy sinks in the PSII repair cycle are discussed.

Introduction

The light-driven splitting of water molecules by photosystem II (PSII) and the subsequent flow of electrons to photosystem I (PSI) provide the electrons and protons that drive the formation of NADPH and adenosine triphosphate (ATP), respectively. These two products together drive photosynthetic conversion of CO₂ to carbohydrates (carbon assimilation), with oxygen released as a by-product. PSII, having to perform a highly energy-demanding reaction in biology while utilizing minimal photochemical potential (or minimal 'over-potential'), is inevitably inactivated by visible light (Barber & Andersson, 1992; van Gorkom & Schelvis, 1993; Vass, 2011). Photoinactivation of PSII typically takes place in a manner that depends on the light dose (Jones & Kok, 1966; Nagy *et al.*, 1995; Chow *et al.*, 2005). It occurs under any light environment, ranging from low light (Keren *et al.*, 1997) to saturating light (e.g. Kou *et al.*, 2012), and even occurs in widely-spaced repetitive flashes (Keren *et al.*, 1995). Regarding gross photoinactivation itself, at least two mechanisms, driven by different light-absorbers (light-harvesting

photosynthetic pigments vs the Mn₄CaO₅ cluster in PSII), are responsible for the photoinactivation of PSII (Oguchi *et al.*, 2011; Zavafer, 2021).

The entire PSII population in a leaf will be photoinactivated over the course of a sunny day if ongoing repair does not occur at the same time (Kou *et al.*, 2012). A mechanism has evolved to repair PSII by synthesis of chloroplast proteins (Greer *et al.*, 1986). Photoinactivation of PSII leads to damage of a site on the D1 protein that binds the secondary quinone electron acceptor in the PSII reaction centre. After photodamage of D1, a new D1 is synthesized to replace the damaged D1 (Mattoo & Edelman, 1987; Aro *et al.*, 1993; Baena-González & Aro, 2002). While ongoing repair of PSII maintains a population of functional PSII, any inactive PSII that may be present will absorb light but not utilize it for photochemical conversion; consequently, overall photosynthetic efficiency is compromised (Powles, 1984; Raven, 2011), though the diminished delivery of electrons from PSII to PSI helps to protect PSI against permanent photodamage (Tikkanen *et al.*, 2014).

The cycle of PSII photodamage and repair involves the coordinated regulation of degradation and synthesis of the D1 protein (Baena-González & Aro, 2002; Li *et al.*, 2018). The energy cost

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of repair of PSII following photoinactivation is estimated to be considerably greater than, for example, the ‘avoidance’ option in which leaflets fold during exposure to sunflecks with unfolding afterwards (Raven, 1989). However, it has been estimated that < 0.5% of the ATP produced in the light is used in the replacement of photodamaged D1 protein (Miyata *et al.*, 2012), which in turn provides the added benefit of retaining the photosynthesis that would otherwise have been passed up due to loss of photosynthetic efficiency (Raven, 2011).

Processes that require ATP in the repair cycle have been considered (Murata & Nishiyama, 2018): activation of FtsH protease and degradation of the D1 protein; phosphorylation of D1 protein and other proteins in the PSII core complex; transcription of the *psbA* gene that encodes the D1 protein; and synthesis of the D1 protein. Thus, it is estimated that 1304 ATP molecules are required in the turnover of one D1 polypeptide (including 1059 molecules of ATP or guanosine triphosphate (GTP, synthesized at the expense of ATP) in the translation of *psbA* messenger RNA (mRNA) and 240 ATP in the activation of FtsH); in addition, synthesis of one transcript of the *psbA* gene requires 1242 nucleoside triphosphates (including ATP and GTP). That is, a total of 2546 ATP or GTP molecules are required in the turnover of one D1 (Murata & Nishiyama, 2018). Additionally, ATP may also be required to activate heat-shock proteins involved in the degradation and synthesis of D1, or in the activation of other as-yet-unidentified macromolecules in the repair of PSII, but there is no information on the number of ATP molecules required (Murata & Nishiyama, 2018).

Another study (Miyata *et al.*, 2012) calculated the energy cost of replacing D1 to be 2827 ATP/D1, taking into account, for example, the cost of amino acid turnover which seems to be relevant in the long timescale over which repair occurs. In addition to D1, other PSII proteins are also photodamaged and need to be replaced by *de novo* synthesis. For example, the combined loss of D2 (PsbD) and CP43 (PsbC) proteins due to photodamage is comparable to that of D1 in wild-type *Arabidopsis* (Tikkanen *et al.*, 2008). Similarly, D2 protein in diatoms is lost to an extent roughly comparable to that of D1 (Wu *et al.*, 2011, 2012). These proteins other than D1 were not considered by Murata & Nishiyama (2018). However, Miyata *et al.* (2012) considered the ATP consumed in the repair of D2, CP43 and CP47 (PsbB) proteins in addition to the ATP consumption in the repair of one D1 protein (C_{D1}); they concluded that the total ATP consumption for repair of a PSII complex could be 4.1 times C_{D1} , that is, *c.* 1.7% of the total ATP produced by photophosphorylation is consumed in the repair of PSII.

Given that there may still be other factors–processes left out in calculating the energy required for repairing each photoinactivated PSII complex, there is a need to experimentally determine the overall energy cost of repairing each photoinactivated PSII, with minimal assumptions. The objectives of this study were (1) to determine the energy cost of repairing one photoinactivated PSII complex, and (2) to compare the ATP consumed for PSII repair with that used in net or gross photosynthesis during illumination for a period of 7 h at a number of selected irradiances. While higher plants do not usually exhibit repair of PSII in darkness, diatoms maintain extensive cycling

of PSII proteins in the dark (Li *et al.*, 2016). Cotton leaves are unusual among higher plant species in that they are capable of rapid PSII repair in darkness at a substantial rate; further, the repair of PSII in cotton leaves in darkness is inhibited by lincomycin (Hu *et al.*, 2013). Therefore, we took advantage of the ability of cotton leaves to repair PSII in the dark, thereby vastly simplifying the estimation of ATP molecules required in PSII repair, since the ATP requirement can be met by mitochondrial respiration alone. By contrast, attempting to experimentally quantify the ATP needed for PSII repair *in vivo* in the light would require formidable assumptions or measurement of, for example, (1) the gross rate of light-driven ATP synthesis (photophosphorylation) *in vivo* obtained from three quantities, namely the linear as well as cyclic electron transport rates, the $H^+ : e^-$ ratio and the ATP : H^+ ratio; (2) the proportion of the gross photophosphorylation rate devoted to PSII repair; and (3) quantification of the proportion of mitochondrial respiration rate in the light devoted to PSII repair. Such assumptions or approximations would compound the uncertainty in the final estimate of the ATP required for PSII repair. By contrast, our approach based on PSII repair in darkness requires no such assumptions and is, therefore, much simpler to analyse. Using this approach, our results show that, experimentally, the number of ATP molecules required to repair each photoinactivated PSII is considerably larger than that calculated from the breaking and forming of chemical bonds during degradation and synthesis of D1 protein (Murata & Nishiyama, 2018) or of D1 protein plus three other PSII proteins (Miyata *et al.*, 2012). Further, assuming the same energy cost which was obtained for repair in darkness, we found that up to 4.6% of photophosphorylation at moderate to high irradiance is diverted from gross carbon assimilation to PSII repair during 7 h illumination, a percentage which is two- to three-fold greater than that estimated previously (Miyata *et al.*, 2012). It appears that there are other factors–processes–macromolecules that are large ‘dark’ energy sinks involved in the PSII repair cycle; these remain to be identified and the energy costs quantified.

Materials and Methods

Plant material and growth conditions

Cotton (*Gossypium hirsutum* L. cv. Sicot 75) plants were grown in a glasshouse at approximately 28°C : 18°C (day : night) under natural light, the maximum irradiance being *c.* 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The plants were given a nutrient solution of Aquasol weekly (Yates Australia, Pastow, Australia). For measurement of the rate of PSII repair during darkness subsequent to a photoinactivation treatment, leaves attached to the plant were illuminated overnight with light at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, prior to a photoinactivation treatment of leaf discs the next day; this pre-illumination treatment was intended to maintain a nonlimiting carbohydrate reserve in all leaf discs during subsequent repair in darkness.

Uptake of lincomycin

To determine the rate coefficient of PSII photoinactivation in the presence of lincomycin, detached leaves were allowed to take up

lincomycin (1 mM) through the cut petiole overnight in darkness. Overnight illumination was omitted, so as to avoid accumulation of inactive PSII complexes in the presence of lincomycin. Typical average concentrations of lincomycin taken into the leaf tissue were estimated to be *c.* 1 mM. Leaf discs punched from such lincomycin-infiltrated leaves were subjected to a photoinactivation treatment at selected irradiances.

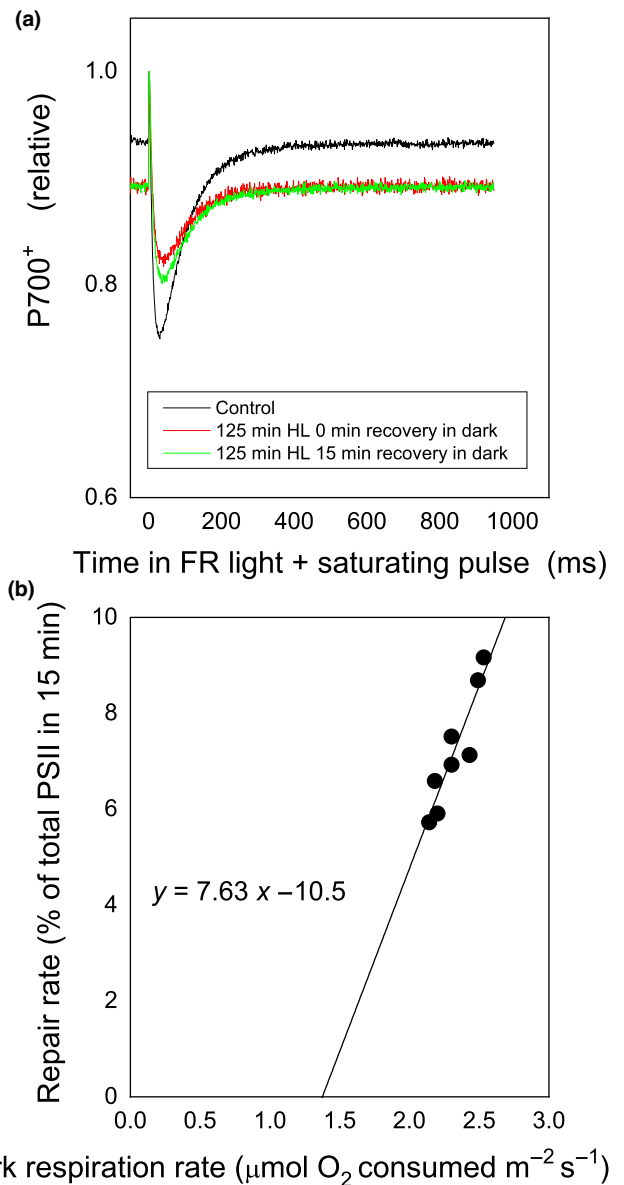
Light treatment

Leaf discs (2.22 cm²), floating with the adaxial side (that normally faces the stem) in contact with water or a solution of lincomycin (1 mM) in a clear plastic Petri dish on a water bath, were illuminated with an array of nine white light-emitting diodes (LEDs, supplying an irradiance as specified in the text), with the light directed upwards at the adaxial surface, while the abaxial surface (with more abundant stomata) was exposed to air. The temperature of the water or lincomycin solution was kept at 26°C.

Photosystem II (PSII) functionality

For a rapid, noninvasive and whole-tissue determination of the functional (i.e. active) fraction of PSII (*f*), we used the signal from P700⁺ (the oxidized primary electron donor in PSI) to monitor the delivery of electrons from functional PSII complexes to P700⁺ following a brief light pulse (0.1 ms duration, 7000 μmol photons m⁻² s⁻¹) superimposed on steady far-red light (50 μmol photons m⁻² s⁻¹) applied to a leaf disc. In the presence of far-red light, which predominantly excites PSI, P700 is largely oxidized. On addition of a brief strong light pulse, the redox kinetics of the P700⁺ signal consist of transient, complete oxidation of the remaining P700 to P700⁺, followed by reduction of P700⁺ to a level below that at steady state in far-red light, and then re-oxidation of P700 to steady state by the continuous far-red light (Fig. 1a). The area bounded by the horizontal line corresponding to steady state and the P700⁺ kinetics curve is here termed the P700 kinetics area, and is, empirically, directly proportional to the content of functional PSII in cotton and other species (Kou *et al.*, 2012; Hu *et al.*, 2013), the latter assayed by the oxygen yield per saturating, single-turnover xenon flash (Chow *et al.*, 1989) (see later in the Materials and Methods section). The ratio of the P700 kinetics area of a leaf disc pre-treated with light to photoinactivate some of the PSII to that of a nonilluminated sample is taken as *f*; the functional fraction of PSII.

The P700⁺ signal itself was measured using a dual-wavelength (810/870 nm) unit attached to a Pulse Amplitude Modulation (PAM) 101/102/103 fluorometer (Walz, Effeltrich, Germany) in the reflectance mode as described previously (Kou *et al.*, 2012; Hu *et al.*, 2013). A multi-channel pulse/delay generator (Model 555; Berkeley Nucleonics Corp., San Rafael, CA, USA) was used to time the start of data acquisition and the application of a strong brief light pulse (from a white LED) that delivers electrons from functional PSII to PSI. The analogue output from the dual-wavelength unit was digitized. Four flashes were given at 0.2 Hz, and the signals were averaged automatically and stored in a computer for later analysis. The P700⁺ signal was measured through



Dark respiration rate (μmol O₂ consumed m⁻² s⁻¹)

Fig. 1 (a) An example of the changes in the P700⁺ signal in *Gossypium hirsutum* leaf discs with time before and after a brief light pulse is added (at time 0) to continuous far-red light. Each trace is normalized to the maximum signal immediately after application of the brief light pulse. The black trace refers to a control leaf disc not pre-exposed to high light (HL), and the red trace a leaf disc pre-exposed to HL (1600 μmol photons m⁻² s⁻¹) for 125 min, and measured promptly afterwards. The green trace refers to a leaf disc pre-exposed to HL for 125 min, and allowed to recover in darkness for 15 min before being promptly measured. (b) A plot of the percentage of the total (functional plus nonfunctional) photosystem II (PSII) repaired in 15 min of darkness against the dark respiration rate for leaf discs pre-exposed to HL for varied durations to induce varied values of both parameters. At a fixed photoinhibition irradiance of 1600 μmol photons m⁻² s⁻¹, a higher rate of repair on the Y axis corresponds to a longer exposure of leaf discs to light at the fixed irradiance. The slope of the linear regression is the change in PSII repair rate per unit change in dark respiration rate.

a multifurcated light guide that was inserted vertically into the same oxygen electrode chamber in which dark respiration (see later in the Materials and Methods section) was measured concurrently.

For a determination of the absolute functional PSII content per unit leaf area, we determined the gross oxygen yield per saturating, single-turnover xenon flash (full width at half height being *c.* 3 μ s). The repetitive flashes were given at 10 Hz to a leaf disc in air enriched with 1% CO₂ in weak background far-red light to help to maintain an oxidized inter-photosystem pool of electron carriers (Chow *et al.*, 1989). This measurement makes the reasonable assumption that, after four saturating flashes, each functional PSII evolves one oxygen molecule in the absence of photorespiration. A slight heating artefact, measured with a green card disc, was taken into account. Oxygen measurement was conducted in a Clarke-type gas-phase oxygen electrode (Hansatech, King's Lynn, UK).

Measurement of dark respiration rate

Oxygen measurements were made using a Clarke-type gas-phase leaf-disc oxygen electrode (Hansatech). The oxygen electrode was used to measure the respiration rates of leaf discs (2.22 cm²) kept in darkness, after pre-illumination (see light treatment mentioned earlier) at varied durations to induce varied extents of photoinactivation of PSII. The same chamber was used to measure the P700⁺ signal (see earlier in the Materials and Methods section), through a multifurcated light guide, the combined end of which was inserted vertically through a port into the oxygen electrode chamber. With the leaf disc perpendicular to the actinic light, both the oxygen and P700⁺ signals were induced under an identical geometry of illumination. Both the P700⁺ and oxygen signals were measured concurrently. An instrument drift signal, measured when a leaf disc was substituted by a black card disc, was subtracted to yield the time course of the change in oxygen concentration in darkness. In the first 1–2 min after placing a leaf disc in the oxygen electrode chamber, there was an initial variability of the net signal because of variable temperature adjustment which affected the diffusion of oxygen through the Teflon membrane to the platinum electrode surface; thereafter, however, the net signal changed linearly over at least 15 min. Therefore, it was assumed that the dark respiration rate was constant over the entire 15-min of measurement during recovery of PSII from photoinactivation.

Measurement of gas exchange to determine the net carbon assimilation rate, P_n

Short-term leaf photosynthetic response to irradiance was measured with a LI-6400 IRGA Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE, USA) to obtain the net rate of photosynthesis (P_n) of leaves (attached to a plant, with or without cling film adhering to the adaxial surface). The cling film was used to approximately simulate the floating of leaf discs with the adaxial side in contact with water. The P_n was measured after equilibration to obtain a steady-state photosynthetic rate at each selected irradiance. Illumination was applied in the following sequence of irradiances: 1952, 1530, 1197, 810, 527, 312, 100 and 0 μ mol photons m⁻² s⁻¹, at 400 μ mol CO₂ (mol air)⁻¹ and 27°C leaf temperature. The actinic illumination was provided by a red-blue LED light source (6400-02B; Li-Cor).

The P_n was also continuously measured with the LI-6400 IRGA Portable Photosynthesis System for 7 h. A leaf attached to the plant was illuminated with red-blue LED light at one of the following irradiances: 1952, 1530, 1197, 810 or 527 μ mol photons m⁻² s⁻¹ for almost 7 h. A cling film adhered to the adaxial surface, at which the red-blue LED light was directed.

Results

Quantifying the functional fraction of PSII during repair

On illumination, a functional PSII delivers electrons downstream towards PSI. Kinetic changes in the P700⁺ signal in PSI can, therefore, be used to nonintrusively and rapidly monitor the delivery of electrons from PSII to P700⁺ (Jia *et al.*, 2014). This can be achieved by adding a brief light pulse to continuous background far-red light which, before the brief flash, photo-oxidizes the majority of primary electron donor (P700) in PSI. Subsequent to the brief flash and after partial reduction of P700⁺ by electrons delivered from PSII, the far-red light re-oxidizes P700 to the steady-state level. Empirically, the 'P700 kinetics area', bounded by the steady-state P700⁺ level (a horizontal line) in continuous far-red light and the P700 redox kinetics curve on adding a brief light pulse, is directly proportional to the oxygen yield per single-turnover, saturating flash during repetitive flash illumination (Kou *et al.*, 2012; Hu *et al.*, 2013). The latter depends on the functional PSII content (Chow *et al.*, 1989), but its measurement using an oxygen electrode is slow and does not offer good time resolution. Therefore, we adopted the P700 kinetics area as a rapid, nonintrusive measure of the relative functional PSII content. Fig. 1(a) displays examples of P700 redox kinetics curves obtained in the presence of continuous far-red light, which photo-oxidizes the majority of the P700 prior to the application of a brief strong light pulse. The top (black) curve refers to a control cotton leaf disc, showing a transient complete oxidation (induced by a brief light pulse, with signal height set to be 1.0) of the remaining (reduced) P700 at time $t = 0$, quickly followed by a 'dip' which indicates electron delivery from PSII to momentarily reduce some of the P700⁺ before continuous far-red light photo-oxidizes P700 to the steady state again. The red curve for a leaf disc pre-treated with high light (HL, 1600 μ mol photons m⁻² s⁻¹ for 125 min) had a much shallower 'dip'; that is, delivery of electrons from PSII to P700⁺ was diminished upon photoinactivating a portion of the PSII with HL pre-treatment. However, some recovery of the P700 kinetics area could already be observed after 15 min recovery in darkness following the HL pre-treatment (green curve). The increase in the P700 kinetics area after 15 min of recovery in darkness, as a percentage of the control, gives the repair rate (percentage of the total (nonfunctional plus functional) PSII repaired in 15 min).

Quantifying the number of ATP molecules required for the repair of one PSII

The rate of PSII repair depends on the content of nonfunctional PSII. In the extreme case, when all PSII complexes are functional,

the repair rate is zero. Following varied durations of HL treatment of discs (punched from a single cotton leaf) to induce varied extents of PSII photoinactivation, the varied repair rate over the first 15 min (900 s) darkness was plotted against the corresponding rate of dark respiration measured concurrently in the same oxygen electrode chamber in an identical geometry of illumination. It should be noted the repair rate is based on the increase in the P700⁺ kinetics area after 900 s of recovery in darkness, relative to the P700⁺ kinetics area of a control leaf disc in which all PSII complexes are assumed to be functional to begin with. That is, the repair rate is relative to the functional PSII content of a control leaf disc or to the total (functional plus nonfunctional) PSII content after photoinhibition treatment. Fig. 1(b) gives an example of such a plot for leaf discs punched from one leaf. It is seen that higher rates of PSII repair corresponded to higher rates of dark respiration. The slope of the linear regression represents the change in repair rate brought about by a unit change in dark respiration rate. For recovery in air, the slope was 6.40 ± 0.94 (SE, $n = 12$ leaves), and for recovery in air enriched with 1% CO₂, the slope was 8.61 ± 0.77 (SE, $n = 12$ leaves) % of total (nonfunctional plus functional) PSII repaired in 900 s per unit change in dark respiration rate (in $\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$). Given the small difference in the two mean values and their large SE, we combined the two sets of similar data; averaged over the 24 cotton leaves, the change in repair rate was 7.50 ± 0.64 (SE) % of the total (nonfunctional plus functional) PSII repaired in 900 s per unit change in dark respiration rate. Using the method of the oxygen yield per single-turnover, saturating flash (Chow *et al.*, 1989), we determined the functional PSII content for control leaves to be 1.76 ± 0.09 (SE, $n = 10$ leaves) $\mu\text{mol m}^{-2}$; this is the total content of functional PSII complexes in control leaf discs, before the start of a photoinhibitory treatment, and it should equal the total functional plus nonfunctional PSII content after a photoinhibitory treatment. The inverse of the slope then simplifies to 6818 oxygen molecules consumed per PSII complex repaired.

There are 29 ATP molecules formed (and six oxygen molecules consumed) per glucose molecule metabolized via the cytochrome pathway (ATP : O₂ = 29 : 6), while 11 ATP molecules are formed per glucose molecule via the cyanide-resistant alternative pathway (ATP : O₂ = 11 : 6) (Noguchi *et al.*, 2001). For high-light-grown plants measured early in the night, the cyanide-resistant alternative pathway accounts for 41% (spinach) or 40% (*Phaseolus vulgaris*) of the total respiratory rate (Noguchi *et al.*, 2001). Taking the alternative pathway as 40% of total respiration, in our cotton leaf discs pre-treated with HL and then placed in the dark for measurement, the overall ATP : O₂ ratio is $(29/6) \times 0.60 + (11/6) \times 0.40 = 3.63$. Therefore, $6818 \text{ oxygen (PSII repaired)}^{-1} = 6818 \times 3.63 \approx 24\,750$ ATP molecules required to repair each PSII complex, here rounded down to 24 000.

The rate coefficient of PSII photoinactivation

In the presence of lincomycin, an inhibitor of PSII repair, the P700 kinetics area relative to that of the control, representing the functional fraction of PSII (f), declined from the initial value of

1.0 exponentially with time t during illumination (Fig. 2a), i.e. $f = \exp(-k_i t)$. The rate coefficient of photoinactivation k_i is directly proportional to irradiance (Tyystjärvi & Aro, 1996; Lee *et al.*, 1999; Kato *et al.*, 2003); this is the case in cotton cv. Deltapine (Hu *et al.*, 2013), and also in cotton cv. Sicot (Fig. 2b). Thus, from Fig. 2(b), at any selected irradiance one can calculate the corresponding k_i ; the rate of PSII photoinactivation

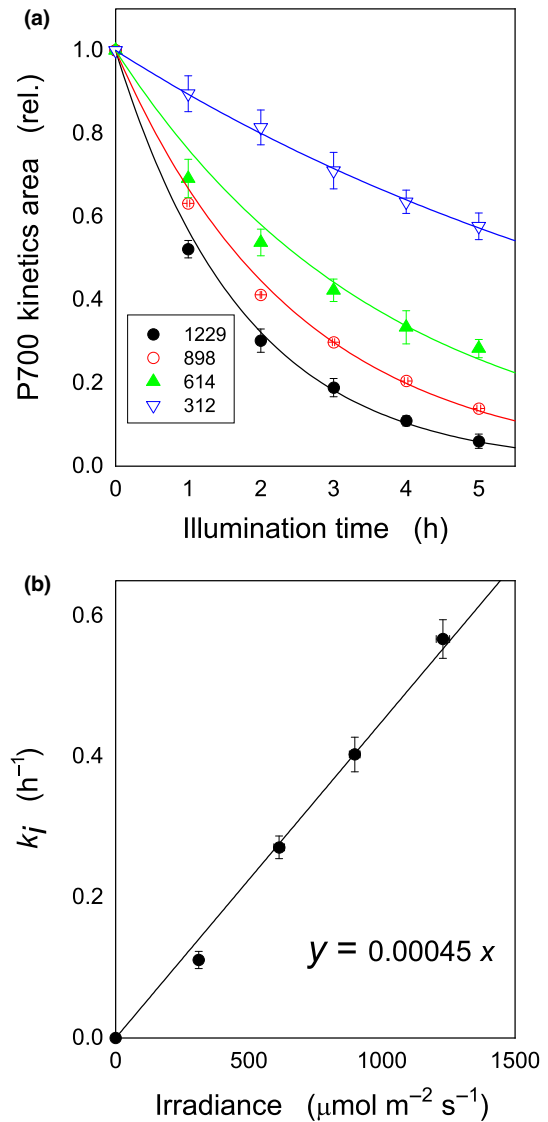


Fig. 2 (a) Exponential decline in the functional fraction of photosystem II (PSII) with time of illumination at varied irradiance in the presence of lincomycin, an inhibitor of chloroplast-encoded protein synthesis, as measured by the relative P700 kinetics area. The relative P700 kinetics area is the area bounded by (a) the horizontal line (not drawn) corresponding to the steady state value before and after the brief light pulse and (b) the P700⁺ kinetics curve which dips down on application of a brief flash, and then increases to a steady-state value in Fig. 1(a). Fitting with a single exponential decay yielded the rate coefficient of photoinactivation of PSII k_i . (b) The rate coefficient of photoinactivation k_i plotted against the photoinhibition irradiance. The linear regression is constrained to pass through the origin. Each data point is the mean of $n = 4$ *Gossypium hirsutum* leaf discs, \pm SE.

can then be obtained as $k_i \times$ the content of functional PSII in cotton cv Sicot.

The time-dependent rate coefficient of PSII repair during illumination and the cumulative content of PSII repaired in 7 h

In the absence of any inhibitor of PSII repair, the functional fraction of PSII (f) is the net result of the concurrent photoinactivation counteracted by the repair. We assume that k_i obtained in the presence of lincomycin remains unchanged in leaf discs illuminated in the absence of lincomycin. In a simple model, the rate of photoinactivation is directly proportional to f , and the rate of repair directly proportional to $(1 - f)$, the 'constants' of proportionality being the rate coefficients k_i and k_r for photoinactivation and repair, respectively. In this simple kinetic model (Wünschmann & Brand, 1992; Hu *et al.*, 2013), the net rate of change of the functional fraction of PSII at time t is given by

$$df/dt = k_r(1 - f) - k_i f \quad \text{Eqn 1}$$

However, in general, at each photoinhibition irradiance, k_r may not be fixed but instead decreases with time of illumination t , described by Fan *et al.* (2016) as

$$k_{r(t)} = k_{r(0)} / (1 + t/\tau) \quad \text{Eqn 2}$$

where τ denotes the time for an initial $k_{r(0)}$ to decrease by half. That is,

$$df/dt = [k_{r(0)} / (1 + t/\tau)](1 - f) - k_i f \quad \text{Eqn 3}$$

Eqn 3 is solved with the aid of a Matlab program (Matlab, R2010b; the MathWorks, Natick, MA, USA) according to Fan *et al.* (2016), yielding the function

$$f(T) = e^{-k_i T} (\tau + T)^{-k_{r(0)\tau}} \left\{ \tau^{k_{r(0)\tau}} + \int_0^T k_{r(0)} \tau e^{k_i t} (\tau + t)^{(k_{r(0)\tau}-1)} dt \right\} \quad \text{Eqn 4}$$

where $f(T)$ represents the running fraction of functional PSII after cumulative photoinhibitory treatment time T , with the boundary condition that $f = 1.0$ at $T = 0$. In this equation, t is the integration variable. Using VISUAL BASIC 6.0 (Microsoft Corp., Redmond, WA, USA), Eqn 4 was employed to fit the data points for varied photoinhibitory irradiances. Fig. 3 depicts the variation of experimental data points with illumination time, and the fitted curves; the parameters $k_{r(0)}$ and τ , obtained after fitting, are displayed in Table 1, column 3, as a pair of numbers. It is noted that $k_{r(0)}$ is halved in *c.* 2–3 h of light treatment.

The absolute rate of repair at time t is $1.76 k_{r(t)} (1 - f(t))$, where $1.76 \mu\text{mol m}^{-2}$ is the total (functional plus nonfunctional) PSII content per unit leaf area, $k_{r(t)}$ is given by Eqn 2, and $(1 - f(t))$ is the nonfunctional fraction of the total PSII content. The cumulative PSII content repaired over a 7 h period,

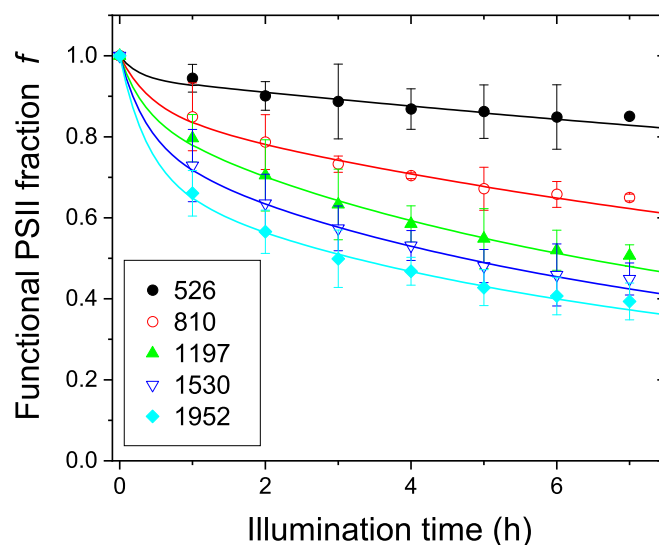


Fig. 3 Decline in the functional fraction of photosystem II (PSII) with time of illumination at varied irradiance in the absence of lincomycin, when photoinactivation and repair of PSII occurred concurrently. The curves were fitted with Eqn 4 with a time-dependent rate coefficient of repair $k_{r(t)}$ as given by Eqn 2, yielding $k_{r(0)}$ and τ (as a pair of numbers in column 3, Table 1) at each photoinhibitory irradiance. Each data point is the mean of $n = 4$ *Gossypium hirsutum* leaf discs, \pm SE.

$\sum \text{PSII}_{\text{repaired}}$, was evaluated by numerical integration of $1.76 k_{r(t)} (1 - f(t))$ with respect to t using Excel (Table 1, column 4) at five pre-treatment irradiances. In each case over the 7-h period, $\sum \text{PSII}_{\text{repaired}}$ exceeded the total functional PSII content present prior to photoinactivation pre-treatment ($1.76 \mu\text{mol m}^{-2}$), implying that each PSII was repaired at least once during 7 h. The total ATP needed for repair of PSII over 7 h was obtained by multiplying $\sum \text{PSII}_{\text{repaired}}$ by 24 000 ATP/PSII_{repaired} (Table 1, column 5).

Net carbon assimilation over 7 h

To determine the cumulative net photosynthesis over 7 h for a comparison with $\sum \text{PSII}_{\text{repaired}}$, we needed to determine the net carbon assimilation rate under conditions comparable to leaf discs floating on water with the adaxial side in contact with water maintained at constant temperature, and the abaxial side facing air while leaf discs were being illuminated on the adaxial side. To this end, we applied a thin transparent 'cling' film to the adaxial side of a leaf attached to a plant, the film adhering to the leaf surface with the help of a small amount of water. By blocking air flow through stomates on the adaxial side with cling film, the net rate of photosynthesis P_n was decreased at high irradiance, compared with the absence of cling film (Fig. 4a). Leaves attached to the plant, with their adaxial side covered by cling film, were illuminated continually on the adaxial side at a chosen irradiance for nearly 7 h. The net rates of photosynthesis P_n are depicted in Fig. 4(b). The cumulative net carbon assimilation was obtained as the area under the curve, with a slight extrapolation to 7 h; values are listed in Table 1, column 6. The ATP needed for the cumulative net photosynthesis (Table 1, column 7) was obtained by

Table 1 Cumulative photosystem II (PSII) repaired ($\sum \text{PSII}_{\text{repaired}}$), cumulative adenosine triphosphate (ATP) required for PSII repair, cumulative net carbon assimilation and ATP needed for net carbon assimilation over a 7-h period of illumination at selected irradiances.

Irradiance ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	k_i (h^{-1})	$k_{r(0)}$, τ (h^{-1}), (h)	$\sum \text{PSII}_{\text{repaired}}$ in 7 h ($\mu\text{mol m}^{-2}$)	ATP for PSII repair in 7 h (mol m^{-2})	Net carbon assimilation in 7 h (mol m^{-2})	ATP used for net carbon assimilation in 7 h (mol m^{-2})	ATP _{PSIIrepair} / ATP _{net carbon assimilation} (%)
526	0.237	3.70, 2.82	2.30	0.055	0.273	0.82	6.7
810	0.365	1.90, 2.75	2.68	0.064	0.415	1.25	5.1
1197	0.539	2.34, 1.61	3.36	0.081	0.432	1.30	6.2
1530	0.689	1.96, 2.14	3.94	0.095	0.450	1.35	7.0
1953	0.878	1.71, 2.75	4.56	0.109	0.484	1.45	7.5
Mean							6.5

The rate coefficient of photoinactivation k_i is obtained from Fig. 2(b). The initial rate coefficient of PSII repair $k_{r(0)}$ and the time τ , which together describe the time-dependent $k_{r(t)}$ according to Eqn 2, are obtained by curve fitting as shown in Fig. 3. The cumulative PSII repaired over 7 h is calculated by numerical integration of $1.76 k_{r(t)} (1 - f(t))$ with respect to time t , where $1.76 \mu\text{mol m}^{-2}$ is the total (functional plus nonfunctional) PSII content per unit leaf area. The cumulative ATP required for repair of PSII during 7 h is obtained by multiplying $\sum \text{PSII}_{\text{repaired}}$ in 7 h by 24 000 ATP/PSII_{repaired}. The cumulative net carbon assimilation is obtained from the area under the curves in Fig. 4(b) over 7 h, and multiplied by three to give the ATP required for the cumulative net carbon assimilation.

multiplying the cumulative net carbon assimilation by three, assuming that three ATP molecules are needed for each CO_2 molecule fixed.

The ratio of the ATP needed for PSII repair to the ATP needed for net carbon assimilation over the 7-h period is displayed in Table 1, column 8. This ratio was approximately constant across the range of irradiance from 526 to 1953 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, with a mean value of 6.5%.

Discussion

This study aimed to (1) determine the energy cost of repairing one photoinactivated PSII complex, and (2) compare the ATP consumed in PSII repair with that used in carbon assimilation. In leaves of cotton, the only higher-plant species we have so far observed to undergo PSII repair at a substantial rate in darkness in a lincomycin-sensitive manner (Hu *et al.*, 2013), the ATP requirement for PSII repair seems to be met by mitochondrial respiration alone; if so, the estimation of the energy cost of repair is vastly simplified.

ATP requirement for repair of one photoinactivated PSII

A change in the PSII repair rate was accompanied by a corresponding change in dark respiration rate (see Fig. 1(b) for leaf discs from one leaf). The repair rate in this figure is relative to the functional PSII content of a control leaf disc or to the total (functional plus nonfunctional) PSII content after a photoinhibition treatment. That is, the absolute repair rate is obtained once the functional PSII content of a control leaf disc is known. The slope of the plot was obtained for 24 leaves, with a mean value of 7.50 ± 0.64 (SE) % of total (functional plus nonfunctional) PSII repaired in 900 s per unit change in dark respiration rate. From the absolute functional PSII content ($1.76 \mu\text{mol m}^{-2}$), and given that *c.* 40% of total respiration occurs via the alternative pathway early in the night (Noguchi *et al.*, 2001), resulting in an overall ATP : O_2 ratio of 3.63, the inverse of the slope simplifies to 24

750 ATP molecules required for each PSII repaired, here rounded down to 24 000. This value is eight- to nine-fold larger than either (1) the 1304 ATP molecules required for the synthesis of D1 and its turnover, plus 1242 nucleoside triphosphate molecules for the synthesis of one transcript of *psbA* mRNA which may be used more than once during its lifetime (Murata & Nishiyama, 2018), or (2) the 2827 ATP molecules for replacement of each D1 protein calculated by Miyata *et al.* (2012).

However, D1 is not the only PSII protein which is photodamaged and which needs to be re-synthesized. When Arabidopsis leaves are illuminated in the presence of lincomycin for 3 h at $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$, the approximate loss of D1 is 80%, D2 50% and CP43 40% (Tikkanen *et al.*, 2008). That is, the combined loss of D2 and CP43 exceeded that of D1. D2 and CP43 have molecular masses similar to or greater than that of D1, implying that their combined replacement cost is at least twice the energy required for replacing D1. In *Spiroidela oligorrhiza*, the rate of degradation of D2 was about one-third of that of D1 (Jansen *et al.*, 1999). In *Spinacia oleracea*, the decrease in the amount of CP47 was *c.* 75% of that of CP43 (Yamamoto & Akasaka, 1995). In diatoms, the D2 protein is lost to an extent roughly comparable to that of D1 (Wu *et al.*, 2011, 2012). Consistent with the earlier reports, Miyata *et al.* (2012) calculated that ATP consumption in the repair of each photoinactivated complex (specifically based on replacement of D1, D2, CP43 and CP47 proteins) could be 4.1 times that required for replacing D1 alone (2827 ATP/D1), so the calculated ATP requirement becomes 11 500 ATP/PSII complex. Thus, our experimental value of 24 000 ATP/PSII_{repaired} is approximately twice as large as that calculated by Miyata *et al.* (2012).

The discrepancy between the present experimental energy cost for repairing an entire PSII and the calculated value reported by Miyata *et al.* (2012) could be due to factors—macromolecules—processes which require inputs of ATP in PSII repair, but which have not been accounted for. Such factors—macromolecules—processes could include (1) extraction of a photodamaged D1 protein from the PSII core; (2) insertion of a newly-synthesized D1;

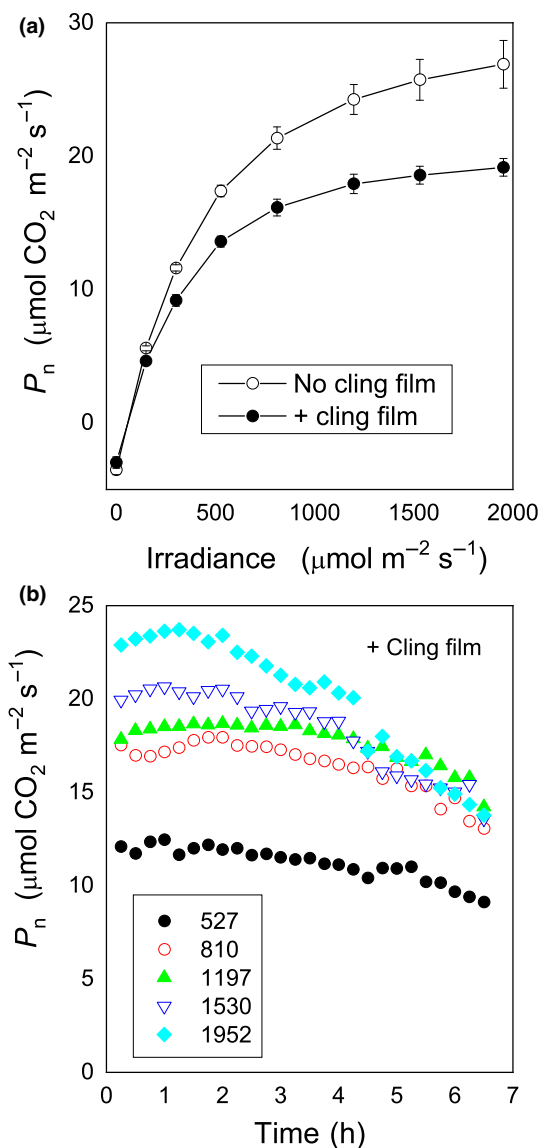


Fig. 4 (a) Light-response curves of the net photosynthetic rate P_n of *Gossypium hirsutum* leaves attached to the plant. The control leaf with no cling film is indicated by open circles. A cling film adhered to the adaxial side with the help of a little water to mimic leaf discs floating on water (closed circles). Each data point is the mean of $n = 3$ leaves, \pm SE. Illumination was directed at the adaxial side. (b) A cling film was applied to the adaxial side of each leaf, one leaf for each irradiance. The net rate of carbon assimilation is plotted against illumination time at each actinic irradiance.

(3) molecular motors needed to overcome viscous resistance in directed motion (Nelson, 2004); (4) work that needs to be done by chaperons; (5) degradation of any mal-translated or mal-folded D1 protein by proteases followed by its replacement by another newly-synthesized D1; (6) maintenance of a high concentration of K^+ (100 mM) and Mg^{2+} (10 mM) in the stroma as required for optimal synthesis of proteins (Bhaya & Jagendorf, 1984), particularly D1; and (7) import of nutrients and solutes and export of waste associated with PSII repair. As a specific example, the unidirectional motion associated with the extraction of a photodamaged D1 protein from the PSII core and the

insertion of a newly-synthesized D1 could be analogous to the ATP-dependent conversion of small motions at the nucleotide-binding site of kinesin and myosin into a power stroke that drives forward motion (Vale & Milligan, 2000).

PSII repair was here studied in the dark in cotton leaf discs for the convenience of deducing the ATP produced by mitochondrial respiration and used in PSII repair. In the light, the ATP required for PSII repair would be mainly supplied by photophosphorylation. In the dark, ATP could in principle be imported from mitochondria into chloroplasts to support PSII repair. Such import of ATP via nucleotide transporters may incur additional expenditure of ATP, which may partly explain the discrepancy between our experimental value (determined during repair in darkness) and that calculated by Miyata *et al.* (2012). However, there is no evidence at present that the expression of nucleotide-transport genes persists in chloroplasts of mature *Arabidopsis* leaf tissue (Voon *et al.*, 2018). The supply of ATP for PSII repair in darkness will, therefore, need to be investigated, including the possibility that nucleotide transporters survive long enough, in the absence of their gene expression in fully-expanded cotton leaves, to allow PSII repair in the dark.

Another factor that may possibly contribute to an overestimate of $\text{ATP/PSII}_{\text{repaired}}$ in our measurement relates to the synthesis of a possible reserve pool of D1 protein. Wettern (1986) reported that in *Chlamydomonas* there is a pool of D1 (*c.* 30–35% of the total D1) which does not bind azido-atrazine and is not incorporated into fully functional PSII complexes. Similarly, Li *et al.* (2016) found in diatoms a large pool of reserve D1 that is not part of active PSII complexes. If some of the D1 protein is synthesized but not incorporated into functional PSII complexes, there are two possibilities. One is that the reserve pool of D1 is subsequently inserted into a functional PSII, in which case, the $\text{ATP/PSII}_{\text{repaired}}$ should not be affected. The other possibility is that the reserve pool of D1 is degraded before incorporation into functional PSII complexes and the energy expended in D1 synthesis is wasted, in which case our observed $\text{ATP/PSII}_{\text{repaired}}$ is an overestimation that includes the wasted ATP.

Yet another factor that may contribute to an overestimate of $\text{ATP/PSII}_{\text{repaired}}$ in our measurement relates to a possible repair-unrelated increase in mitochondrial respiration as the duration of photoinhibition treatment increased (though overnight pre-illumination of an attached leaf before photoinactivation treatment and measurement the next day could have minimized this factor). In that case, the slope in Fig. 1(b) should be greater if only the repair-associated mitochondrial respiration rate were considered; that is, the slope in this figure is an underestimate. Thus, the inverse of the slope (that gives the required $\text{ATP/PSII}_{\text{repaired}}$) could be overestimated. Therefore, the value of 24 000 $\text{ATP/PSII}_{\text{repaired}}$ is an upper limit, while that (11 500 $\text{ATP/PSII}_{\text{repaired}}$) calculated by Miyata *et al.* (2012) is a lower limit.

There are other issues related to PSII repair in darkness. As reviewed by Raven (2020), (re)assembly of the oxygen-evolving complex in PSII requires *c.* 40 mM Cl^- (Vinyard *et al.*, 2019). The accumulation of such a high Cl^- concentration in the thylakoid lumen in the light could be linked in some way to a proton efflux from the thylakoid lumen (e.g. a $\text{Ca}^{2+} : \text{H}^+$ antiport that

accumulates Ca^{2+} in the lumen in exchange for H^+ efflux from the lumen, followed by uptake of Cl^- into the lumen), forgoing some photophosphorylation and incurring an expenditure of energy. In our study, however, PSII repair occurred in darkness, raising the question of how the accumulation of a high $[\text{Cl}^-]$ in the thylakoid lumen might be achieved and whether it demands an energy supply in the dark. Another interesting observation is made by Yamada *et al.* (2020), who showed that inhibition of respiration by myxothiazol under HL decreases k_r of wild-type Arabidopsis leaves by 65% in 400 ppm CO_2 and 45% in 3000 ppm CO_2 , suggesting that inhibition of respiration could (1) increase the production of reactive oxygen species and/or (2) decrease respiratory ATP production, in each case hampering the repair cycle.

The rate coefficient of PSII repair

To prevent dehydration of leaf discs and to regulate the leaf disc temperature during illumination, we floated leaf discs with the adaxial side in contact with water (or with a lincomycin solution when repair was to be prevented) at 26°C, while the stomate-rich abaxial side faced air. However, since the adaxial side did not have free access to air, the supply of CO_2 would have been somewhat limited, resulting in oxidative stress, which is known to impair D1 protein synthesis (Nishiyama *et al.*, 2001) at the elongation step (Nishiyama *et al.*, 2004).

Further, the oxidative stress could have been variable, increasing with prolonged illumination time. Probably, this was one reason why we could not closely fit the data points to a simple model with the photoinactivation rate \propto the functional PSII fraction, and the repair rate \propto the nonfunctional PSII fraction, when the rate coefficient of repair was assumed constant at a given treatment irradiance (Wünschmann & Brand, 1992; Hu *et al.*, 2013). Indeed, the data points tended to be above a fitted curve at a short illumination time, but below the curve at longer illumination time (curve fitting of the data points not shown) when possibly increased oxidative stress exacerbated the disruption of PSII repair (Nishiyama *et al.*, 2001, 2004). A similar phenomenon was observed in spinach leaf discs, and a modified model, in which the repair rate coefficient decreased with time, was introduced to obtain good fitting (Fan *et al.*, 2016). Here, we used a time-dependent $k_{r(t)}$ (described by Eqn 2) to fit the data in Fig. 3, yielding the parameters $k_{r(0)}$ and τ that allowed the cumulative content of repaired PSII to be conveniently obtained by integration (see later in the Discussion section). Nevertheless, it should be pointed out that even the use of a time-dependent $k_{r(t)}$ still utilizes the same simplified assumption used by Wünschmann & Brand (1992) about mass action with only two reactions. A more elaborate model has been proposed by Tyystjärvi *et al.* (1994) and applied by Fan *et al.* (2016) in which, besides k_i , and instead of k_r , rate coefficients for the degradation (k_d) and synthesis (k_s) were introduced. Even so, the model is still an oversimplification, since these rate coefficients refer to the overall damage, degradation and synthesis of various proteins, not just the D1 protein, in the whole PSII complex, as suggested by the large energy cost of repair in the present study.

The cumulative content of PSII centres repaired and the cumulative net carbon assimilation over 7 h

Knowing $k_{r(0)}$ and τ allowed us to determine the rate of repair as $1.76 k_{r(t)} (1 - f(t))$ where $k_{r(t)}$ is expressed in terms of $k_{r(0)}$ and τ according to Eqn 2. The cumulative content of repaired PSII, $\sum \text{PSII}_{\text{repaired}}$, is given by numerical integration of the rate of repair with respect to time. In Table 1, $\sum \text{PSII}_{\text{repaired}} \geq 2.3 \mu\text{mol m}^{-2}$ over 7 h, exceeding the total initial content of functional PSII ($1.76 \mu\text{mol m}^{-2}$) before HL treatment. Thus, each PSII was repaired at least once during 7 h; if PSII had not been repaired, photochemical efficiency and capacity would have been totally lost, since when more than half of PSII complexes are photoinactivated, the light- and CO_2 -saturated photosynthetic capacity decreases linearly with decrease in f (Lee *et al.*, 1999).

As an upper limit, *c.* 24 000 ATP molecules are needed to repair one PSII complex during recovery of PSII in darkness. Assuming that the ATP requirement for repair in the light is the same as in the dark, knowing the cumulative content of repaired PSII over 7 h of illumination (Table 1, column 4) allows the cumulative ATP requirement to be determined (Table 1, column 5). As irradiance increased, the required ATP increased steadily; similarly, the cumulative net carbon assimilation, and hence the ATP used in net carbon assimilation, also increased. Interestingly, the ratio of ATP required for PSII repair to the ATP used in net carbon assimilation was relatively steady from 526 to $1953 \mu\text{mol m}^{-2} \text{s}^{-1}$, with a mean value of 6.5% (Table 1, last column). Miyata *et al.* (2012) calculated that the repair of the PSII complex require *c.* 1.7% of ATP produced by gross photophosphorylation, at least three-fold smaller than we have observed here for repair in darkness.

So far, we have considered the net carbon assimilation rate P_n or its integral with respect to time, both directly measurable. By contrast, since we did not measure the gross photosynthetic rate P_{gross} , we can only estimate it. If the rates of photorespiration and mitochondrial respiration in the light were *c.* 30% and 10% of P_n , respectively, P_{gross} would be *c.* 1.4 times larger than P_n . Then the ratio of ATP required for PSII repair to the ATP used for gross carbon assimilation would be *c.* 4.6% of ATP synthesis required to support P_{gross} over 7 h. If total photophosphorylation supports not only P_{gross} but also other processes, then the ratio of ATP required for PSII repair to total photophosphorylation would be smaller and somewhat closer to that (1.7%) calculated (Miyata *et al.*, 2012). There is another possible reason for the discrepancy. In our leaf discs, blockage of export of photosynthate may cause feedback inhibition of photosynthesis, resulting in increased photoinactivation of PSII and necessitating greater expenditure of ATP for repair. This factor may be one reason for the discrepancy between (1) our estimated ratio (4.6%) of cumulative ATP used in PSII repair (determined in leaf discs) to that used in gross photosynthesis (measured in attached leaves) over 7 h of illumination and (2) the ratio (*c.* 1.7%) calculated by Miyata *et al.* (2012). Therefore, the value of 4.6% may represent an upper limit, and 1.7% a lower limit.

It is instructive to compare ATP requirement in PSII repair with oxidative phosphorylation. Computations on *Synechococcus* 6301 suggest that protein synthesis to balance the gross

photodamage (at $f = 0.5$) consumes up to 20% of oxidative phosphorylation (Raven & Samuelsson, 1986). In cotton leaves (Fig. 3a; Table 1), taking irradiance = $1530 \mu\text{mol m}^{-2} \text{s}^{-1}$, $1 - f \approx 0.5$ at time 7 h, and $k_{r(7 \text{ h})} \approx 0.46 \text{ h}^{-1}$, the PSII repair rate is equivalent to $1.76 \mu\text{mol PSII m}^{-2} \times 0.5 \times 0.46 \text{ h}^{-1} \times 6818 \mu\text{mol O}_2 (\mu\text{mol PSII})^{-1} / (3600 \text{ s h}^{-1}) = 0.76 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$. Taking the dark respiration rate to be $2.2 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$, the rate of repair uses 34% of the equivalent ATP from mitochondrial respiration in cotton, a value about twice the upper limit computed by Raven & Samuelsson (1986) for *Synechococcus* 6301. From Fig. 3(a) and Table 1, at $526 \mu\text{mol m}^{-2} \text{ s}^{-1}$, $1 - f \approx 0.14$ at time 7 h, and $k_{r(7 \text{ h})} = 1.06 \text{ h}^{-1}$; the rate of PSII repair is equivalent to $0.50 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$, *c.* 22% of the dark respiration rate, closer to the upper limit computed by Raven & Samuelsson (1986).

In an environment of high vapour pressure deficit and irradiance, PSII repair may be particularly demanding of energy input. In such an environment, decreasing the incoming light by 40% does not decrease P_n but instead lowers the air temperature and transpiration, resulting in an improvement in water-use efficiency (Campi *et al.*, 2020). Further, photoprotection and photoinactivation are both attenuated; more dry matter per peach tree accumulates, presumably partly due to a saving on the energy diverted to PSII repair (Losciale *et al.*, 2011). Notwithstanding the significant energy cost of PSII repair in any harsh environment, however, there is great benefit in maintaining photosynthetic capacity and/or efficiency, effected by repairing photoinactivated PSII complexes. Without repair, the entire population of PSII would be photoinactivated during a sunny day, resulting in the complete loss of linear photosynthetic electron transport.

Concluding remarks

Experimentally, up to 24 000 ATP molecules are required to repair each photoinactivated PSII complex. This value is likely to be an upper limit, since repair in darkness may, for example, incur further expenditure of energy in the import of ATP into chloroplasts. However, that (11 500) calculated by Miyata *et al.* (2012) is a lower limit, since certain ATP-requiring factors–processes may not have been taken into account, as discussed earlier. The ratio of ATP required for PSII repair to that used in gross carbon assimilation (estimated to be 4.6%) over 7 h of illumination, appear to be between two- and three-fold greater than the value (*c.* 1.7%) calculated by Miyata *et al.* (2012), thus setting the upper and lower limits. These discrepancies between experiment and calculation suggest that certain ‘dark’ energy sinks for ATP in the PSII repair cycle remain to be identified and quantified.

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Author contributions

D-YF, X-GZ, PL, Y-LZ, W-FZ and WSC designed research; X-PY, H-SY and D-YF performed research; X-PY, H-SY, D-YF and WSC analysed data; and X-PY, H-SY, D-YF and WSC wrote the article. X-PY and H-SY contributed equally to this work.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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