Separation of Light-induced Linear, Cyclic and Stroma-sourced Electron Fluxes to P700$^+$ in Cucumber Leaf Discs after Pre-Illumination at a Chilling Temperature

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Pre-illumination of cucumber leaf discs at 4°C with low-irradiance white light (i) led to a marked decrease in the extent of photo-oxidation of P700 (the special chlorophyll pair in the PSI reaction center) in actinic light at room temperature and (ii) hastened the post-illumination re-reduction of P700$^+$. Quantifying the linear, cyclic and stroma-sourced electron fluxes to P700$^+$ in two actinic light regimes, we found that there was no increase in cyclic or linear electron fluxes to account for these changes. Rather, we observed a decrease in the maximum extent of P700 photo-oxidation assayed by a strong flash superimposed on continuous, background light of wavelength 723 nm, which we interpret to represent a loss of stable charge separation in PSI due to enhanced charge recombination as a result of the pre-illumination treatment. The funneling of electrons towards fewer non-damaged PSI complexes could explain the hastened post-illumination re-reduction of P700$^+$, aided by a slight increase in a stroma-sourced electron flux after prolonged pre-illumination at 4°C. Quantifying the separate fluxes to P700$^+$ helps to elucidate the effects of chilling of cucumber leaf discs in the light and the reasons for the hastened post-illumination re-reduction of P700$^+$.

Keywords: Cucumis sativus — Cyclic electron flow — P700 — Photoinactivation — Photoinhibition — PSI.

Introduction

Photoinhibition, defined as a decline in photochemical efficiencies resulting from exposure to excessive light that cannot be utilized by photosynthesis, is widely acknowledged to occur at PSI at normal temperatures. In contrast, PSI seems to be better protected against photoinhibitory damage under favorable growth temperatures. For example, there is no decrease in the photo-oxidizable amount of P700 following high-light stress, in shade- or sun-acclimated leaves; this photoprotection strategy may be due to a high proportion of P700$^+$ acting as an efficient quencher of excitation energy (Barth et al. 2002). Alternatively, cyclic electron transport, mediated by the putative enzyme ferredoxin-quinone oxidoreductase (FQR) and/or ferredoxin-NAD(P)H oxidoreductase (FNR), establishes a trans-thylakoid membrane H$^+$ gradient. The H$^+$ gradient in turn down-regulates PSI and limits the delivery of electrons to the acceptor side of PSI (Heber and Walker 1992). Only under special circumstances such as a lack of the key enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is PSI damaged by illumination at favorable temperatures (Allahverdiyeva et al. 2005).

At a chilling temperature, however, PSI is sometimes more vulnerable than PSII under illumination. Terashima et al. (1994) demonstrated that low irradiance, combined with a chilling temperature, can differentially decrease the photochemical efficiency of PSI in cucumber leaves, providing direct evidence that the main site of photoinhibition in cucumber leaves is PSI, not PSII. Similar findings were reported for potato leaves by Havaux and Davaud (1994). The extent of photoinhibition of PSI, however, depends on the acclimation history of winter rye, specifically the excitation pressure on PSII (Ivanov et al. 1998). Although preferential photoinhibition of PSI is not a general phenomenon in chilling-sensitive plants (Barth and Krause 1999), a number of investigations have revealed that photoinhibition of PSI can occur in the field, not only in leaves of chilling-sensitive plants, but also in those of winter barley (Teicher et al. 2000), suggesting a physiological relevance of this phenomenon in the field.

The underlying mechanism for PSI photoinhibition at low temperatures may operate at the acceptor side of...
PSI (Sonoike et al. 1995, Tjus et al. 1998, Scheller and Haldrup 2005). At a low temperature, the over-reduction of electron carriers at the acceptor side of PSI is mainly caused by the low activity of Calvin cycle enzymes, such as Rubisco, during chilling of tomato leaves (Sassenrath and Ort 1900) or stromal bisphosphatases even on rewarming (Sassenrath et al. 1990). The over-reduction of electron carriers at the acceptor side of PSI can stimulate the production of reactive oxygen species (ROS), exacerbated by the lower activity of ROS-scavenging enzymes, particularly Cu/Zn superoxide dismutase (Choi et al. 2002) and ascorbate peroxidase (Terasima et al. 1998) at such a temperature. ROS subsequently destroy the iron–sulfur centers, leading to proteolysis and disintegration of PSI subunits (Sonoike et al. 1995, Tjus et al. 1998, Scheller and Haldrup 2005). Additionally, ROS can damage Calvin cycle enzymes (e.g. Rubisco; Nakano et al. 2006), thereby aggravating the over-reduction state of the acceptor side of PSI. Significantly, unlike PSII, a damaged PSI needs a much longer time (days) for repair (Kudoh and Sonoike 2002, Zhang et al. 2004) to recover partial normal physiological function.

An interesting observation is that the post-illumination re-reduction of $P700^+$ in cucumber leaves pre-illuminated with low light at a chilling temperature was completed in a shorter time with an increase in pre-illumination time (Sonoike 1998). The increased rate coefficient of $P700^+$ re-reduction could be due to a number of causes, including enhanced cyclic electron flow after the pre-illumination at a chilling temperature (Sonoike 1998). For example, cyclic electron flow could be enhanced by the accumulation of stromal reductants during pre-illumination; these reductants equilibrate with the plastoquinone pool, perhaps via NAD(P)H dehydrogenase complex (Joët et al. 2002), to give a proper poising required for efficient operation of FQR-mediated cyclic electron flow (Joët et al. 2002, Govindachary et al. 2007). Alternatively, the increased rate coefficient of $P700^+$ re-reduction could be a result of an increased rate of electron donation from stromal reductants to $P700^+$ following photoinhibition at a chilling temperature (Bukhov et al. 2004).

To our knowledge, however, there has been no reporting of the magnitudes of cyclic, linear and stroma-sourced electron fluxes in leaves that had been photo-inhibited at a chilling temperature. Therefore, this study aimed to quantify the various electron fluxes to $P700^+$, and to investigate how they have changed following pre-illumination of cucumber leaf discs at a chilling temperature. Joliot and Joliot (2005, 2006) developed a method to quantify the fraction of PSI centers that operate in the cyclic or linear mode, the method being based on the onset kinetics of $P700$ photo-oxidation during illumination of dark-adapted leaves. The kinetics of $P700$ photo-oxidation, however, are extremely variable among dark-adapted leaves (Joliot and Joliot 2005). Therefore, we chose instead to deduce the electron fluxes from the $P700^+$ re-reduction kinetics (Chow and Hope 2004) on cessation of illumination with low-irradiance far-red light alone or with moderate-irradiance far-red + red light. In either light regime, we found that there was little or no increase in the cyclic electron flux, but rather a decrease at long durations of illumination at a chilling temperature. There was some increase in the stroma-sourced electron flux to $P700^+$, starting from a low value of the control that had not been pre-illuminated at 4°C. The linear electron flux decreased when assayed in either light regime, presumably being limited by photodamage to the acceptor side of PSI and to PSII. Such damage was indicated by the decrease in the maximum extent to which $P700$ could be photo-oxidized by a flash superimposed on continuous LED (light-emitting diode) light at 723 nm, presumably due to enhanced charge recombination in PSI. The hastened post-illumination re-reduction of $P700^+$ after the pre-illumination treatment at 4°C could be explained by the funneling of electrons towards fewer non-damaged PSI complexes.

**Results**

*Electron fluxes assayed with low-irradiance far-red light*

Leaf discs were illuminated for 60 s to produce a steady [$P700^+$] with low-irradiance far-red light, and the post-illumination re-reduction kinetics of $P700^+$ were measured. Fig. 1 shows examples of post-illumination $P700^+$ signals normalized to the maximum value of control non-inhibited samples. The maximum value of non-inhibited samples was obtained by a flash superimposed on continuous low-irradiance LED light at a wavelength of 723 nm (Chow and Hope 2004). Each signal, therefore, indicates the fraction of total $P700$ in the oxidized form, varying as a function of time of relaxation. During illumination with low-irradiance far-red light (before time 0), the concentration of $P700^+$ at any time was set by the rate of photo-oxidation counteracted by re-reduction by electrons from various sources. With an increase in the duration of pre-illumination of leaf discs at 4°C, the amplitude of the signal decreased. Further, depending on infiltration of leaf discs with water or methyl viologen (MV) ± DCMU, different electron fluxes were permitted to occur. In water-infiltrated samples, all three (linear, cyclic and stroma-sourced) electron fluxes occurred; in the presence of MV, the cyclic electron flux was eliminated, while in DCMU + MV, only the stroma-sourced electron flux remained.

On abruptly turning off the actinic light, photo-oxidation of $P700$ ceased immediately, but the re-reduction of $P700^+$ continued for some time. The initial rate of re-reduction of $P700^+$, therefore, should equal the electron
flux to P700$^{+}$ immediately before cessation of actinic illumination. Hence, our objective was to determine the initial rate of re-reduction of P700$^{+}$ on abruptly turning off the low-irradiance far-red actinic light. The re-reduction kinetics (Fig. 1) were well fitted by the sum of two negative exponentials as in previous reports (Bukhov et al. 2002, Bukhov et al. 2004), with normalized amplitudes $A_1$ and $A_2$, and rate coefficients $k_1$ and $k_2$, yielding the total initial rate of re-reduction of P700$^{+}$ (i.e. electron flux) $A_1k_1 + A_2k_2$. The units of an electron flux are electrons per P700 per second.

Fig. 2A shows the electron fluxes assayed with low-irradiance far-red light as a function of the duration of pre-illumination with white light at 4°C. The flux in H$_2$O-, MV- or (DCMU + MV)-infiltrated leaf discs increased at first and then decreased upon prolonged pre-illumination at a chilling temperature. The difference between H$_2$O and MV infiltration gives the cyclic electron flux, since MV accepts electrons at a site before soluble ferredoxin (Fd) (Kobayashi and Heber 1994). It is apparent that the cyclic electron flux increased little, if anything, and indeed decreased upon prolonged pre-illumination (Fig. 2B). The small linear flux in low-irradiance far-red light, obtained as the difference between MV- and (DCMU + MV)-infiltrated samples, also decreased. Only the stroma-sourced electron flux, obtained in the presence of (DCMU + MV), showed a consistent increase, albeit with a decline at longer pre-illumination times (Fig. 2B). It should be noted that these fluxes were low because a low far-red irradiance was used. When expressed as a fraction of the total electron flux obtained with water-infiltrated samples, both the cyclic and linear fluxes declined with pre-illumination, while the stroma-sourced flux increased steadily (Fig. 2C).

The rate coefficients for the re-reduction of P700$^{+}$ (fast and slow components) both increased with pre-illumination time (Fig. 3). The rate coefficients did not solely determine the electron fluxes, however, since the amplitude of each component was also a contributing factor.

Electron fluxes assayed with moderate-irradiance red + far-red light

In moderate-irradiance red + far-red actinic light, the linear electron flux was dominant in control leaf discs (Fig. 4A). The linear electron flux, however, declined markedly with an increase in the duration of pre-illumination at 4°C, decreasing by a factor of about 6 after 5h. The cyclic flux showed little or no increase initially, decreasing slightly at longer pre-illumination times. Starting from a low value, the stroma-sourced electron flux increased several-fold, but its magnitude remained much less than those of the linear and cyclic fluxes even upon prolonged pre-illumination. At longer pre-illumination times, the linear and
cyclic electron fluxes were comparable (~40–50% of the total), the stroma-sourced flux making up the remainder at about 10% of the total (Fig. 4B).

In the case of moderate-irradiance red + far-red actinic light, the re-reduction kinetics of P700+ were better fitted as the sum of three negative exponentials instead of two. Thus, we obtained three rate coefficients, \( k_{\text{fast}} \), \( k_{\text{mid}} \) and \( k_{\text{slow}} \) for the fast, middle and slow phases, respectively. The rate coefficient \( k_{\text{fast}} \) remained relatively constant as the pre-illumination time increased, both in water- and in MV-infiltrated samples (Fig. 5A). For the middle phase, \( k_{\text{mid}} \) was approximately constant in the presence of MV but increased in water-infiltrated samples as the pre-illumination time increased (Fig. 5B). The rate coefficient \( k_{\text{slow}} \) increased in all three cases, including the case of DCMU + MV which only permitted a slow phase (Fig. 5C). Again, it should be noted that the rate coefficients alone did not determine the electron fluxes.

The loss of stable charge separation in PSI

The signal in leaf discs infiltrated with H2O or MV, obtained with a flash superimposed on steady LED light at wavelength 723 nm, is a measure of the maximum extent of P700 photo-oxidation. A loss of maximum amplitude of the...
P700$^+$ signal means that a strong xenon flash, combined with steady LED light at 723 nm, was unable to induce a stable electron donation from P700 in some of the PSI complexes. In the particular case of leaf discs infiltrated with (DCMU + MV), where only a stroma-sourced electron flux was permitted, the steady-state [P700$^+$] (the signal level before the flash at \( t = 50 \text{ ms} \)) in LED light at 723 nm decreased with pre-illumination at 4°C for 0–5 h, and even a strong flash was unable to stabilize the charge separation to any great extent on the time scale of milliseconds (Fig. 6).
This demonstrates the damage to PSI due to pre-illumination of leaf discs at 4°C.

In contrast, PSII was less photoinhibited by the pretreatment. Fig. 7 depicts the slow decrease of \( \frac{1}{F_o} - \frac{1}{F_m} \) with pre-illumination time. This chlorophyll fluorescence value is linearly correlated with the fraction of functional PSII in cucumber leaf discs, though the correlation intercepts the y-axis above zero (Kim et al. 2001).

**Discussion**

Following pre-illumination of cucumber leaf segments at a chilling temperature, re-reduction of P700\(^+\) takes less time to complete when assayed at room temperature (Sonoike 1998, Kim et al. 2001, Bukhov et al. 2004); this observation is also apparent from the time courses in Fig. 1. Suggestions have been made that chilling in the light may have primed the leaf segments for enhanced cyclic electron flow (Sonoike 1998), that the accumulation of stromal reductants during pre-illumination enhances the feeding of electrons towards the rapid re-reduction of P700 (Bukhov et al. 2004) or that accumulated stromal reductants give a better redox poise for the operation of cyclic electron flow mediated by FQR (Govindachary et al. 2007). To test these possibilities, we quantified the separate electron fluxes that converge to reduce P700\(^+\), using two actinic light regimes: a low-irradiance far-red light that mainly stimulated PSI and a moderate-irradiance red + far-red light that excited both PSI and PSII.

**Cyclic electron flux**

The cyclic electron flux was obtained as the difference between the electron fluxes in H\(_2\)O- and MV-infiltrated samples. Assayed in both light regimes, the cyclic electron fluxes appeared to be stable for the first 2h of pre-illumination, but decreased greatly in low-irradiance far-red light (Fig. 2B) or slightly in moderate red + far-red light (Fig. 4B). There was no obvious increase in the absolute cyclic electron flux in either actinic light, though the cyclic flux as a proportion of the total increased with progressive photoinhibition. For example, in red + far-red light, it reached ~50% of the total electron flux after 5h pre-illumination (Fig. 4B), mainly due to a large decrease in the linear electron flux.

Actually, one would not necessarily expect an increase in the absolute cyclic electron flow. Photodamage to the F\(_A\)/F\(_B\) iron-sulfur centres or upstream components in PSI (Sonoike et al. 1995, Tjus et al. 1998, Scheller and Haldrup 2005) would inhibit electron flow to Fd. Since FQR-mediated cyclic electron transport depends on Fd\(^-\), it may be limited by the availability of Fd\(^-\).

If there was no increase in cyclic electron flow, why then did the post-illumination re-reduction of P700\(^+\) take less time to complete? We suggest that as more and more PSI complexes were damaged on the acceptor side, a greater proportion of PSI complexes contained reduced P700 on the donor side due to rapid charge recombination (see below).
Then electrons from PSII and/or stromal reductants would be funneled to fewer PSI complexes that did have P700$^+$ on the donor side to accept electrons. Therefore, we should observe a shorter half-time for re-reduction of P700$^+$ in leaf discs pre-illuminated at 4°C. Our observation of increasing rate coefficients for re-reduction of P700$^+$ (Figs. 3, 5) is consistent with the funneling of electrons to fewer sites containing P700$^+$. Further, we observed that the total electron flux per functional PSI increased by a factor of 1.3 in red + far-red light and 1.5 in low-irradiance far-red light after 5 h pre-illumination at 4°C (data not shown).

It should be pointed out that an electron flux is the product of an amplitude and a rate coefficient, or the sum of two or more such products. It is not valid to assume that a flux is only dependent on the rate coefficient (or, inversely, the half-time), as often the amplitude also varies.

**Linear electron flux**

The linear electron flux was obtained as the difference between MV- and (DCMU + MV)-infiltrated samples. It was understandably small in low-irradiance far-red light which was preferentially absorbed by PSI, not PSII. In moderate red + far-red light, on the other hand, the linear flux made up >70% of the total flux in control samples. Interestingly, it decreased greatly with pre-illumination time, much more than the cyclic electron flux did (Fig. 4A). By 5 h, the linear flux was exceeded by the cyclic component. The linear electron flux in leaf discs after pre-illumination at 4°C could have been limited by a bottle-neck on the photodamaged acceptor side of PSI. For example, a photodamaged F$_A$/F$_B$ center in a PSI could have kept P700 in a reduced state in the actinic light due to charge recombination (see below). The electrons supplied from water splitting in PSII would not be transferred to the P700 in such a photodamaged PSI. They could only be funneled to the non-damaged PSI complexes which, therefore, had to handle more electron traffic. Indeed, the total electron flux per remaining functional PSI increased with the duration of pre-illumination of leaf discs at 4°C (data not shown). Conceivably, the redox poising of cyclic electron transfer components was improved as a result of the funneling effect, with potential enhancement of the cyclic electron flux around the non-damaged PSI complexes. Indeed, there was a hint of an increase in cyclic electron flow at 1 h (Fig. 4B) or 2 h (Fig. 2B) of pre-illumination at 4°C.

**Stroma-sourced electron flux**

Asada et al. (1992) reported a method for (i) demonstrating that stromal components donate electrons to P700$^+$ through the intersystem chain in chloroplasts of C3 plants, and (ii) quantifying the functional size of the stromal pool size of electrons that can be donated to P700$^+$ after illumination of intact leaves. Further, they showed that the stromal pool size of electrons after actinic illumination was much greater in the C4 plant maize than in C3 plants (Asada et al. 1993). Here we obtained the stroma-sourced electron flux at the instant of cessation of illumination in the presence of MV and DCMU, which eliminated cyclic and linear electron flow, respectively. In low-irradiance far-red light, its value was comparable with those of the other two fluxes in control samples ($\sim$0.2 e$^-$/P700$^+$ s$^{-1}$, Fig. 2B); by 5 h pre-illumination, however, the stromal flux constituted almost the total flux (Fig. 2C). In moderate red + far-red light, the stroma-sourced electron flux was relatively insignificant in control samples (Fig. 4A); it increased as pre-illumination time increased, while the cyclic and linear fluxes decreased, reaching approximately 10% of the total electron flux at 5 h (Fig. 4B). Possibly, pre-illumination at 4°C led to an accumulation of reducing equivalents in the stroma when carbon assimilation and photorespiration were both much diminished. These stromal reductants could subsequently inject electrons into the plastoquinone pool, thereby being partially responsible for the hastened re-reduction of P700$^+$ after pre-illumination at 4°C (Bukhov et al. 2004).

The stroma-sourced electron flux to P700$^+$ could have been mediated by the NAD(P)H dehydrogenase (NDH) complex (Bukhov et al. 2004, Govindachary et al. 2007), though the NDH-dependent cyclic pathway is probably more important in C4 photosynthesis, while the Fd- and PGR5-dependent cyclic pathway is more important in C3 plants such as Arabidopsis and tobacco (Shikanai 2007). The multi-phasic relaxation of P700$^+$ has sometimes been used to suggest various possibilities for the different routes of electron flow: (i) populations of PSI with different properties (Albertsson 1995); (ii) the same enzyme located in different membrane domains (Bukhov et al. 2002); or (iii) different enzymes mediating different routes (Chow and Hope 2004). In this study, we have not attempted to identify the post-illumination re-reduction phases with particular pathways of electron flow. Instead, we have focused on determining the electron flux flowing to P700$^+$, using MV to inhibit the cyclic flux or DCMU to inhibit the linear flux. In this way, we were able to separate the electron fluxes.

In Fig. 6, the flash-induced further photo-oxidation of P700 (the rapid rise of the signal after a flash), in the presence of background LED light at 723 nm as well as DCMU and MV, was very small in control leaf discs, but increased in extent up to 2 h of pre-illumination, and then decreased on further pre-illumination. This is because the steady-state level of P700$^+$ before the flash represented a balance between photo-oxidation by the 723 nm light and...
reduction of P700$^+$ by the stroma-sourced electron flux, which was the only electron flux remaining in the presence of DCMU and MV; a higher stromal electron flux led to increased reduction of P700$^+$ and a lower steady-state level of P700$^+$ before the flash and, therefore, a greater extent of flash-induced photo-oxidation of P700$^+$. If the stroma-sourced electron flux happened to change on prolonged illumination, then flash-induced further photo-oxidation of P700 would change. Assayed in the low-irradiance far-red actinic light, the stroma-sourced electron flux indeed increased and then decreased during pre-illumination (Fig. 2B, triangles).

**Charge recombination**

Donation of electrons from stromal reductants indirectly to P700$^+$ could in principle maintain a low steady-state [P700$^+$] in continuous LED light at 723 nm wavelength (Fig. 6), such that longer pre-illumination treatments gave lower steady-state [P700$^+$] values. However, a strong flash superimposed on the LED light at 723 nm did not give a much higher [P700$^+$]. Therefore, we propose that charge recombination in PSI complexes that had suffered photodamage on the acceptor side could return an electron to P700$^+$ very soon after charge separation took place. In that case, we would not be able to observe any stable charge separation on the time scale of milliseconds. Specifically, charge recombination should occur from a component before $F_{A/F_B}$ to P700$^+$, otherwise MV present at 0.3 mM (Fig. 6) would effectively inhibit any $F_{A/F_B}$ to P700$^+$ charge recombination (Sonoike and Terashima 1994). Charge recombination events from components prior to $F_{A/F_B}$ are fast: 1 ms for $F_{X}$ to P700$^+$ (Goldbeck 1995) and 10–250 $\mu$s for $A_1$ to P700$^+$ (Vassiliev et al. 1997). The faster charge recombination event in barley leaves after illumination at a chilling temperature was in fact a 5 $\mu$s component, the amplitude of which was much greater than that of the 1 ms component (Tjus et al. 1998, Teicher et al. 2000). We suggest that the fast return of an electron to P700$^+$ resulted in the loss of net photo-oxidation of P700 in damaged PSI complexes monitored in the millisecond time range.

As shown in Fig. 6, after 5 h pre-illumination at 4°C, the maximum magnitude, P700$^+_{\text{max}}$, of flash-induced photo-oxidation of P700 in the presence of continuous background 723 nm light was only about 30% of the control. Judging from the drastic diminution of the P700$^+_{\text{max}}$ after 5 h pre-illumination at 4°C (Fig. 6), about 70% of the PSI complexes must have been damaged, which is consistent with the data from previous studies (Sonoike and Terashima 1994, Terashima et al. 1994, Kudoh and Sonoike 2002). The photodamage in cucumber leaf discs must have been widespread, and charge recombination a main event. By comparison, the damage to PSI induced by chilling in the light for 8 h was about 32% in Arabidopsis thaliana (Zhang and Scheller 2004) and 15–20% in barley (Tjus et al. 1998, Teicher et al. 2000).

Our interpretation is that charge recombination in photodamaged PSI after pre-illumination of cucumber leaf discs at 4°C was responsible for the decreased [P700$^+$]_{max} (Fig. 6). We do not think, however, that charge recombination accounted for the shorter time for the complete post-illumination re-reduction of P700$^+$ in the time range of milliseconds and longer (Fig. 1), contrary to our earlier interpretation (Kim et al. 2001). The quicker completion of re-reduction of P700$^+$ was probably due to a slightly increased stroma-sourced electron flux, but, more importantly, to a funneling of electrons towards fewer non-damaged PSI complexes where charge separation could be stabilized and where charge recombination was not occurring to any great extent.

**Functionality of PSII**

The loss of active PSII complexes in cucumber leaf discs was much less than that of active PSI. The chlorophyll fluorescence parameter $1/F_o - 1/F_m$ is linearly correlated with the fraction of active PSII complexes and, despite a non-zero intercept in the linear correlation, gives an approximate measure of the functional fraction of PSII (Kim et al. 2001). It is a necessary substitute for the usual technique of assaying functional PSII by the oxygen yield per single-turnover flash; the latter technique underestimates the functional PSII content when the inhibition of PSI results in fewer electrons being stably transferred out of PSI than are delivered from PSI into the plastoquinone pool on each consecutive flash (Kim et al. 2001). Fig. 7 indicates that a substantial fraction of functional PSII complexes, indicated by $1/F_o - 1/F_m$, remained even after 5 h pre-illumination at 4°C, consistent with previous reports (Havaux and Davaud 1994, Terashima et al. 1994).

**Conclusions**

Following pre-illumination of cucumber leaf discs at 4°C, the cyclic electron flux did not increase when assayed at room temperature with either far-red or red + far-red actinic light. Instead, it declined after prolonged pre-illumination. Therefore, there was no evidence for enhanced cyclic electron flow following pre-illumination at 4°C. The linear electron flux similarly decreased in either actinic light, presumably hampered by damage to the acceptor side of PSI. In either actinic light, the stroma-sourced electron flux increased with the duration of pre-illumination at 4°C; its magnitude relative to the total electron flux depended on the actinic light, being only a small fraction in moderate-irradiance red + far-red light but a substantial fraction in low-irradiance far-red light. It appears that none of the three electron fluxes could adequately explain the hastened post-illumination re-reduction of P700$^+$. Rather, we suggest
that charge recombination is widespread in photodamaged PSI complexes following pre-illumination at 4 °C, since the amount of stable, photo-oxidizable P700 decreased drastically; with fewer PSI complexes in which stabilized charge separation took place, post-illumination electrons from PSII and the stroma reduced the remaining P700⁺ in a shorter time, an observation that has been erroneously interpreted to imply enhanced cyclic electron flow.

Materials and Methods

Growth of plants

Cucumber (Cucumis sativus L. cv. Lebanese) plants were grown at 24/21 °C (day/night) with a 10 h photoperiod (200 μmol photons m⁻² s⁻¹ supplied by a combination of metal halide and incandescent lamps). The potting mixture was supplemented by a slow-release fertilizer (‘Osmorec’, Scotts Australia Pty Ltd, Castle Hill, Australia).

Photooxidation treatment and vacuum infiltration of leaf discs

Discs (2 cm²) were punched from a leaf and placed on wet filter paper in a thin plastic dish floating on water refrigerated at 4 °C in a cold room, with the adaxial side illuminated at 120 μmol photons m⁻² s⁻¹ for up to 5 h. The light source was a compact, ‘natural’ fluorescent lamp with a reflector (Nelson Lamps, Rowville, Australia). At various times, leaf discs were taken and infiltrated with H₂O, 0.3 mM MV or 0.1 mM DCMU + 0.3 mM MV (all with 0.5% ethanol, v/v). They were blotted with absorbent paper, and allowed to evaporate off excess intercellular water in darkness for 40 min at room temperature, and then placed in a humidified container for a further 20 min in darkness (total dark time 60 min) before measurement of light-induced P700 kinetics at room temperature.

Measurement of post-illumination re-reduction kinetics of P700⁺

Redox changes of P700 in cucumber leaf discs were observed with a dual-wavelength (820/870 nm) unit (ED-P700DW) attached to a pulse amplitude modulation fluorometer (Walz, Effeltrich, Germany) used in the reflectance mode (Chow and Hope 2004). To obtain the maximum signal corresponding to the total amount of photo-oxidizable P700 (P700⁺max), a steady-state was first sought by illumination with LED light at 723 nm wavelength (12 μmol photons m⁻² s⁻¹, 102-FR, Walz) for > 20 s. Then a single-turnover xenon flash (XST 103 xenon flash) was applied to the adaxial side of the leaf disc. Flashes were given at 0.1 Hz, and 16 consecutive signals were averaged (time constant = 95 μs). The maximum signal amplitude immediately after the flash was taken as the total amount of photo-oxidizable P700, and its average value for control leaf discs obtained in the same geometry was 0.5 s for low-irradiance far-red actinic light, and 0.1 s for moderate-irradiance for red + far-red actinic light. Signals from 4–6 separate leaf discs (each illuminated once) were averaged.

All post-illumination P700⁺ signals were normalized to the P700⁺max of control leaf discs obtained in the same geometry (described earlier) to give the fraction of oxidized P700 at any instant. During illumination, the concentration of P700⁺ was set by the rate of photo-oxidation counteracted by re-reduction by electrons from various sources. On abruptly turning off the actinic light, photo-oxidation ceased immediately, but the re-reduction continued for some time. The initial rate of re-reduction of P700⁺, therefore, should equal the electron flux to P700⁺ immediately before cessation of illumination. Hence, our objective was to determine the initial rate of re-reduction of P700⁺ on abruptly turning off the actinic light. In low-irradiance far-red actinic light, the re-reduction kinetics were well fitted by the sum of two negative exponentials, with normalized amplitudes k₁ and k₂, and rate coefficients k₁ and k₂, yielding the total initial rate of re-reduction of P700⁺ (electron flux) A₁k₁ + A₂k₂. In moderate-irradiance red + far-red actinic light, the sum of three negative exponentials was necessary to fit the re-reduction kinetics (except in the case of MV + DCMU). Curve fitting of each kinetic trace used the software ORIGIN 7.0 (Origin Lab Corporation, Northampton, MA, USA), allowing A₁, A₂, k₁, k₂ and k₈ to vary from their initial guess values and to converge after the iterations. The remaining amplitude A₃ was also allowed to vary, but only as given by 1 − A₁ − A₂. The units of an electron flux (initial rate of P700⁺ re-reduction) are electrons s⁻¹ P700⁺⁻¹. An example of curve fitting and the residuals can be found in Fig. 1 of Chow and Hope (2004). If the total P700 content is known, either on a leaf area basis or on a chlorophyll basis, then the absolute electron flux can be expressed on these bases.

Our method based on post-illumination kinetics of P700 re-reduction is akin to that used by others to estimate cyclic electron flow in isolated thylakoids (Cleland and Bendall 1992), in algae (Maxwell and Biggins 1976) and leaves (Clarke and Johnson 2001). The main difference is that we determined the total flux of electrons flowing to P700⁺ in the presence of H₂O₂ (which permits the linear, cyclic and stroma-sourced electron fluxes), MV (which abolishes the cyclic flux) or DCMU + MV (which abolishes both the linear and cyclic fluxes). In this way we were able to separate the linear, cyclic and stroma-sourced electron fluxes.

There is a shortcoming of this method, which should be kept in mind. The introduction of MV and DCMU could potentially perturb the state of the chloroplasts in the leaf, thereby altering the electron fluxes we were trying to measure. An obvious example is the case where linear electron flow is limited downstream by the coupled Calvin–Benson cycle. When MV is present, the limitation is mitigated, and the linear flow is not the same as in the absence of MV. Nevertheless, we think that at the relatively low
irradiances used in this study, electron fluxes were not limited by processes downstream of the acceptor side of PSI. In that case, whether MV or NADP⁺ acted as the electron acceptor was not critical, so long as electron transport was limited by light absorption or by upstream electron transfer steps such as the oxidation of plastoquinol at the cytochrome f complex. Indeed, using the same moderately strong, red-far-red light as in this study, Fan et al. (2007) showed in spinach leaf discs that the linear electron transport rate through PSI as measured by chlorophyll fluorescence was identical for MV- or H₂O-infiltrated samples illuminated for 60 s or longer.

**Measurement of chlorophyll fluorescence yields Fᵢ and Fₘ**

After leaf discs were transferred from the chilling treatment in the light, they were dark-adapted for 30 min at room temperature. The Fᵢ and Fₘ (minimum and maximum fluorescence yields corresponding to open and closed reaction center traps, respectively) were measured with a Plant Efficiency Analyzer (Hansatech, King’s Lynn, UK) operated at 100% maximum excitation light intensity. To use 1/Fᵢ and 1/Fₘ as a measure of functional PSI complexes (Kim et al. 2001), all fluorescence yields were normalized to the mean Fᵢ value of controls (= 1.00).

**Funding**

The National Natural Science Foundation of China (No. 30770346 to D.F.); the Major State Basic Research Development Program (2006CB403206 to D.F.); an ARC grant (DP 0664719 to W.S.C.); a Visiting Fellowship from the Research School of Biological Sciences, Australian National University (to A.B.H.).

**References**


Electron fluxes to P700+ after photoinhibition


(Received January 7, 2008; Accepted April 15, 2008)