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# Flexible coupling between light-dependent electron and vectorial proton transport in illuminated leaves of $C_3$ plants. Role of photosystem I-dependent proton pumping

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**Abstract.** The role of cyclic electron transport has been re-examined in leaves of $C_3$ plants because the bioenergetics of chloroplasts ($H^+/e = 3$ in the presence of a Q-cycle; $H^+/ATP = 4$ of ATP synthesis) had suggested that cyclic electron flow has no function in $C_3$ photosynthesis. After light activation of pea leaves, the dark reduction of P700 (the donor pigment of PSI) following far-red oxidation was much accelerated. This corresponded to loss of sensitivity of P700 to oxidation by far-red light and a large increase in the number of electrons available to reduce $P700^+$ in the dark. At low $CO_2$ and $O_2$ molar ratios, far-red light was capable of decreasing the activity of photosystem II (measured as the ratio of variable to maximal chlorophyll fluorescence, $F_v/F_m$) and of increasing light scattering at 535 nm and zeaxanthin synthesis, indicating formation of a transthylakoid pH gradient. Both the light-induced increase in the number of electrons capable of reducing far-red-oxidised P700 and the decline in $F_v/F_m$ brought about by far-red in leaves were prevented by methyl viologen. Antimycin A inhibited $CO_2$-dependent $O_2$ evolution of pea leaves at saturating but not under limiting light; in its presence, far-red light failed to decrease $F_v/F_m$. The results indicate that cyclic electron flow regulates the quantum yield of photosystem II by decreasing the intrathylakoid pH when there is a reduction in the availability of electron acceptors at the PSI level (e.g. during drought or cold stresses). It also provides ATP for the carbon-reduction cycle under high light. Under these conditions, the Q-cycle is not able to maintain a $H^+/e$ ratio of 3 for ATP synthesis: we suggest that the ratio is flexible, not obligatory.

**Key words:** $C_3$ plant (electron transport) – Chlorophyll fluorescence – Cyclic electron transport – Photosynthesis – Photosystem I – Photosystem II

## Introduction

Almost 40 years ago, in classic studies, Daniel Arnon and co-workers discovered and characterised the light-dependent phosphorylation of ADP in what, at the time, were termed intact chloroplasts (Arnon et al. 1967; Arnon and Chain 1975). Using electron acceptors such as ferricyanide and-or electron carriers such as phenazine methosulfate they distinguished cyclic from non-cyclic photophosphorylation. The former was driven by PSI and inhibited by antimycin A (AA) whereas the latter required the cooperation of PSII and PSI and was not inhibited by AA. Later, it was shown that carbon assimilation of intact chloroplasts is sensitive to AA, suggesting that cyclic photophosphorylation contributes ATP for carbon reduction (Heber et al. 1978). When it had become established that a transthylakoid proton gradient can provide the energetic basis for the phosphorylation of ADP (Mitchell 1977), this view gained ground because linear electron transport appeared to provide insufficient ATP for carbon assimilation. Early measurements indicated that in linear electron transport the ratio of protons to electrons, the $H^+/e$ ratio, is 2, whereas the ratio of protons leaving the thylakoids during ATP synthesis, the $H^+/ATP$ ratio, was usually thought to be 3, although values of 2 and 4 were also published (Trebst 1974; Hall 1976). With $H^+/e = 2$ and $H^+/ATP = 3$, less than 3 molecules of ATP are synthesised when 4 electrons reduce 2 molecules of NADP and, finally, one molecule of $CO_2$. Since the ATP/NADPH ratio of carbon reduction on the Calvin cycle is 3, extra ATP was obviously required for carbon assimilation.

However, doubts arose when Peter Mitchell proposed a Q-cycle for electrons going through the cytochrome *b*/*c*

Abbreviations: AA = antimycin A; $F_v$ = variable chlorophyll fluorescence; $F_m$ = maximum chlorophyll fluorescence; FR = far-red light; MV = methyl viologen; P700 = donor pigment of PSI; PPFD = photosynthetically active photon flux density

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complex to oxygen during mitochondrial electron transport (Mitchell 1977). The chloroplast cytochrome *b*/*f* complex is very similar to the mitochondrial cytochrome *b*/*c* complex. In a Q-cycle, the $H^+$/e ratio is 2 for each electron passing the cytochrome on the way to a final acceptor. Since the oxidation of water by chloroplasts leaves a proton inside the thylakoids for each liberated electron, a Q-cycle would shift $H^+$/e from 2 to 3.

At the present state of information, $H^+/e = 3$ and $H^+/ATP = 4$ appear to be favoured values (Rumberg et al. 1990; Rich 1991; Kobayashi and Heber 1995). In this situation, as many protons are transported into the intrathylakoid space as are released from this space during ATP synthesis. This places restrictions on the magnitude of the transthylakoid proton gradient. Increased thylakoid acidification is required for the de-epoxidation of violaxanthin to zeaxanthin which, together with a low intrathylakoid pH, facilitates the efficient dissipation of excess excitation energy (Horton et al. 1996; Björkman and Niyogi 1998). This controls electron flow through PSII thereby preventing the photoinhibition of chloroplast electron transport. Increased thylakoid acidification may be brought about by different reactions. Candidates presently discussed are linear electron transport to oxygen in the Mehler reaction (Osmond and Grace 1995; Biehler and Fock 1996) and cyclic electron transport (Wu et al. 1990; Heber et al. 1995; Ivanov et al. 1998). Oxygen reduction is accompanied by the production of highly toxic oxygen species such as the superoxide radical and hydrogen peroxide which require rapid enzymatic detoxification, which is difficult to accomplish at low temperatures when enzymatic reactions are slowed down. Cyclic electron transport has no such restriction.

In the present work it was investigated whether cyclic electron flow occurs in $C_3$ plants, and whether coupled cyclic electron transport around PSI can control the activity of PSII.

## Materials and methods

*Plant material.* *Pisum sativum* L. was grown in a growth chamber (day/night cycle of 16 h/8 h at 22 °C during the day and 18 °C during the night) or outdoors, and *Spinacia oleracea* L. in a greenhouse (day/night cycle of 11 h/13 h). Leaves of *Prunus avium* L. or branches from *Thuja occidentalis* L. were from trees growing in the Botanical Garden of the University of Würzburg.

*Chlorophyll fluorescence and P700 oxidation.* Modulated chlorophyll fluorescence and the redox state of P700, the donor pigment of PSI were monitored either as fluorescence emission in the red region or as 820 nm absorption of the cation radical of P700 by a pulse amplitude modulation fluorometer (Walz, Effeltrich, Germany), using suitable emitter-detector units (Schreiber et al. 1986, 1988). A broad or a narrower band of red light [filters: RG 630 (Schott, Mainz, Germany) and Calflex X (Balzers, Liechtenstein) for the broad band, with the addition of DT-Cyan (Balzers) for the narrow band] from a halogen source was used for actinic illumination. The half bandwidth of the broad band ranged from 630 to 760 nm and that of the narrow band from 630 to 692 nm. Far-red light capable of exciting mainly PSI was provided by an RG9 filter (Schott) and a Calflex C filter (Balzers). The half bandwidth was from 710 to 760 nm. At the highest PFD possible, PSII excitation by a tail component of the far-red light (FR) caused a low rate of carbon assimilation (0.8 μmol $m^{-2}$ $s^{-1}$ or about 5% of maximum carbon assimilation in air). The FR quantum fluxes were measured using a custom-calibrated S 1336–44BK photodiode (Hamatsu; kindly provided by Bernard Genty Université de Paris XI, Lab. d'Ecophysiologie Végétale, Orsay, France). The FR light absorbed by PSI was estimated from the absorption spectra of the leaves.

The number of electrons available to reduce $P700^+$ was determined as follows. Single (ST) and multiple (MT) turnover flashes from XST 103 and XMT 103 lamps (Walz) were applied to transiently reduce photooxidized P700 with electrons from PSII (50-ms MT flashes were shown to largely saturate the available intersystem electron carriers with electrons). After the flashes, the background FR reoxidized P700. Rates of reoxidation and areas formed by reduction and oxidation were measured as in Asada et al. (1992) and the area ratios were related to one another. The ratio of the MT area to the ST area represents the functional pool of intersystem electrons per PSII reaction center.

*Gas exchange.* Exchange of $CO_2$ and $H_2O$ was measured using a Binos infrared gas analyzer (Haereus, Hanau, Germany). Gas flow through a sandwich-type cuvette which enclosed part of a leaf was 500 ml $min^{-1}$. Oxygen evolution was measured with a leaf-disc oxygen electrode (Hansatech, Norfolk, UK). Different $CO_2$ molar ratios in air were provided, using mass-flow controllers, by mixing gases from cylinders. The flow rate passing through the electrode was 20 ml $min^{-1}$. Care was taken to maintain $CO_2$ at a saturating molar ratio during the oxygen measurements (from 3 to 4% $CO_2$). Light was provided by a LS2 white light source (Hansatech). The photosynthetically active photon flux density (PPFD) was changed using neutral filters.

*Light scattering and 505-nm absorption changes.* Changes in light scattering by leaves were recorded as changes in apparent absorbance (Heber 1969). Absorption changes at 505 nm served to monitor changes in the zeaxanthin content of leaves (Bilger and Björkman 1990).

*Uptake of inhibitors.* Methyl Viologen (MV) was infiltrated into the apoplast of leaves using the technique described in Jakob and Heber (1998). Excess infiltrate was removed by centrifugation. Carbon assimilation and transpiration indicated unimpaired gas exchange in control experiments in which water was used for infiltration. In some cases atrazine uptake by leaves was accelerated by wilting the leaves for 3 h before placing them on a solution of $5 \times 10^{-4}$ M atrazine. After feeding atrazine, the leaves had once again become turgescent. A similar procedure ensured the uptake of diuron and AA. In the case of AA, a 0.5 μM solution was used and the approximate concentration inside the leaves was estimated from the amount of solution taken up and the water content of the leaves. Alternatively, AA (2 μM) was infiltrated just like MV.

Whenever dark adaptation of leaves was required, care was taken to use leaves only after at least 4–5 h darkening.

## Results

*Photooxidation of P700 under FR as influenced by previous illumination of leaves with actinic light.* A 5-min exposure of dark-adapted pea leaves to strong actinic illumination caused a large decrease in steady-state P700 oxidation by low-intensity FR (Fig. 1). Reduction in the dark after oxidation by FR was accelerated. The extent of the decreased photooxidation by FR was a function of the intensity of the previous actinic illumination and of the PFD of FR (Fig. 2A). The effect of actinic illumination on the sensitivity of P700 to oxidation by FR was much diminished at 24 °C

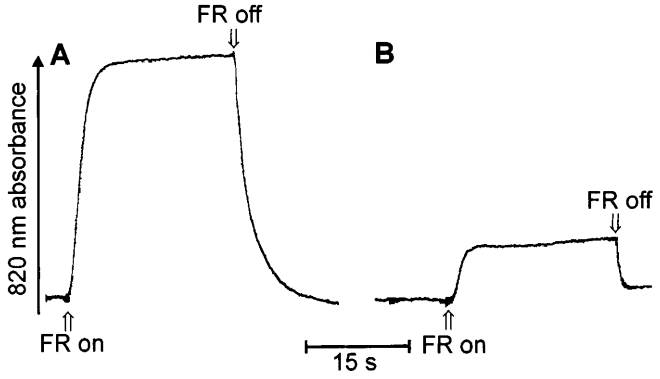


**Fig. 1A,B.** Photooxidation of P700 (as shown by increased absorption at 820 nm) by low-intensity FR before (**A**) and after (**B**) a 5-min exposure of a dark-adapted pea leaf to a PPFD of 2300 μmol $m^{-2}$ $s^{-1}$. Steady-state oxidation is decreased and dark reduction accelerated after exposure to actinic light

after electron flow between PSII and PSI had been inhibited by atrazine (Fig. 2B) or diuron (not shown).

A 2-min exposure to actinic light was enough to saturate the loss of sensitivity to P700 photooxidation measured under low FR (Fig. 3). High sensitivity of P700 to photooxidation was recovered slowly. It is interesting to note that a 1-min exposure of the leaf to

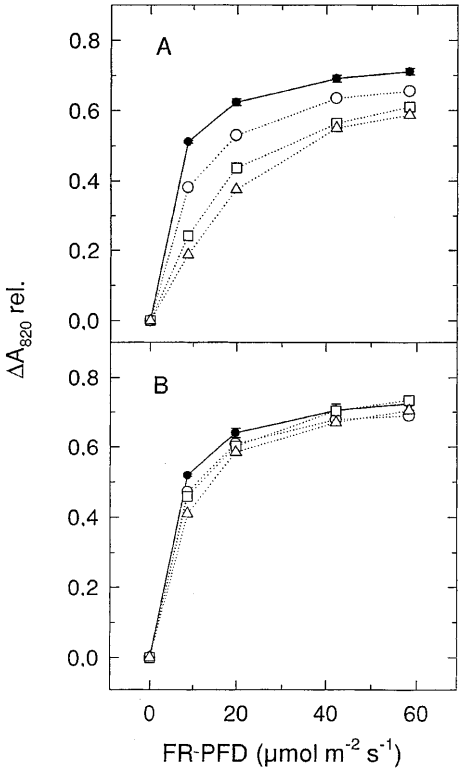


**Fig. 2A,B.** Steady-state P700 photooxidation as shown by $\Delta A_{820}$ as a function of the intensity of FR after long predarkening of a pea leaf or after 5 min exposure of the leaf to different PPFDs of actinic light (**A**). Atrazine (**B**) prevents the modulation by actinic light of subsequent P700 photooxidation by FR. Data from a representative experiment. ●, darkened leaf; ○, after PPFD = 770 μmol $m^{-2}$ $s^{-1}$; □, after PPFD = 1500 μmol $m^{-2}$ $s^{-1}$; Δ, after PPFD = 2000 μmol $m^{-2}$ $s^{-1}$

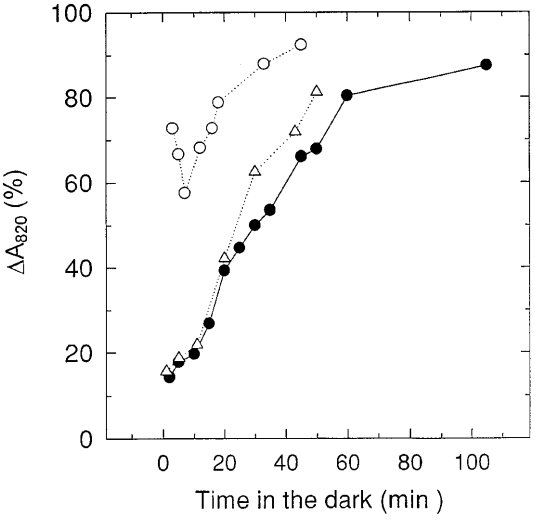


**Fig. 3.** Recovery of sensitivity of P700 to photooxidation by FR after exposure of a dark-adapted leaf of *Pisum sativum* to a PPFD of 1080 μmol $m^{-2}$ $s^{-1}$ for 1 min (○), 2 min (△) and 5 (●) min. Data in percent of the ΔA820 observed before exposure of a pea leaf to actinic light

actinic light produced a substantial loss of sensitivity to P700 photooxidation which continued for 5 min in the dark before recovery set in. Continuous actinic illumination was not necessary to cause desensitization of P700 to oxidation by FR: strong light pulses lasting 1 s separated by 30-s dark periods were sufficient for an appreciable response (data not shown).

The results suggest that the reduction in the $P700^+$ signal is due to the production of reducing equivalents during the period of actinic illumination since atrazine prevented the decline in the FR signal.

Figure 4 shows the extent of P700 photooxidation in a pea leaf which was illuminated in air containing either 350 or 45 μl $l^{-1}$ $CO_2$. After having reached the steady state at different PPFDs, the leaf was briefly darkened. The resulting rapid reduction of $P700^+$ permitted the

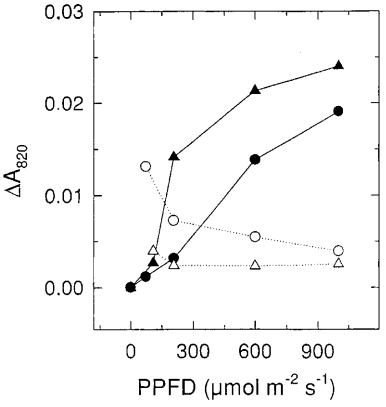


**Fig. 4.** Steady-state P700 photooxidation in a leaf *of Pisum sativum* as a function of actinic illumination in air containing either 350 (●) or 45 (▲) μl $l^{-1}$ $CO_2$ (*solid lines*) and subsequent P700 photooxidation after actinic illumination by a constant subsaturating intensity of FR [350 (○) or 45 (△) μl $l^{-1}$ $CO_2$, *dotted lines*]

**Table 1.** First order reaction constants of the dark reduction of $P700^+$ after oxidation by FR. Effects of the previous exposure of a dark-adapted leaf to 7 min illumination with PPFDs ranging from 60 to 640 μmol $m^{-2}$ $s^{-1}$ actinic light. One min after the actinic light was turned off, oxidation of P700 by FR was attained and then the kinetics measured in the dark. For treatments with MV, leaf segments were punched out and incubated for 5 h in the dark on a solution of 5 mM MV. All experiments were done with individual segments of the same leaf

| | Untreated leaf segments | | | MV-treated leaf segments | | |
|---|---|---|---|---|---|---|
| | Slow reaction (% of total) | $k_{slow}$ ($s^{-1}$) | $k_{fast}$ ($s^{-1}$) | Slow reaction (% of total) | $k_{slow}$ ($s^{-1}$) | $k_{fast}$ ($s^{-1}$) |
| Dark-adapted leaf | 64 | 0.064 | 0.30 | 80 | 0.056 | 0.33 |
| Light, 60 μmol $m^{-2}$ $s^{-1}$ | 52 | 0.13 | 0.68 | 84 | 0.046 | 0.36 |
| 180 μmol $m^{-2}$ $s^{-1}$ | 55 | 0.32 | 1.47 | 82 | 0.046 | 0.29 |
| 640 μmol $m^{-2}$ $s^{-1}$ | 65 | 0.43 | 2.17 | 82 | 0.045 | 0.30 |

measurement of the extent of P700 photooxidation in the light. Photooxidation of P700 increased in a sigmoid fashion with increasing intensities of actinic light.

After 60 s in the dark, the leaf received a constant PFD of subsaturating FR. As expected from Fig. 1, steady-state oxidation levels under FR illumination varied according to the PPFDs of previous exposures to actinic light. They were low when P700 oxidation under strong actinic illumination had been considerable, and they were high when the actinic light had been insufficient to produce much P700 photooxidation.

When the experiment, which was initially performed at a $CO_2$ concentration of 350 μl $l^{-1}$, was repeated with the same leaf, but at a $CO_2$ molar ratio approximating that of the $CO_2$ compensation point (45 μl $l^{-1}$), more P700 was photooxidized under actinic illumination compared to photosynthesis at 350 μl $l^{-1}$ $CO_2$, suggesting increased control of electron flow at the levels of PSII and of the cytochrome *b*/*f* complex. In contrast, steady-state P700 oxidation under subsequent FR illumination was reduced compared to the experiment in which 350 μl $l^{-1}$ $CO_2$ had supported considerable net carbon reduction.

*Kinetics of the dark reduction of photooxidized P700; effect of MV*. After P700 had been photooxidized by FR, semilog plots of dark reduction versus time revealed two phases of recovery of reduced P700, usually a smaller fast phase followed by a larger slow phase. Occasionally both phases were comparable in magnitude. Both obeyed first-order kinetics. The fast phase was not due to electrons coming from PSII as it was insensitive to 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) or atrazine (Manuel et al. 1999). In the experiments described in Table 1, dark-adapted leaves were first submitted to 7 min of actinic illumination followed by 1 min in the dark before FR was turned on. After steady-state oxidation of P700 was attained, FR was turned off and the kinetics of dark reduction of P700 were measured. The rate constants of both phases increased as the PPFD of the actinic light was increased. The acceleration of the reduction of photooxidized P700 after dark-adapted leaves had been exposed to actinic irradiation explains why the extent of steady state photooxidation of P700 by FR was decreased after leaves had been illuminated (Figs. 1–4).

Brief actinic light pulses separated by 30-s dark periods were also sufficient to increase the rate constant of the two phases measured after FR illumination (not shown).

It is known that MV catalyses a rapid light-dependent depletion of chloroplast reductants including NADPH (Ort and Izawa 1973). In the presence of air levels of oxygen it is effective in suppressing cyclic electron transport in isolated intact chloroplasts (Kobayashi and Heber 1994; Ivanov et al. 1998). Table 1 shows that in leaves it also prevents the acceleration of the dark reduction of FR-oxidized $P700^+$ after the leaves had been illuminated with actinic light.

*Control of PSII by PSI*. The experiments shown in Fig. 5 were done with pea leaves at 1 °C to decrease traffic of electrons to downstream acceptors. In this situation, cyclic electron flow is expected to be favoured. Flashes of actinic light (1 s) given every 30 s were used to measure the quantum yield of PSII. Figure $5A_1$ shows that in 21% $O_2$ FR caused a strong and transient quenching of maximum chlorophyll fluorescence ($F_m$) even though it is only weakly absorbed by the leaf. The ratio of variable to maximal chlorophyll fluorscence ($F_v/F_m$) decreased from 0.76 at the beginning of FR illumination to a minimum of 0.64. In this situation the 1-s flashes were effective at keeping the intersystem electron-carrier system partially reduced even under FR, as shown by the slow and incomplete relaxation of chlorophyll fluorescence towards steady-state fluorescence after the flashes. When increased oxidation of intersystem electron carriers was indicated by a fast decline in flash-induced fluorescence, $F_v/F_m$ increased, indicating loss of electrons to an external acceptor, perhaps oxygen. In this situation, electrons are drained from the cyclic pathway. Observations similar to those made in the experiment of Fig. $5A_1$ for a pea leaf were also made with leaves of *Spinacia oleracea*, *Prunus avium* and *Thuja occidentalis* (not shown).

When the oxygen molar ratio of the atmosphere was not 21% but 1% (Fig. $5B_1$), or when air was replaced by nitrogen (data not shown), the suppression of charge separation in PSII by FR was a much more permanent effect than shown in Fig. $5A_1$ ($F_v/F_m$ decreased from 0.75 to 0.56). In Fig. $5B_1$, nonphotochemical fluorescence quenching relaxed only after admission of 21% oxygen.

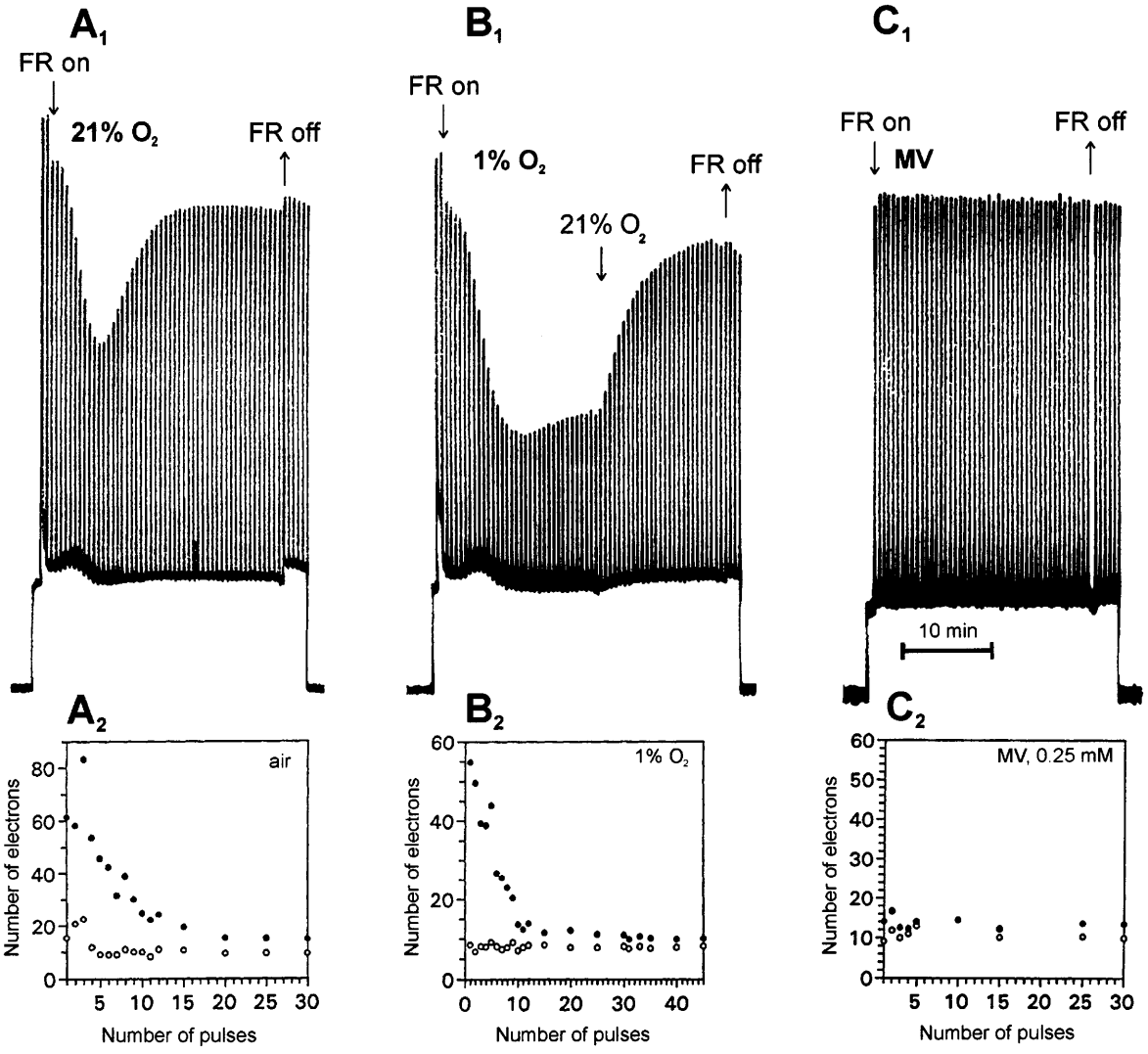


**Fig. 5.** **$A_1$** Recordings of modulated chlorophyll fluorescence emitted by a pea leaf under the influence of FR illumination in air at 1 °C. Saturating light pulses of 1 s were given once every 30 s. Fluorescence was excited by very weak red light (PPFD 1.5 μmol $m^{-2}$ $s^{-1}$). Absorbed FR was about 25 μmol $m^{-2}$ $s^{-1}$. **$A_2$** Number of electrons passing through PSI per 50-ms flash (○) and per 1-s light pulse (●). **$B_1$** Same as **$A_1$**, but 1% oxygen initially; 21% oxygen was admitted as indicated. **$B_2$** Same as **$A_2$**, but 1% oxygen initially; 21% oxygen was admitted as indicated. **$C_1$** Same as **$A_1$**, but leaf infiltrated with 250 μM MV for several min. Excess infiltrate was removed by centrifugation. **$C_2$** Same as $A_2$, but leaf treated with MV

It is important to emphasize that the effects demonstrated in Fig. $5A_1$,$B_1$ indicate control of PSII by coupled cyclic electron transport around PSI rather than being a consequence of residual PSII excitation by FR. To rule out the latter possibility, the very slow rate of carbon reduction possible under FR illumination was measured at 1% oxygen and 380 μl $l^{-1}$ $CO_2$. Subsequently, the intensity of short-wavelength red light was adjusted so as to produce the same low rate of carbon reduction. When used instead of FR, this short-wavelength band, unlike FR (Fig. $5A_1$,$B_1$), failed completely to decrease charge separation in PSII (data not shown).

After leaf disks were infiltrated with 0.25 mM MV and the infiltrate was removed by centrifugation, FR was no longer effective in decreasing charge separation in PSII in air (Fig. $5C_1$). This experiment also shows that, under our conditions, the linear electron flow supported by MV could not create a ΔpH large enough for energy-dependent fluorescence quenching.

After a leaf was infiltrated with 2 μM AA and excess AA was removed, flashing experiments similar to those shown in Fig. 5 yielded results that resembled those shown for the MV-treated leaf. Whereas MV diverted electrons from the cyclic pathway, AA was effective in inhibiting cyclic electron transport.

*Effects of MV and temperature on the number of electrons available for oxidation by FR.* Figure $5A_2$ shows that the number of electrons available for the reduction of $P700^+$ in the intersystem chain of a dark-adapted leaf was usually about 10 after a 50-ms saturating actinic pulse. This was calculated according to Asada et al. (1992). After a 1-s light pulse, this number rose to 60, which is far beyond the capacity of the intersystem pool of electron carriers for electron donation. Apparently,

electrons were cycling around PSI. As the dark-adapted leaf continued to receive 1-s light pulses in air, the number of cycling electrons decreased. Simultaneously, control of PSII increased as shown in Fig. $5A_1$. As nonphotochemical quenching of PSII fluorescence approached its transient maximum, the number of electrons liberated by the 1-s light pulses had decreased to about 15. This lower number was maintained even while nonphotochemical fluorescence quenching slowly relaxed, indicating opening of the acceptor side of PSI. This permitted some linear electron transport to occur. This electron transport, by itself insufficient to maintain control of PSII, appeared to inhibit coupled cyclic electron transport.

When the experiment was repeated with a leaf that was kept in 1% oxygen (Fig. $5B_2$), the number of cycling electrons decreased in a fashion similar to that shown in Fig. $5A_2$ while control of PSII was more pronounced than in the air experiment of Fig. $5A_1$. Admission of 21% oxygen slightly reduced the number of electrons liberated by a flash while control of PSII relaxed.

After MV infiltration of a leaf, the number of electrons liberated after a dark-adapted leaf received light pulses was much decreased compared to that shown in Fig. $5A_2$ and $B_2$.

Similar measurements were also performed after steady-state illumination of a pea leaf (Fig. 6). Whereas about 11 electrons were available in the intersystem pool of electron carriers to reduce photooxidized P700 after a 50-ms flash, both at 1 and 25 °C, a similar number reduced FR-oxidized P700 only immediately after weak steady-state illumination. Stronger actinic light made more electrons available. After exposure for 15 min to PPFDs close to sunlight, the number of electrons passing through PSI was three times larger than the number of electrons released by a 50-ms saturating flash. Low temperature increased this number and brief infiltration of a leaf with 250 μM MV reduced it to or below the number measured by a saturating 50-ms flash.

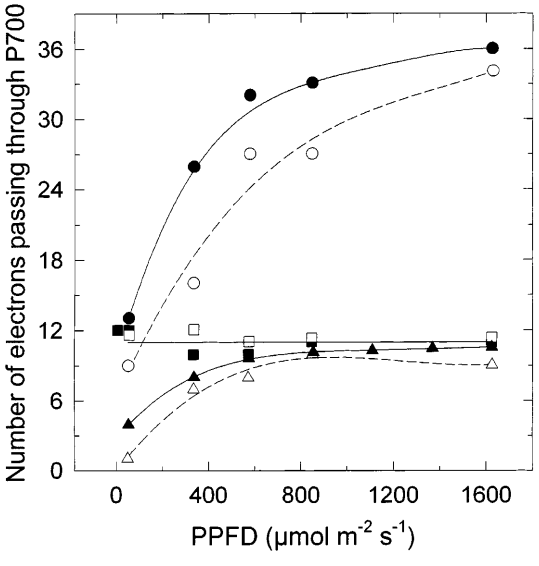


**Fig. 6.** Number of electrons passing through PSI of a pea leaf either immediately after exposure (15 min) to the PPFDs shown on the abscissa (*circles*) or after a saturating 50-ms flash following light adaptation (*squares*). The experiments were performed either at 1 °C (*closed symbols*) or at 25 °C (*open symbols*). *Triangles* show the number of electrons going through PSI after the leaf had been infiltrated with 250 μM MV. Excess infiltrate was removed by centrifugation

*Relation between light scattering and the control of PSII by PSI.* Figure 7 shows changes in zeaxanthin content as indicated by 505 nm absorbance and in light scattering at 535 nm of a leaf in parallel with changes in the control of PSII as measured by modulated chlorophyll fluorescence at 20 °C. The changes were produced under illumination with FR (that contained a small addition of red light) by altering the gas composition of the atmosphere. Far-red illumination neither changed zeaxanthin content nor light scattering of a leaf maintained in air containing 380 μl $l^{-1}$ $CO_2$ while causing only insignificant changes in $F_m$ (not shown). Also, removal of $CO_2$ had almost no effect at 21% oxygen. However, decreasing not only assimilatory but also photorespiratory electron transport by lowering the $O_2$ molar ratio to 1% increased zeaxanthin and light

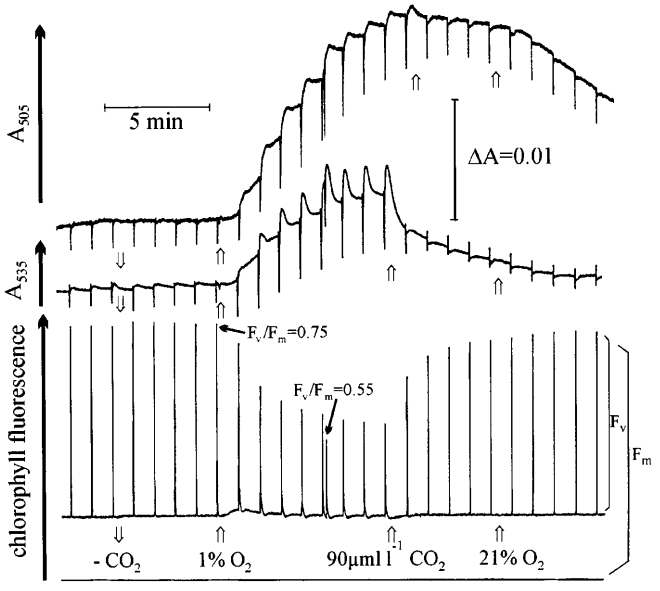


**Fig. 7.** Simultaneous recordings of light scattering as measured by changes in the apparent absorbance of a leaf at 535 nm (middle trace) and of modulated chlorophyll fluorescence (lower trace). The upper trace shows changes in 505-nm absorbance in a subsequent experiment performed with the same leaf. The slow component in 505 nm absorbance indicates changes in zeaxanthin levels. Illumination was with strong FR (absorbed PFD about 25 μmol $m^{-2}$ $s^{-1}$) plus weak red light (PPFD 1.5 μmol $m^{-2}$ $s^{-1}$). The leaf also received strong 1-s red light pulses (2500 μmol $m^{-2}$ $s^{-1}$) every 60 s. Temperature was 20 °C. The experiments started with 1% $O_2$ without $CO_2$, then 90 μl $l^{-1}$ $CO_2$ were added followed by an elevation of $O_2$ to 21%. Carbon assimilation in air containing 380 μl $l^{-1}$ $CO_2$ was 1.1 μmol $m^{-2}$ $s^{-1}$ or about 7% of its light-saturated rate at the beginning of the experiment. Owing to the compensation of respiration, assimilation was not negligible at 1% $O_2$ even when $CO_2$ was removed (about 0.4 μmol $m^{-2}$ $s^{-1}$). Fast negative spikes in $A_{535}$ are unrelated to the much slower light scattering changes. In $A_{505}$, negative spikes are caused in part by a light leak

scattering. Simultaneously, $F_m$ decreased. Under these conditions the 1-s pulses of strong red light caused appreciable light-scattering transients which were correlated with particularly strong PSII control (see the effect of an additional light pulse during this phase): As a consequence the ratio $F_v/F_m$ then decreased from initially 0.75 to 0.55. Apparently, the light pulses injected electrons into the electron transport chain. This contributed to FR-dependent cyclic electron flow around PSI (Katona et al. 1992) and lowered the intrathylakoid pH as seen by stepwise increases in zeaxanthin (upper trace in Fig. 7) and increased scattering (middle trace). Together with zeaxanthin, the low intrathylakoid pH facilitated the radiationless dissipation of absorbed light energy (Niyogi 1999) which is shown as decreased $F_v/F_m$ (Fig. 7, lower trace).

Adding 90 $\mu l\, l^{-1}$ $CO_2$ to the atmosphere diverted electrons from the cyclic pathway to $CO_2$. It caused an immediate increase in $F_m$ and a decrease in light scattering but not zeaxanthin levels. Increasing the oxygen concentration to 21% had only small additional effects on light scattering and chlorophyll fluorescence, but it decreased zeaxanthin levels. It is known that the epoxidation of zeaxanthin to violaxanthin requires oxygen.

Figure 7 shows that reducing the availability of electron acceptors stimulates both coupled cyclic electron transport and zeaxanthin synthesis. Together, they control the activity of PSII. Zeaxanthin alone is ineffective.

*Inhibition of $CO_2$-dependent oxygen evolution of pea leaves by AA*. Figure 8 shows $CO_2$-dependent oxygen evolution of pea leaves as a function of incident PPFD. Leaflets from the same leaf were either used as controls or were permitted to take up some AA, a known inhibitor of cyclic electron transport. The approximate AA concentration inside the leaf, calculated from solute uptake and assuming homogeneous repartition in different leaf compartments, was not higher and probably appreciably lower than 0.15 μM. Photosynthesis was partially inhibited by AA only at high PPFDs. At limiting light it remained unchanged. This observation is similar to the response of protoplasts to AA reported by Furbank and Horton (1987). It strongly suggests that cyclic electron flow provides ATP to the photosynthetic carbon-reduction cycle at high, but not at low light intensities. Apparently, ATP becomes limiting for carbon reduction only at high light. Such limitation would be surprising if the Q cycle were obligatory. At presently accepted stoichiometric $H^+/e$ and $H^+/ATP$ ratios, linear electron transport provides ATP and NADPH at a ratio of 1.5/1 (Rumberg et al. 1990; Rich 1991; Horton et al. 1996). This is also the ratio of consumption of ATP and NADPH by the carbon cycle and by photorespiration. Thus, together with the earlier results of Heber et al. (1978) and Furbank and Horton (1987), the experiment of Fig. 8 indicates that the Q cycle is not able to maintain $H^+/e = 3$ when protons must be transported into the intrathylakoid space against a high internal proton concentration.

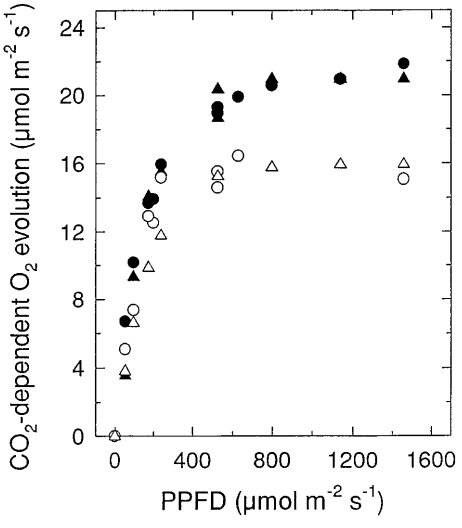


**Fig. 8.** Carbon dioxide-dependent $O_2$ evolution of pea leaflets as a function of incident PPFD. One leaflet served as control while the opposite one was treated with AA: in this case the detached leaflet was first allowed to wilt in dim light. After loss of about 30% of its water it was permitted to rehydrate on 0.5 μM AA solution. Controls ensured that the rates of $CO_2$-dependent $O_2$ evolution of opposite leaflets of a leaf were similar. Different symbols refer to measurements on two different plants. *Open symbols*, treated leaflets; *closed symbols*, control leaflets. The same experiment repeated on younger plants (having two to four leaves) yielded similar results. The temperature was 25 °C and the $CO_2$ molar ratio in the air was saturating (from 3 to 4%)

## Discussion

*Cyclic electron flow operates in leaves of $C_3$ plants*. After light activation of leaves, dark reduction of $P700^+$ (by electrons from two kinetically distinguishable sources) following oxidation by FR is much accelerated (Table 1). Apparently, prior exposure of leaves to actinic illumination opens a path for electrons from the reducing side of PSI back to the oxidizing side. Under FR, this permits cyclic electron transport to occur and explains the decreased sensitivity of steady-state oxidation of P700 under FR (Figs. 1, 2). As 60 s of actinic light was enough to produce a substantial decline in the effect of limiting FR on leaf absorbance at 820 nm (Fig. 3), we argue that the decline in the PSI signal cannot be attributed to photoinhibition as concluded by Havaux and Davaud (1994) for potato leaves. In the presence of atrazine there was no effect of actinic light on the subsequent dark reduction of PSI by FR (Fig. 2). It is concluded that PSII activity is necessary to activate this path which is fully operational after only a few minutes of light (Fig. 3).

The sensitivity of P700 to FR recovers slowly in the dark, suggesting that the pools of electrons available for PSI reduction decrease under these conditions. Feild et al. (1998) measured an increase of $F_o$ (the basic level of chlorophyll fluorescence usually associated with an oxidized plastoquinone pool) in the dark starting about 200 s after an actinic light period. They attributed it to an increased amount of $Q_A^-$ as a consequence of plastoquinone reduction in the dark during chlororespi-

ration. On a kinetic basis it is possible to distinguish between the plastoquinone dark reduction they observed and what we are reporting here since Fig. 3 shows that the reducing equivalent available for the reduction of FR-oxidized P700 decreased in the dark after the actinic light was turned off.

Consistently, the number of electrons available to $P700^+$ during strong actinic light is more than the number measured in the intersystem chain. This higher number increased with actinic quantum flux both at 25 and 1 °C (Fig. 6). This is similar to what was reported by Asada et al. (1992) and demonstrates that components from the reducing side of PSI donate electrons to $P700^+$. Results on isolated intact chloroplasts in which AA was used to inhibit cyclic electron flow show indeed that cyclic electron flow is increased when quantum flux is increased (Woo 1983). Methyl Viologen prevents the light-induced increase in the availability of electrons to $P700^+$ since it depletes chloroplast reductants (Fig. $5C_2$).

As shown by Genty et al. (1990), there is a near linear relationship between the quantum efficiencies of PSI and PSII in carbon assimilation. This could be explained by an absence of cyclic electron transport. However, it could also mean that cyclic electron transport is coupled to the rate of linear electron flow to $CO_2$ (Kramer and Crofts 1996). Gerst et al. (1995) have shown that there is a large departure from a linear relationship under $CO_2$ depletion when the stomata of a leaf are closed. We conclude that cyclic electron flow can be operative in vivo in $C_3$ plants. For a full understanding of the coupling between this flux and the linear fluxes information about the redox situation of the stromal NADP system is required (Ivanov et al. 1998).

*Cyclic electron flow leads to a decrease in lumenal pH.* There are two main mechanisms by which light-activated cyclic electron flow could occur. One is charge recombination that would not be accompanied by energy conservation via the formation of a transthylakoid proton gradient. The other one is coupled cyclic electron transport.

The experiments documented in Figs. $5A_1$,$B_1$ and 7 strongly argue in favour of electron flow coupled to the formation of a transthylakoid proton gradient. Far-red light was capable of both suppressing charge separation in PSII and of inducing considerable nonphotochemical fluorescence quenching. This effect was more pronounced at a low oxygen concentration or under nitrogen than in air. Measurements of the transfer of electrons through PSI after the 1-s light pulses, which served simultaneously to indicate the state of PSII by the response of modulated chlorophyll fluorescence and to supply electrons to the intersystem pool of electron acceptors, clearly indicated cycling of electrons around PSI (Fig. $5A_2$,$B_2$). Again, the number of electrons going from the oxidizing to the reducing side of PSI was far larger than the number of electrons supplied by PSII to the intersystem electron carriers (Fig. $5A_2$,$B_2$). The cyclic electron transfer occurring transiently under FR illumination in air, and even more clearly at low oxygen, was coupled to vectorial proton transfer into the intrathylakoid space. This conclusion is based on a large body of published evidence showing that the nonphotochemical fluorescence quenching demonstrated in Fig. $5A_1$,$B_1$ requires the formation of a transthylakoid proton gradient (for example, see reviews by Kramer and Crofts 1996; Owens 1996). Quenching relaxed in air when a low steady-state fluorescence level indicated opening of the acceptor side of PSI (Fig. $5A_1$). This suggests that the Mehler reaction could have a poising role in $C_3$ leaves rather than a direct one in controlling PSII activity. Apparently, by itself, the Mehler reaction could not support a proton gradient sufficient to suppress charge separation in PSII under our experimental conditions whereas FR was effective as long as the reducing side of PSI was sufficiently closed to prevent photoassimilatory or photorespiratory electron transport.

The capacity of cyclic electron flow to lower the intrathylakoid pH is also shown in Fig. 7 where nonphotochemical fluorescence quenching was measured by giving actinic light pulses of 1 s duration every 60 s in the presence of FR. An increase in light scattering occurred together with an increase in nonphotochemical fluorescence quenching when the $O_2$ molar ratio was shifted from 21% to 1%. Of particular interest is the demonstration that there was a strongly increased control of PSII during the large and rapid increase in the light-scattering transient which was caused by the 1-s high-light pulse. This clearly demonstrated the efficiency of the light flash to induce a $\Delta$pH in combination with FR background light. Increased light scattering requires the presence both of zeaxanthin and $\Delta$pH (Björkman and Niyogi 1998).

*Roles for cyclic electron flow in $C_3$ plants.* For an understanding of the role of cyclic electron transport in $C_3$ photosynthesis under field conditions, redox poising in vivo must be considered. The need for a balanced redox situation was shown decades ago by Arnon (1969) and has been confirmed by many studies on isolated intact chloroplasts.

Deductions are possible from experiments such as those shown in Figs. 5 and 7. As cyclic electron flow is favoured when the availability of electron acceptors in the stroma limits linear electron flow, its main role will be to downregulate PSII through a build-up of a low lumenal pH rather than to provide ATP which is less needed in this situation (Heber and Walker 1992). Figure 4 shows that, at a $CO_2$ molar ratio which roughly approximates that of a $C_3$ leaf $CO_2$ compensation point, actinic light was more effective to oxidize P700 at very low levels of $CO_2$ than at air levels of $CO_2$ whereas, after a period of actinic illumination, the opposite was true for FR. We conclude that at low $CO_2$ coupled cyclic electron transport contributed more to total electron transport than at high $CO_2$. By its contribution to the transthylakoid proton gradient it controlled PSII so that under actinic light P700 could remain in a more oxidized state than at air levels of $CO_2$. Thus we expect the cyclic electron flow to play a crucial role in the regulation of PSII quantum yield in dehydrating leaves where the $CO_2$

molar ratio is low in the light because of stomatal closure (Katona et al. 1992; Cornic and Massacci 1996). In fact, Gerst et al. (1995) have shown that during stomatal closure of a dehydrating leaf the activity of PSI decreases much less than that of PSII.

Strong oxidation of P700 is known to exist in sunlight, even in the cold, while the acceptor side of PSII is largely reduced (Manuel et al. 1999), net carbon assimilation is slow and photorespiration may be absent. Figure 6 shows that under these conditions more electrons actually passed through PSI than at 25 °C when steady-state actinic illumination was turned off and electron donation from PSII suddenly ceased. Their number was far above electron availability within the pool of electron acceptors between PSII and PSI. These electrons were not available when MV served as electron acceptor (Fig. $5C_2$). From the experiments shown in Fig. $5A_1$,$B_1$ it must be concluded that these electrons were cycling around PSI, and that they contributed to the control of the activity of PSII.

During sunny, frosty periods in winter, the stomata of evergreens are firmly closed, restricting entry of external electron acceptors. Strong excitation of PSI and limited availability of electron acceptors are the conditions which permit cyclic electron flow to occur. In the experiments of Fig. 5, strong light had to be replaced by much less effective FR to demonstrate cyclic electron transport unequivocally in leaves, because sunlight makes it impossible to distinguish between the effects of linear and cyclic electron transport. In other respects, strong oxidation of P700 and limited availability of electron acceptors are mimicked in the experiments of Fig. 5. As long as electrons were cycling around PSI, charge separation in PSII was suppressed.

Apart from this role in the regulation of PSII photochemistry, cyclic electron flow around PSI also appears to provide energy for $CO_2$ fixation. Figure 8 shows inhibition of carbon assimilation by AA at high, but not at low PPFDs. Furbank and Horton (1987) have published very similar observations for barley protoplasts, and Heber et al. (1978) for isolated intact chloroplasts. The data had been interpreted to show that linear electron transport is unable to provide sufficient ATP for carbon assimilation when rates of photosynthesis are high. Later, it was shown that a Q-cycle permits deposition of protons inside the thylakoids during linear electron transport at a ratio of $H^+/e = 3$ (Rich 1991; Kobayashi and Heber 1995). As chloroplast ATP synthesis appears to require four protons per ATP (Rumberg et al. 1990), an obligatory Q-cycle should be able satisfy the ATP requirements of carbon assimilation. Since this is not the case at high rates of assimilation, we take the observations as evidence that the Q-cycle is not obligatory but flexible.

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