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Journal:	<i>Functional Plant Biology</i>
Manuscript ID:	FP13010.R1
Manuscript Type:	Research paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Kou, Jiancun; Northwest A&F University, College of Animal Science & Technology Takahashi, Shunichi; Australian National University, Research School of Biology Oguchi, Riichi; Australian National University, Research School of Biology Fan, Da-Yong; The Chinese Academy of Sciences, Institute of Botany Badger, Murray; Australian National University, Research School of Biology Chow, Wah Soon; Australian National University, Research School of Biology
Keyword:	Photosystem I, Photosynthesis, Irradiance, Water stress physiology, Temperature stress

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Estimation of the steady-state cyclic electron flux around Photosystem I in spinach leaf discs in white light, CO₂-enriched air and other varied conditions

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Running title: Estimation of cyclic electron flux around PS I

Abbreviations: ATP, adenosine triphosphate; CEF, cyclic electron flux around Photosystem I; Chl, chlorophyll; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; ETR1, the total electron transport rate through PS I; ESC, electrochromic signal; f_i , the fraction of absorbed light partitioned to PS I; F_m or F_m' , maximum fluorescence in the fully relaxed dark state or in the light; F_o or F_o' , minimum fluorescence in the fully relaxed dark state or in the light; F_s' , relative steady-state Chl fluorescence yield in the light; F_v or F_v' , variable fluorescence in the fully relaxed dark state or in the light, where $F_v = F_m - F_o$ and $F_v' = F_m' - F_o'$; F_v/F_m or F_v'/F_m' , ratio of variable to maximum Chl fluorescence yield in the dark-relaxed state or during illumination, respectively; FR, far-red light; LEF_{Fl} , the linear electron transport rate through PS II estimated by Chl fluorescence; LEF_{O_2} , the linear electron flux through PS II estimated by gross O_2 evolution; MV, methyl viologen; NAD(P), nicotinamide adenine dinucleotide (phosphate); NPQ, non-photochemical quenching; P700, special chlorophyll dimer acting as the primary electron donor in PS I; PAM, pulse amplitude modulation fluorometer; ϕ_{NPQ} , the fraction of light absorbed by the PS II antennae that is dissipated thermally in a light-dependent manner; $\phi_{PS II}$, photochemical yield of PS II; P_m , P_m' , maximum P700⁺ signal in weak FR light or actinic light, respectively; Q_A , primary quinone electron acceptor in PS II; qP , photochemical quenching parameter; RWC, relative water content; Y(I), photochemical yield of PS I.

Abstract

Cyclic electron flux (CEF) around Photosystem I (PS I) is essential for efficient photosynthesis and aids photoprotection, especially in stressful conditions, but the difficulty in quantifying CEF is non-trivial. The total electron flux through PS I (ETR1) and the linear electron flux (LEF_{O_2}) through both photosystems in spinach leaf discs were estimated from the photochemical yield of PS I and the gross oxygen evolution rate, respectively, in CO_2 -enriched air. $\Delta Flux = ETR1 - LEF_{O_2}$ is an upper estimate of CEF. (1) Infiltration of leaf discs with 150 μM antimycin A did not affect LEF_{O_2} , but decreased $\Delta Flux$ 10-fold. (2) $\Delta Flux$ was practically negligible below 350

44 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, but increased linearly above it. The following results were obtained at
 45 $980 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. (3) ΔFlux increased 3-fold as the temperature increased from 5°C to
 46 40°C . It did not decline at high temperature, even when LEF_{O_2} decreased. (4) ΔFlux increased
 47 by 80% as the relative water content of leaf discs decreased from 100% to 40%, when LEF_{O_2}
 48 decreased 2-fold. The method of using ΔFlux as a non-intrusive upper estimate of steady-state
 49 CEF in leaf tissue appears reasonable when photorespiration is suppressed.

50

51 **Key words:** Antimycin A; cyclic electron flow; linear electron flow; P700; Photosystem I

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53

54 Introduction

55 Arnon *et al.* (1955) demonstrated photophosphorylation on illumination of isolated thylakoids
 56 in the presence of vitamin K via a cyclic electron flux (CEF) around Photosystem I (PS I).
 57 Since CEF is essential for efficient photosynthesis (Munekage *et al.* 2004) and for
 58 photoprotection (Takahashi *et al.* 2009), there have been sustained efforts to elucidate the
 59 mechanisms and roles of this cyclic electron flow (for reviews, see Bendall and Manasse, 1995;
 60 Allen, 2003; Bukhov and Carpentier, 2004; Johnson, 2005; Joliot and Joliot, 2006; Shikanai
 61 2007; Alric 2010; Miyake 2010; Kramer and Evans 2011). Achieving such an understanding,
 62 however, has been hampered by the difficulty of quantifying CEF in physiological conditions
 63 due to the absence of a net product of cyclic electron flow.

64 Methods for measuring/infering CEF can be grouped into two categories, as
 65 summarized in Table 1. In Category (A), CEF is monitored directly, in at least three ways. (1)
 66 One way is to measure the transient increase in chlorophyll (Chl) fluorescence yield on
 67 cessation of actinic illumination; the increase reflects electron donation to the plastoquinone

68 pool from reductants accumulated during illumination, and is suppressed by antimycin A (Mano
69 *et al.* 1995). This way of monitoring CEF, however, is basically qualitative (Gotoh *et al.* 2010).
70 (2) A second way is to use an elaborate sequence of redox poisoning by pre-illumination of
71 thylakoids in anaerobic conditions, followed by the addition of 3-(3,4-dichlorophenyl)-1,1-
72 dimethyl-urea (DCMU) to eliminate linear electron flow, and finally illumination with actinic
73 light to monitor redox changes of ferredoxin in a light-to-dark transition (Cleland and Bendall,
74 1992). It was possible to deduce a value of CEF, but only in the absence of linear electron flow
75 and in anaerobic conditions. (3) A third way is to use a modulated photoacoustic signal (Malkin
76 and Canaani, 1994), induced by modulated actinic light, that has an amplitude depending on the
77 absence or presence of a saturating light pulse; it has been used to indicate cyclic electron flow
78 leading to energy storage in far-red actinic light (e.g. Herbert *et al.* 1990; Joët *et al.* 2002;
79 Jeanjean *et al.* 2008). This method, however, is limited to the use of far-red actinic light, unless
80 inhibitors are used to prevent the linear electron flux in white light (Canaani *et al.* 1989;
81 Veeranjanyulu *et al.* 1998), in which case the magnitude of CEF is altered because the redox
82 poise is altered by inhibition of LEF.

83 In Category (B) ETR1, the total electron flux through PS I, is first estimated. Then by
84 inhibiting/suppressing LEF, or estimating LEF independently without inhibiting it, or inhibiting
85 CEF by methyl viologen (MV), a CEF can be obtained by difference. In this category, ETR1
86 can be measured in three ways. (i) One way is to use the electrochromic signal (ECS) in
87 continuous actinic light of a particular spectral quality (reviewed by Bailleul *et al.* 2010) by
88 monitoring the dark-interval relaxation kinetics (DIRK, Sacksteder and Kramer, 2000) or the
89 “dark pulse” kinetics (Joliot and Joliot, 2002) of the proton current immediately after cessation
90 of illumination. Joliot and Joliot (2002) used the post-illumination ECS kinetics to estimate the
91 cyclic electron flow through PS I upon inhibition of PS II by DCMU, but the CEF so obtained is
92 different from that in the absence of DCMU because of altered redox poisoning. In order to
93 estimate CEF while the linear electron flux is occurring, Bailleul *et al.* (2010) subtracted a linear
94 electron flow through PS II (LEF_{FI} , obtained from Chl fluorescence) from the proton flux

obtained from the post-illumination ESC kinetics. However, the fluorescence signal reports from a certain depth of the leaf tissue (Oguchi *et al.* 2011). By contrast, the ECS signal indicates a whole-tissue property: the fast rise of the ECS signal can be used to obtain a stoichiometry of the two photosystems that agrees with that obtained from the entire population of the two photosystems (Fan *et al.* 2007a). Therefore, the difference between the post-illumination proton flux and LEF_{FI} as a measure of CEF is subject to considerable uncertainty.

Subtraction of LEF_{FI} from the total proton current can be avoided by simply comparing the proton current between a drought-stress treatment and a control (Kohzuma *et al.* 2009), or a mutant and the wild type (Livingston *et al.* 2010) at the same LEF_{FI} . The extra proton current at a given LEF_{FI} is taken to be due to CEF. However, LEF_{FI} at a given irradiance that is greater than $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ is different between the treatment and control, or between a mutant and the wild type, so the method is restricted to low irradiance, where CEF is small anyway. Another draw-back is that since the proton current obtained by the ECS method is in units s^{-1} , calculating the CEF on a leaf area basis requires further information, such as the content of PS I reaction centres per unit leaf area.

(ii) A second way in Category B for measuring ETR1, in a number of variations, measures the total electron flux through PS I by monitoring the light-to-dark redox changes of P700 (the special chlorophyll dimer acting as the primary electron donor in PS I), recently with improved signal-to-noise ratio and time resolution (Klughammer and Schreiber, 1994; Laisk *et al.* 2010). This method, based on post-illumination $P700^{+}$ re-reduction kinetics, sometimes uses DCMU to abolish linear electron flow (Maxwell and Biggins, 1976; Golding *et al.* 2004; Cardol *et al.* 2009), such that CEF remains as the predominant electron flow through PS I. Unfortunately, such CEF is different from that occurring in the absence of DCMU. In order to avoid the use of DCMU, Laisk *et al.* (2005) assayed the total electron flux through P700 (ETR1, obtained from a light-to-dark $P700^{+}$ re-reduction signal transient), and used chlorophyll fluorescence to determine the rate of PS II electron transport, with the obvious problem of

121 comparing different regions of the leaf tissue. In another variation, methyl viologen (MV) was
122 used to eliminate CEF, while allowing linear electron flow to occur (Fan *et al.* 2007b; Jia *et al.*
123 2008). However, MV itself retards linear electron flow to some extent during continuous
124 illumination (Jia *et al.* 2008; Fan *et al.* 2009), necessitating a correction of LEF in the presence
125 of MV before CEF is obtained as the difference in the total electron flux through PS I in the
126 absence and presence of MV. The correction factor is based on the assumption that the ratio of
127 LEF_{FI} estimated by chlorophyll fluorescence in the absence of MV to that in the presence of MV
128 is the same everywhere within the whole leaf tissue. Based on this assumption, the linear
129 electron flux through PS I in the presence of MV is multiplied by a *single* correction factor to
130 yield the corrected LEF, which is then subtracted from ETR1 to give CEF. However, since the
131 chlorophyll fluorescence signal is a localized indicator at a specific depth of the leaf tissue,
132 while the P700 redox signal is a whole-tissue signal (Oguchi *et al.* 2011), the assumption may
133 lead to an error. Another short-coming of using MV is that the duration of illumination at high
134 irradiance has to be limited in order to minimize the production of reactive oxygen species that
135 may cause damage. A further variation of the method based on the $P700^+$ light-dark signal uses
136 far-red actinic light to examine cyclic electron flow with little linear electron flow during
137 photosynthetic induction (Joliot and Joliot, 2005; Vredenberg and Bulychev, 2010), thereby
138 avoiding the use of DCMU. While the use of far-red actinic light is non-intrusive, it does not
139 give the CEF in white light which normally drives photosynthesis in nature. Laisk *et al.* (2010)
140 determined the total electron flux through PS I from the post-illumination kinetics of $P700^+$ re-
141 reduction and the linear electron flux by the rate of oxygen evolution in high CO_2 , both being
142 whole-tissue measurements. However, because far-red actinic light was necessary to sustain a
143 substantial oxidation level of $P700^+$ for measurement of post-illumination re-reduction kinetics,
144 the study did not use white light.

145 (iii) A third way in Category B is to determine ETR1 from the photochemical yield of
146 PS I multiplied by the irradiance, the leaf absorptance and the fraction of light partitioned to PS
147 I (f_i). Miyake *et al.* (2005) and Huang *et al.* (2011) used this approach, but had to estimate the

linear electron flux (LEF_{FI}) by Chl fluorescence. The difference between $ETR1$ and LEF_{FI} is taken to be CEF. However, in this approach, different parts of the leaf tissue are again sampled in measurements of $ETR1$ and LEF_{FI} .

From the comparisons above, it is clear that quantification of CEF is not a trivial task. In this study a method (iii in Table 1) was chosen that (a) estimates $ETR1$ from the PS I photochemical yield, and LEF_{O_2} from the gross rate of oxygen evolution under identical illumination, both being whole-tissue measurements which, though often used individually in other contexts, have not been applied in combination for estimating CEF; (b) is non-intrusive; (c) uses white actinic light to simulate sunlight, since coloured light alters CEF; and (d) took note of the important finding that a proper determination of the photochemical yield of PS I for calculating the total electron flux through PS I requires strong far-red light immediately before and during the application of a saturating light pulse (Siebke *et al.* 1997). This method is not yet ideal, but probably yields a closer quantitative estimate of CEF than others so far. In this study, it was applied to spinach leaf discs in a variety of treatments expected to influence CEF and/or LEF. The overall aim of this study was to achieve a reasonable quantitative estimate of CEF that will help to elucidate the roles and regulation of cyclic electron flow *in vivo* in leaf tissue under white light.

Materials and methods

Plant growth

Spinacea oleracea L. (cv. Yates hybrid 102) plants were grown in a glasshouse (approximately 30/15°C, day/night). A nutrient mix (Aquasol, Hortico, Clayton, Australia) was supplemented by a slow release fertilizer ('Osmocote', Scotts Australia Pty Ltd, Castle Hill).

Vacuum infiltration of leaf discs

171 When required, leaf discs (1.5 cm²) were immersed in water or a selected concentration of
172 antimycin A, vacuum-infiltrated using a water-driven pump for about 30 s, blotted with
173 absorbent paper, and allowed to evaporate off the excess intercellular water in darkness for a
174 total of 30 min before measurement.

175 **Water stress treatment**

176 Leaf discs were allowed to lose water in a desiccator, aided by a battery-powered fan. Relative
177 water content was calculated as:

$$178 \text{ RWC} = 100 \times (\text{Fresh weight} - \text{Dry Weight}) / (\text{Turgid weight} - \text{Dry weight}) (\%).$$

179 Each leaf disc was subjected to cumulative losses of water content, with measurements made at
180 each chosen stage of dehydration.

181 **Linear electron flux measured by O₂ evolution**

182 O₂ evolution was measured in a gas-phase oxygen electrode (Hansatech, King's Lynn, UK)
183 chamber, thermostatted at 25°C unless specified otherwise, that accepted a multifurcated light
184 guide, and contained 1% CO₂ supplied by fabric matting moistened with 1 M NaHCO₃/Na₂CO₃
185 (pH 9). White incandescent light from a projector halogen lamp filtered by a Calflex C heat-
186 reflecting filter (Linos Photonics, Göttingen, Germany) and neutral-density filters was used to
187 illuminate a leaf disc. O₂ evolution was measured over several minutes until steady state. The
188 post-illumination drift was subtracted algebraically from the steady-state net oxygen evolution
189 rate, and the *gross* oxygen evolution rate so obtained was multiplied by four to give the linear
190 electron flux, LEF_{O₂}. For calibration of the oxygen signals, 1 mL of air at 25°C (taken to
191 contain 8.05 μmol O₂) was injected into the gas-phase O₂ electrode chamber that was
192 thermostatted at a selected temperature.

193 **Measurement of redox kinetics of P700**

194 Redox changes of P700 were observed with a dual wavelength (820/870 nm) unit (ED-P700DW)
 195 attached to a PAM fluorometer (Walz, Effeltrich, Germany) in the reflectance mode (response
 196 time constant = 95 μ s). The reflectance mode (with a metal mesh under the leaf sample to
 197 reflect the near infra-red radiation,) was chosen for convenience. Previously, it was shown that
 198 the variation of the relative size of the P700⁺ signal with far-red irradiance was practically
 199 identical for measurements in the reflectance or transmission modes (Kim *et al.* 2001). Oguchi
 200 *et al.* (2011) also showed that the reflectance mode measures the whole tissue. Before
 201 measurements, a leaf disc was brought to steady-state photosynthesis by illuminating it with
 202 white actinic light for about ten minutes for simultaneous measurement of O₂ evolution and Chl
 203 fluorescence yields. To retain steady state for P700⁺ measurements, immediately after these
 204 simultaneous measurements, each leaf disc was re-illuminated with the same actinic light for
 205 9.016 s, using an electronic shutter controlled by one terminal of a pulse/delay generator (Model
 206 555, Berkeley Nucleonics, USA).

207 During each 9.016-s illumination, at time $T = 8.80$ s (corresponding to the time point t
 208 = -50 ms in Fig. 1a), data acquisition (using software written by the late A. B. Hope) was
 209 started by a second terminal of the pulse/delay generator. At $T = 8.85$ s, a strong far-red light
 210 (FR, $\sim 2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) from a light-emitting diode array (741 nm $\pm$ 13 nm, LED735-66-60,
 211 Roithner LaserTechnik, Vienna, Austria) was triggered on for 100 ms using a third terminal of
 212 the pulse/delay generator. The strong FR light depleted electrons from the inter-system chain,
 213 so that the subsequent saturating pulse (see below) oxidized P700 maximally (Siebke *et al.*
 214 1997). While the strong FR light was on, at $T = 8.90$ s, a saturating light pulse ($\sim 9000 \mu\text{mol m}^{-2}$
 215 s^{-1}) was applied for 10 ms by a pulse from a fourth terminal of the pulse/delay generator,
 216 yielding the maximally-oxidized P_m' signal in Fig. 1a. Finally, the white actinic light was
 217 turned off by the electronic shutter (at $T = 9.016$ s). Data acquisition continued for 85 ms after
 218 cessation of actinic illumination to obtain the baseline corresponding to complete reduction of

219 P700. Immediately after completion of one cycle of illumination and data acquisition (at $T =$
 220 9.101 s), another 9.016-s illumination was restarted, thereby maintaining steady-state
 221 photosynthesis. Nine traces were averaged automatically to improve the signal-to-noise ratio.

222 Next, we determined the maximum photo-oxidizable P700 content. We established a
 223 steady state by illumination with weak continuous far-red light ($\sim 12 \mu\text{mol m}^{-2} \text{s}^{-1}$, 723 nm) for
 224 > 10 s (Fig. 1b). Then we superimposed a saturating single-turnover flash. Flashes were given
 225 at 0.2 Hz; 9 consecutive signals were automatically averaged. The maximum signal, P_m ,
 226 immediately after the flash was used to normalize the signal interval b in Fig. 1a. The
 227 photochemical yield of PS I is then given by $Y(I) = b/P_m$ (Klughammer and Schreiber 2007).
 228 ETR1 was calculated as

$$229 \quad \text{ETR1} = Y(I) \times I \times 0.85 \times 0.5 \quad (1)$$

230 where I is the irradiance, 0.85 is the assumed absorptance and 0.5 the assumed fraction (f_I) of
 231 absorbed white light partitioned to PS I. An experimental estimation of f_I in special cases is
 232 given in Table 2.

233 The rate of electron transport through PS II measured by Chl fluorescence

234 Before measurement of O_2 evolution, we measured the relative Chl fluorescence yield (blue
 235 excitation light, detection of all fluorescence > 660 nm) in the dark-acclimated state (F_o) and
 236 after complete closure of traps (F_m) on application of a 0.8-s saturating light pulse ($\sim 9000 \mu\text{mol}$
 237 $\text{m}^{-2} \text{s}^{-1}$). Then, simultaneously with measurement of O_2 evolution, the relative Chl fluorescence
 238 yield at steady state (F_s) and the maximum relative yield during illumination (F_m') were
 239 measured using a PAM 101 fluorometer (Walz, Effeltrich, Germany). The effective quantum
 240 yield of PS II photochemistry (Genty *et al.* 1989), $\phi_{\text{PS II}} = (1 - F_s/F_m')$, was used to calculate the
 241 rate of electron transport through PS II (LEF_{FI}) as $\text{LEF}_{\text{FI}} = (1 - F_s/F_m') \times I \times 0.85 \times 0.5$ with the
 242 same values of leaf absorptance and partitioning of light energy between PS II and PS I as used
 243 in calculating ETR1.

Photochemical quenching (qP) was calculated as $(F_m' - F_s)/(F_m' - F_o')$ (Krause and Weis 1991), where F_o' was calculated according to Oxborough and Baker (1997). The fraction of light absorbed by the PS II antennae that is dissipated thermally via ΔpH - and xanthophylls-regulated processes (ϕ_{NPQ}) was calculated as $(F_s/F_m') - (F_s/F_m)$, (Hendrickson *et al.* 2004).

All measurements of oxygen evolution, P700 kinetics and chlorophyll fluorescence were made on a leaf disc placed in a gas-phase O_2 electrode chamber thermostatted at 25°C or at the temperatures specified.

Results

Estimation of the fraction of absorbed light (f_i) partitioned to PS I

Estimation of the total electron flux through PS I according to Equation 1 requires information on f_i , the fraction of absorbed light partitioned to PS I. To estimate f_i , we consider some special cases in which CEF is likely to be small or negligible (Table 2). These special cases occur where antimycin A was used to inhibit CEF (see Fig. 2 below) or when light at low irradiance was used to drive linear electron flow but possibly little CEF (see Fig. 3 below). Consider leaf discs infiltrated with 150 μM antimycin A and exposed to 980 μmol photons $m^{-2} s^{-1}$. Assuming that inhibition of CEF by antimycin A was complete, ETR1 would approximately equal LEF_{O_2} . The measured $Y(I)$ was 0.403, from which Equation 1, with f_i replacing 0.5 and LEF_{O_2} ($= 165 \mu mol e^- m^{-2} s^{-1}$) replacing ETR1, could be used to estimate f_i to be 0.49. Similarly, infiltration with 200 μM antimycin A and exposure to 980 μmol photons $m^{-2} s^{-1}$ gave an estimated $f_i = 0.47$. At moderately low irradiance such as 236 and 352 μmol photons $m^{-2} s^{-1}$, assuming that CEF was negligible in CO_2 -enriched air, we estimated f_i to be 0.45 and 0.48, respectively (Table 2). As an approximation, we assumed f_i to be 0.50 in the calculation of ETR1 according to Equation 1, in all the results below.

267 **Electron fluxes in response to antimycin A**

268 Antimycin A inhibits the ferredoxin-dependent CEF (Moss and Bendall, 1984; Ivanov *et al.*
269 1998). We monitored the electron fluxes as a function of [antimycin A] at $980 \mu\text{mol m}^{-2} \text{s}^{-1}$
270 (Fig. 2a). ETR1 decreased towards an asymptotic value. However, the inhibitor had no effect
271 on LEF_{O_2} assayed by gross O_2 evolution. The difference (ΔFlux) between the two fluxes
272 decreased steadily with increase in [antimycin A], eventually down to about 10% of the control.
273 As ΔFlux decreased, the fraction of excitation energy dissipated non-photochemically in a light-
274 regulated manner (ϕ_{NPQ}) decreased by about 34% (Fig. 2b), *despite* the constancy of LEF_{O_2} .

275 **Electron fluxes in response to irradiance of white light**

276 ETR1, the total electron flux through PS I, did not show saturation even at the highest irradiance
277 of white light used (Fig. 3). By contrast LEF_{O_2} , assayed as the gross rate of O_2 evolution
278 multiplied by 4, peaked at about $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The maximum LEF_{O_2} was about
279 $188 \mu\text{mol electrons m}^{-2} \text{s}^{-1}$ (gross O_2 evolution rate, $47 \mu\text{mol m}^{-2} \text{s}^{-1}$). The difference between
280 ETR1 and LEF_{O_2} ($= \Delta\text{Flux}$), was negligibly small below $350 \mu\text{mol m}^{-2} \text{s}^{-1}$; above this threshold,
281 it increased approximately linearly with irradiance (Fig. 3). At the highest irradiance, ΔFlux
282 almost equalled LEF_{O_2} .

283 The gas-phase O_2 electrode chamber also allowed us to examine LEF_{FL} , the linear
284 electron flux obtained from Chl fluorescence, under identical illumination conditions. LEF_{FL}
285 roughly matched LEF_{O_2} evolution at irradiance $< 250 \mu\text{mol m}^{-2} \text{s}^{-1}$; however, at higher
286 irradiance it was less than the whole-tissue linear electron flux measured by gross O_2 evolution,
287 the discrepancy increasing with irradiance.

288 **Electron fluxes in response to temperature**

At 980 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, ETR1 increased steadily with temperature, peaking at about 30°C but decreasing above this temperature (Fig. 4). LEF_{O_2} assayed by O_2 evolution followed a similar pattern, though peaking slightly below 30°C. The difference, ΔFlux , increased steadily with temperature, showing no decline at 40°C. Notably, at the extreme temperatures, ΔFlux approached LEF_{O_2} . At all the temperatures tested, LEF_{O_2} exceeded LEF_{FI} ; this was particularly true at low temperatures.

Electron fluxes in response to loss of leaf water content

At 980 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, as the water content of leaf discs decreased from 100% to 55%, ETR1 increased slightly but decreased at water content < 55% (Fig. 5). LEF_{O_2} , assayed by gross oxygen evolution, remained fairly constant between 100% and 55%; it then decreased at leaf water content < 55%. Consequently, ΔFlux increased by about 80% as the water content decreased from 100% to 40%.

DISCUSSION

Notable features of our method that gives ΔFlux as an upper estimate of CEF

(1) Advantages

Our method measures (1) the total electron flux through PS I (ETR1) via the photochemical yield of PS I and (2) LEF_{O_2} by the gross rate of oxygen evolution (multiplied by four) *under identical white-light illumination*. By difference ΔFlux was obtained as an upper estimate of CEF, since ETR1 includes not only CEF, but also electron fluxes associated with the water-water cycle, electron donation from stromal reductants and direct charge recombination in PS I, though photorespiration can be excluded in 1% CO_2 . An important aspect of our method is the introduction of a strong far-red light pulse before and during a saturating pulse for measuring total electron flux thorough PS I (Fig. 1a); without the strong far-red light pulse, the

photochemical yield (and hence ETR1) could be under-estimated by as much as 18% (Siebke *et al.* 2000). Another aspect is the use of a saturating single-turnover flash superimposed on weak continuous far-red light to obtain the maximum photo-oxidizable P700 signal (Fig. 1b), in order to avoid the use of a long saturating pulse which may cause some reduction of P700⁺ while being used to photo-oxidize P700 fully. Our method offers some advantages.

First, both the P700⁺ signal and O₂ evolution were measured under identical illumination conditions, in which the intra-leaf gradient of light should be also identical. The intra-leaf gradient of light determines the rate of excitation of both photosystems at different depths of tissue, but both measurements were affected in parallel as the depth increased. Further, both are whole-tissue measurements. That the P700⁺ signal is a whole tissue measurement is demonstrated by the observation that the cumulative delivery of electrons from PS II to P700⁺ (Σ) following a saturating single-turnover flash was well correlated with the oxygen yield per saturating single-turnover flash, when both parameters were varied by a photoinhibition pre-treatment (Losciale *et al.* 2008). The same “calibration” straight line applied to all the plant species tested, regardless of differences in intra-leaf anatomy; by contrast, the correlation of the Chl fluorescence parameter F_v/F_m with the oxygen yield per saturating single-turnover flash varied greatly among the same samples (Losciale *et al.* 2008). Further, following a photoinhibition pre-treatment, a simple integration akin to Σ was affected to the same relative extent (compared with a control), regardless of whether the P700⁺ signal was measured from the adaxial or abaxial side of the leaf (Oguchi *et al.* 2011). Indeed, that the P700⁺ signal is a whole-tissue measurement is to be expected. Since an 820-nm measuring photon is hardly absorbed by neutral Chl molecules and other pigments, such that leaf absorptance is negligible above 750 nm (McCree 1972), it undergoes multiple scattering within the leaf tissue, visiting many chloroplasts before being absorbed by a P700⁺.

By contrast, Chl fluorescence is a localized reporting signal, generally indicating shallower depths in the tissue, where the actinic light is attenuated less drastically and where

any fluorescence is less readily re-absorbed on its way to the detector. The comparatively high light in the shallower parts of the tissue tends to (1) reduce Q_A more, thereby decreasing qP and (2) decrease F_v'/F_m' , the photochemical efficiency of open PS II traps, by virtue of the greater trans-thylakoid ΔpH in a higher-irradiance local environment (Terashima *et al.* 2009). Consequently, LEF_{FI} becomes an under-estimation because its calculation includes ϕ_{PSII} ($= qP \times F_v'/F_m'$). This is supported by the observation that the electron transfer rate measured via chlorophyll fluorescence with red measuring light is lower than the rate measured with gas-exchange measurement (Tsuyama *et al.* 2003). The difference between $ETR1$ and LEF_{FI} is, therefore, expected to over-estimate CEF even more. Indeed, if we had used LEF_{FI} instead of LEF_{O_2} as a measure of linear electron flow, CEF would have been even more over-estimated in all of our treatments (Fig. 3). Further, the depth of fluorescence signal detection may vary with, for example, (1) the actinic irradiance which determines the intra-leaf light gradient, (2) plant species having different intra-leaf anatomy, (3) the colour of the measuring light and (4) the extent of photoinhibition which affects the relative contribution of different depths of tissue to the total signal. Therefore, the difference between $ETR1$ and LEF_{FI} as an estimate of CEF presents considerable uncertainty.

Second, our method allows measurement under non-intrusive conditions, using white light that resembles sunlight. It is free, for example, from special conditions used in the past to quantify cyclic electron flow, including (1) far-red-enriched light to ensure substantial photo-oxidation of P700 for measurement of the post-illumination $P700^+$ re-reduction kinetics (e.g. Jia *et al.* 2008; Fan *et al.* 2009; Laisk *et al.* 2010); (2) the use of MV to abolish CEF (e.g. Jia *et al.* 2008; Fan *et al.* 2009; Kohzuma *et al.* 2009); (3) correction for partial inhibition of PS II by MV (Jia *et al.* 2008; Fan *et al.* 2007b, 2009); and (4) the use of DCMU to inhibit LEF so that the residual electron flux through PS I could be attributed to cyclic flow alone (e.g. Maxwell and Biggins 1976; Golding *et al.* 2004). Such restrictive conditions do not yield the magnitude of

363 CEF *in vivo* under white light in physiological conditions where linear electron flow occurs
364 concurrently.

365 (2) *Assumptions and approximations*

366 Our method, however, does have its assumptions and limitations. First, it assumes that light is
367 partitioned equally between the two photosystems when calculating ETR1; that is, the fraction
368 of light partitioned to PS I (f_I) is assumed to equal 0.50 in all conditions. This assumption, in
369 widespread use in the literature for calculating LEF_{F_I} from Chl fluorescence measurements, is
370 probably reasonable for steady-state photosynthesis (Schreiber 2004), particularly in white light.
371 The values of f_I was estimated under some special conditions in which CEF was expected to be
372 minimal (Table 2). The values of f_I so estimated were not far from 0.50.

373 Second, our method assumed that oxygen-consumption by photorespiration and Mehler
374 reaction is negligible in the conditions of the present study. Photorespiration was suppressed by
375 high (1%) CO₂ during the measurements. On the other hand, our method is limited to situations
376 where photorespiration is inhibited. A potential improvement in the method is to measure
377 oxygen evolution while allowing photorespiration to occur, and to quantify the photorespiration
378 rate so that it can be taken into account when calculating LEF_{O_2} .

379 As for the potentially oxygen-consuming Mehler reaction, the reactive oxygen produced
380 by the reaction is scavenged by the enzymes of the water-water cycle, which leads to no net
381 release or uptake of O₂ (Miyake 2010). Therefore, there should be no significant effect of the
382 Mehler reaction on the LEF_{O_2} measurements, particularly in the steady-state and when cyclic
383 electron flow is unimpeded. However, unlike LEF_{O_2} , ETR1 should include (pseudo-cyclic)
384 electron flow associated with the water-water cycle. Therefore, the difference between ETR1
385 and LEF_{O_2} would include such pseudo-cyclic electron flow, and would over-estimate CEF, but
386 not by as much as when LEF_{F_I} assayed by Chl fluorescence is used in the subtraction.
387 Nevertheless, pseudo-cyclic electron flow should normally be relatively small, particularly in

CO₂-enriched air. By using mass spectrometry with wild-type tobacco, Ruuska *et al.* (2000) reported an ¹⁸O₂ uptake rate of 3.4 μmol m⁻² s⁻¹ in the dark and 4.6 μmol m⁻² s⁻¹ in the light (970 μmol photons m⁻² s⁻¹), with 20% O₂ and 1% CO₂ in both the dark and the light. Taking the respiration rate in the light to be 36% of that in darkness (Atkin *et al.* 2000), a respiration rate of (3.4 × 0.36) = 1.2 μmol m⁻² s⁻¹ in the light is obtained; consequently, the ¹⁸O₂ uptake not due to respiration in the light (attributable to the Mehler reaction) is 4.6 – 1.2 = 3.4 μmol m⁻² s⁻¹, which is only about 8% of the (gross) ¹⁶O₂ evolution rate in the light (Ruuska *et al.* 2000). Similarly, Vredenberg and Bulychev (2010) calculated that the Mehler reaction declines during photosynthetic induction. Further, the water-water cycle in leaves is not a major alternative electron sink for dissipation of excess excitation energy even when CO₂ assimilation is restricted (Driever and Baker 2012). These findings imply that the effect of the Mehler reaction on ETR1 is relatively small in the steady state. The residual antimycin-insensitive ΔFlux in the presence of high [antimycin A], *ca.* 10% of the control value (Fig. 2a), could include such pseudo-cyclic electron flow. Equally, it could also include NAD(P)H dehydrogenase-dependent cyclic electron flow or direct charge recombination within PS I.

Under extreme conditions where the Mehler reaction overwhelms the enzymes of the water-water cycle, the O₂ uptake by the Mehler reaction could lead to an under-estimation of the LEF_{O2} measured by net O₂ evolution. Then the difference between ETR1 and LEF may further over-estimate ferredoxin-dependent CEF.

If the assumptions and approximations of this method can be further validated, and if photorespiration can be reliably measured, it should be feasible to make a portable version of the equipment with the appropriate software for use in the field.

Tests of the method in treatments expected to vary CEF and/or LEF

Antimycin A

Antimycin A is often used as an inhibitor of ferredoxin-dependent cyclic electron flow (Endo *et al.* 1997; Ivanov *et al.* 1998). As expected, it decreased ΔFlux to a low value (about 10% of the control) at 150 and 200 μM (Fig. 2a). This observation suggests that the ferredoxin-dependent cyclic pathway is the main form of cyclic electron flow, and that the NAD(P)H dehydrogenase-dependent pathway may be relatively small. Interestingly, with the inhibition of cyclic electron flow, ϕ_{NPQ} also decreased (by about 33%, Fig. 2b) when LEF_{O_2} was constant. Possibly, cyclic electron flow contributed to the trans-thylakoid ΔpH , such that inhibition of CEF diminished the ΔpH - and xanthophyll cycle-dependent non-photochemical quenching. Indeed, CEF-dependent generation of a ΔpH helps to alleviate photoinhibition by at least two distinct photoprotective mechanisms (Takahashi *et al.* 2009). Any pseudo-cyclic electron flux associated with the Mehler reaction would also contribute to the trans-thylakoid ΔpH and hence to ϕ_{NPQ} . However, the *decrease* in ϕ_{NPQ} in the presence of antimycin A cannot be attributed to pseudo-cyclic electron flow, since, if anything, pseudo-cyclic electron flow should increase when CEF is inhibited.

Increasing irradiance

ΔFlux was negligibly small at irradiances $< 350 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 3). This is expected since the carbon reduction cycle was able to use the majority of NADPH at low irradiance, leaving little spare reduced ferredoxin for poisoning cyclic electron flow. At maximum LEF_{O_2} , however, more reduced ferredoxin would be available for competition between NADP^+ reduction and poisoning of cyclic electron flow (Okegawa *et al.* 2008), and ΔFlux was almost as large as LEF_{O_2} .

Using another method, Jia *et al.* (2008) observed that even at low irradiance ($\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$) of red/far-red actinic light (centred at 697 nm), CEF was as high as 40% of the linear rate (corrected for an inhibitory effect of methyl viologen on PS II). Most probably, the spectral distribution of the actinic light favoured cyclic electron flow over linear electron flow in that study. The white incandescent actinic light used in this present study, more akin to sunlight,

induced negligible ΔFlux below $350 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. This demonstrates the significant effect of the spectral distribution of actinic light on the CEF being investigated. The present method, which allows the use of white incandescent light, should give a reasonable estimate of the magnitude of CEF in natural light.

Increasing temperature

Suggestions have been made that cyclic electron flow is activated by high temperature (Bukhov *et al.* 1999; Clarke and Johnson, 2001). In our study, ΔFlux increased almost 3-fold when the temperature was raised from 5°C to 40°C (Fig. 4). Even when LEF_{O_2} declined between 32.5°C and 40°C , ΔFlux did not decrease (Fig. 4). It has been reported that the temperature dependency of LEF is not correlated with the temperature dependency of PS I activity but with that of activity around PS II (Yamasaki *et al.* 2002; Yamori *et al.* 2008). Yamasaki *et al.* 2002 suggested, according to fluorescence decay analyses, that the temperature dependency of LEF resides in electron transport between Q_A and PS I. Although the carboxylation rate decreases at high temperature, the oxygenation rate normally increases. The ATP/NADPH consumption ratio is higher in oxygenation than in carboxylation. Therefore, CEF may be stimulated at high temperature so as to meet the higher demand for ATP or compensating for the decrease in ATP production caused by the decrease in activity around PS II, particularly if high temperature facilitates the diffusion of ferredoxin or accelerated enzymatic reactions involved in CEF. It is also possible that the increase in leakage of protons from the thylakoid lumen to the stroma at a higher temperature caused the reversible activation of cyclic electron transport, as suggested by Bukhov *et al.* (1999).

Loss of leaf water content

ΔFlux increased with water stress, here defined as the loss of tissue water (Fig. 5). Golding *et al.* (2004) reported that water stress increased CEF when measured in the presence of DCMU and

extremely high light. Similarly, Kohzuma *et al.* (2009) reported an increase in proton current at a given LEF_{FI} measured in red light (626 nm, $\leq 300 \mu\text{mol m}^{-2} \text{s}^{-1}$), in response to long-term drought. By contrast, little change in CEF was observed by Jia *et al.* (2008) even when leaf tissue had lost about 60% of its water; however, Jia *et al.* (2008) relied on a correction for the partial inhibition of PS II by methyl viologen. A problem could have arisen because the correction was based on Chl fluorescence. That CEF (as approximated by ΔFlux) increased, certainly did not decrease, during severe water stress, despite the extreme macromolecular crowdedness in the shrunken chloroplast stroma, is notable; it may be attributable to the involvement of relatively small enzyme complexes in cyclic electron flow (Jia *et al.* 2008).

In conclusion, a simple *non-intrusive* method is here presented that estimates *whole-tissue* ΔFlux under *white* light, ΔFlux being an upper estimate of CEF. Application of the method in several treatments of spinach leaf discs yielded variations of the ΔFlux and, by implication, CEF in ways that are expected. The estimates of CEF also showed a significant difference between the present method using gross oxygen evolution to estimate the linear electron flux and the previously-used method using Chl fluorescence to estimate the linear electron flux; the previous method significantly over-estimates CEF even more.

Acknowledgements

This work was supported by a China Scholarship Council Fellowship (to JK), a JSPS Postdoctoral Fellowship for Research Abroad (21-674) to R.O., a Knowledge Innovation Programme of the Chinese Academy of Sciences grant (KSCX2-EW-J-1) to D.-Y. F., and grants from the Australian Research Council to WSC (DP1093827) and to the CoE in Plant Energy Biology (CE056195 to MRB). We thank Dr W. Yamori for his helpful comments.

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656

For Review Only

657 **Table 1.** Various ways of monitoring the cyclic electron flux (CEF).

	Using Inhibitor(s)	Un- natural light	Subtracting LEF from combined (LEF + CEF) measurement:	
			LEF _{F1} by Chl fluorescence as an estimate of LEF	LEF _{O2} by O ₂ evolution or CO ₂ fixation
(A) CEF measurement				
(1) Post-illumination Chl fluorescence transient (qualitative)	a			
(2) post-illumination ferredoxin redox kinetics	b	-	-	-
(3) Photoacoustics	c	d	-	-
(B) Combined (LEF + CEF) measurement				
(i) Electrochromic signal (DIRK)	e	f	f	-
(ii) P700 ⁺ reduction kinetics	g	h, j	i	j
(iii) P700 ⁺ for PS I photochemical yield	-	-	k	The present study

658

659 Different ways of monitoring CEF can be grouped into Categories A and B as described in the

660 Introduction. (a): Mano *et al.* 1995; Gotoh *et al.* 2010. (b): Cleland and Bendall 1992. (c):

661 Canaani *et al.* 1989; Veeranjanyulu *et al.* 1998. (d): Herbert *et al.* 1990; Joët *et al.* 2002;

662 Jeanjean *et al.* 2008. (e): Joliot and Joliot (2002). (f): Kohzuma *et al.* 2009; Bailleul *et al.* 2010;

663 Livingston *et al.* 2010. (g): Maxwell and Biggins 1976; Golding *et al.* 2004; Fan *et al.* 2007b;

664 Jia *et al.* 2008; Cardol *et al.* 2009; Fan *et al.* 2009. (h): Joliot and Joliot 2005; Vredenberg and

665 Bulychev 2010. (i): Laisk *et al.* 2005. (j): Laisk *et al.* 2010. (k): Miyake *et al.* (2005) and Huang

666 *et al.* (2011).

Table 2. An estimation of the fraction of absorbed light (f_i) partitioned to PS I

Antimycin A (μM)	Irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Y(I)	LEF _{O₂} ($\mu\text{mol e}^{-} \text{ m}^{-2} \text{ s}^{-1}$)	f_i
150	980	0.403 ± 0.019	165 ± 6	0.49
200	980	0.397 ± 0.016	156 ± 5	0.47
0	236	0.747 ± 0.014	67 ± 5	0.45
0	352	0.738 ± 0.013	107 ± 5	0.48

Y(I) is the photochemical yield of PS I, and LEF_{O₂} the linear electron flux determined by the gross rate of O₂ evolution. Values are mean $\pm$ s.e. (n = 14-15 leaf discs).

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675 **Fig. 1.** An example of the measurement of the photochemical yield of PS I in two parts. (a)
676 Steady-state photosynthesis in white light ($980 \mu\text{mol m}^{-2} \text{s}^{-1}$) was occurring with a steady
677 concentration of P700^+ , when strong far-red light (FR, $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) was turned on at time
678 $t = 0$ for 100 ms to further photo-oxidize P700. At $t = 50$ ms, a 10-ms saturating light pulse (SP,
679 $9000 \mu\text{mol m}^{-2} \text{s}^{-1}$) was applied to fully photo-oxidize P700. FR was turned off at $t = 100$ ms,
680 followed by cessation of actinic illumination at $t = 166$ ms. The signal relaxed to a baseline
681 corresponding to fully reduced P700. The signal interval b represents the P700 still available to
682 be photo-oxidized during illumination at a particular irradiance. The signal is an average of 9
683 traces (< 1 s between two consecutive repeats). (b) The maximum amount of photo-oxidizable
684 P700 was obtained by first illuminating a leaf disc with weak continuous far-red light to attain
685 about 88% oxidation in the steady state. Then a single-turnover (ST) saturating xenon flash was
686 applied to photo-oxidize the remainder of the P700. The peak immediately after the flash was
687 taken to be P_m . The signal is an average of 9 traces. The ratio b/P_m is the photochemical yield
688 of PS I (Klughammer and Schreiber 2007).

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Fig. 2. (a) Variation of steady-state electron fluxes with [antimycin A] used to infiltrate leaf tissue. A fixed irradiance of $980 \mu\text{mol m}^{-2} \text{s}^{-1}$ was used. Values are means $\pm$ s.e. ($n = 15$ leaf discs). (b) ϕ_{NPQ} , the fraction of light absorbed by the PS II antennae that is dissipated thermally via ΔpH - and xanthophylls-regulated processes is plotted against [antimycin A].

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702 **Fig. 3.** Response of steady-state electron fluxes to irradiance of white light. ETR1 represents
703 the total electron flux through PS I, and LEF_{O₂} (the gross oxygen evolution rate multiplied by
704 four) represents the linear electron flux through both photosystems, both being whole-tissue
705 steady-state measurements. CEF_{O₂} = ETR1 – LEF_{O₂}. LEF_{FI} is a measure of the electron flux
706 through PS II based on Chl fluorescence emitted from a certain depth in the leaf tissue. Each
707 leaf disc was exposed to an irradiance that increased from 0 to the maximum using white
708 halogen light filtered through neutral density filters. The leaf disc was maintained at each
709 irradiance for about 10 min to reach steady-state photosynthesis. Oxygen evolution and Chl
710 fluorescence were first measured simultaneously. Immediately afterwards P700 kinetics were
711 measured while maintaining steady state. The temperature was 25°C. Values are means ± s.e.
712 (n = 14 leaf discs).

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717 **Fig. 4.** Variation of steady-state electron fluxes with leaf temperature, at $980 \mu\text{mol photons m}^{-2}$
 718 s^{-1} . The leaf temperature was controlled by thermostating the temperature of the gas-phase O_2
 719 electrode chamber at the desired temperature. Other conditions are described in Fig. 3. Values
 720 are means $\pm$ s.e. ($n = 14$ leaf discs).

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725 **Fig. 5.** (a) Variation of steady-state electron fluxes with the relative water content of leaf tissue,
726 at $980 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The straight line is a linear regression line through the points.
727 Other conditions are described in Fig. 3.

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