FUNCTIONAL PLANT BIOLOGY

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|---|---|
| Journal: | *Functional Plant Biology* |
| Manuscript ID | FP18039.R1 |
| Manuscript Type: | Research paper |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Zhang, Meng-Meng; Northeast Forestry University, Harbin, College of Life Science<br>Fan, Da-Yong; Australian National University, Research School of Biology<br>Sun, Guang-Yu; Northeast Forestry University, Harbin, College of Life Science<br>Chow, Wah Soon; Australian National University, Research School of Biology |
| Keyword: | Chlorophyll fluorescence, Electron transport, Photosystem I, Photosystem II, Bioenergetics, Light reactions |
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# Optimizing the linear electron transport rate measured by chlorophyll *a* fluorescence to empirically match the gross rate of oxygen evolution in white light: Towards improved estimation of the cyclic electron flux around Photosystem I in leaves

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**Running title**: Optimizing matching of fluorescence- and $O_2$-based electron transport rates

**Abbreviations**: AA, antimycin A; CEF, cyclic electron flux around PS I; Chl, chlorophyll; ΔFlux, difference between ETR1 and ETR2 as an estimate of CEF; ETR1, the electron flux through PS I; ETR2, the electron flux through PS II (and PS I in series) measured as either $LEF_{fl}$ or $LEF_{O2}$; $f_I$, and $f_{II}$, the fraction of absorbed light partitioned to PS I and PS II, respectively; $F_m$ and $F_m'$, the maximum Chl fluorescence yield in a dark-adapted state and a light-adapted state, respectively; $F_s$ and $F_o'$, the steady-state and minimum Chl fluorescence yield in a light-adapted state, respectively; $I$, irradiance; $LEF_{fl}$, the linear electron flux through both photosystems, measured by chlorophyll fluorescence; $LEF_{O2}$, the linear electron flux through both photosystems, measured by the gross rate of oxygen evolution; MV, methyl viologen; NDH, nicotinamide adenine dinucleotide dehydrogenase-like complex; P700, the special Chl pair acting as the primary electron donor in PSI; PGR5, proton gradient regulation 5 protein; $P_m$ and $P_m'$, signal corresponding to the maximum extent of oxidizable P700 in weak far-red light and in actinic light, respectively; PS I and PS II, Photosystem I and II, respectively; $qP$, the photochemical quenching parameter; Y(I) and Y(II), the photochemical yield of PS I and PS II, respectively.

**Summary Text for the Table of Contents**

Cyclic electron flow around Photosystem I (CEF) was discovered in isolated chloroplasts more than six decades ago, but its quantification has been elusive. We devised a method capable of estimating CEF in intact leaves attached to a plant. The method involves measurement of (1) the total electron flux through Photosystem I by

a near-infra-red signal, and (2) the linear electron flux through both photosystems in series by optimizing conditions of excitation and detection of chlorophyll fluorescence.

**Abstract**

The cyclic electron flux (CEF) around photosystem I (PSI) was discovered in isolated chloroplasts more than six decades ago, but its quantification has been hampered by the absence of net formation of a product or net consumption of a substrate. We estimated *in vivo* CEF in leaves as the difference (ΔFlux) between the total electron flux through PSI (ETR1) measured by a near infra-red signal, and the linear electron flux through both photosystems by optimized measurement of chlorophyll *a* fluorescence ($LEF_{fl}$). Chlorophyll fluorescence was excited by modulated green light from a light-emitting diode at an optimal average irradiance, and the fluorescence was detected at wavelengths >710 nm. In this way, $LEF_{fl}$ matched the gross rate of oxygen evolution multiplied by 4 ($LEF_{O2}$) in broad-spectrum white actinic irradiance up to half (spinach, poplar and rice) or one-third (cotton) of full sunlight irradiance. This technique of estimating CEF can be applied to leaves attached a plant.

**Introduction**

Although discovered six decades ago (Arnon *et al.* 1955, Shikanai 2007), the cyclic electron flux around Photosystem I (PSI) is difficult to quantify because there is neither net formation of a product nor net consumption of a substrate. Currently, a

reasonable estimation involves measuring the total electron flux through PSI (ETR1) and the linear electron flux through both photosystems in series under identical illumination and sampling conditions. That is, ETR1 is measured by the redox signal of P700, the primary electron donor in PSI in the form of a chlorophyll (Chl) dimer in leaves. To obtain ETR1, the photochemical yield of PSI, Y(I), given by the fraction of P700 that can potentially be photo-oxidized under a given set of measurement conditions (Klughammer and Schreiber 1994, 2007), was first determined; Y(I) can then be used to calculate ETR1 by taking into account the irradiance, leaf absorptance and the fraction of absorbed light partitioned to PSI ($f_I$), as well as assuming the quantum efficiency of photochemical conversion to be 1.0. The linear electron flux through PSII and PSI in series (ETR2) was measured by the gross rate of oxygen evolution using a Clarke-type oxygen electrode in $CO_2$-enriched air in which photorespiration is suppressed ($LEF_{O2}$). Both ETR1 and $LEF_{O2}$ are measurements representative of the whole leaf tissue: oxygen is evolved from PSII complexes that absorb light anywhere within the tissue, while the measuring beam for detecting the $P700^+$ signal (820 nm, with reference wavelength 870 nm), being only weakly absorbed by $P700^+$ and hardly absorbed by neutral chlorophyll (Chl) molecules, is scattered repeatedly in the whole tissue until it is absorbed by $P700^+$ (Oguchi *et al*. 2011).

The difference ETR1 − $LEF_{O2}$ = ΔFlux is approximately the cyclic electron flux (CEF) if photorespiration and the local electron cycle in PSI in the form of charge recombination are negligible. Alternatively, if oxygen evolution is assayed

by membrane-inlet mass spectrometry, the oxygen uptake in photorespiratory conditions can be taken into account; the method can then be used in ambient air (although the draw-down of $CO_2$ in a closed chamber rapidly changes the [CO2] in ambient air), provided charge recombination in PSI is negligible. However, membrane-inlet mass spectrometry is not as readily available as a Clarke-type oxygen electrode.

Another disadvantage associated with oxygen measurement is that a leaf segment is cut and placed in a chamber, either that of an oxygen electrode or that of a mass spectrometer, making *in situ* measurement of leaves attached to a plant impossible. A further disadvantage is that, since oxygen measurements are generally slow, there is limited time resolution to monitor the time course of CEF, for example, during photosynthetic induction. Thus, assaying linear electron flux by oxygen measurement can present a number of shortcomings (Fan *et al*. 2016).

The linear electron flux (ETR2) through both photosystems can also be measured by Chl *a* fluorescenc, which yields the photochemical yield of PSII, Y(II). Y(II) can then be used to calculate ETR2 (specifically termed $LEF_{fl}$ here) by taking into account the irradiance, leaf absorptance and $f_{II}$ the fraction of absorbed light partitioned to PSII (Genty *et al*. 1989; Evans *et al*. 2017). Chl fluorescence can be measured on a leaf attached to a plant, in photorespiratory conditions and with good time resolution. Unfortunately, without optimizing the conditions of Chl fluorescence measurement, ETR2 so obtained can be considerably less than $LEF_{O2}$ in broad-spectrum white

actinic light, when the modulated measuring (excitation) light is blue (Kou *et al*. 2013). This underestimation of the linear electron flux is due to the inherently localized detection of the Chl fluorescence signal from a shallow depth of the leaf tissue (Oguchi *et al*. 2011). Under other conditions, where the *actinic* light is blue but the modulated measuring light is red, Chl fluorescence-based ETR2 can be considerably *greater* than the linear electron transport rate obtained from the gross rate of $CO_2$ assimilation (Evans *et al*. 2017); this observation can be explained by a multilayer photosynthesis model (Evans 2009; Evans *et al*. 2017). The aim of this present study is to select conditions in which $LEF_{fl}$ is more representative of the whole tissue, so that it can match $LEF_{O2}$, both measured in broad-spectrum white light; $LEF_{fl}$ can then be subtracted from ETR1 to yield a reasonable estimate of CEF. This method allows us to estimate CEF in leaves attached to a plant even in photorespiratory conditions, at least up to a certain actinic irradiance, as demonstrated in four plant species.

## Materials and methods

### Plant growth

Four plant species, *Spinacia oleracea* L. (cv. Yates hybrid 102), *Populus canadensis* cv. 'Evergreen 65-1', *Oryza sativa* L. (spp. Japonica) and *Gossypium hirsutum* L. (cv. Sicot 75), were grown in a glasshouse (~30/15 ºC, day/night); they represent, respectively, various plant types: herbaceous, woody, monocot, and a perennial plant that is usually grown as an annual crop. A nutrient mix (Aquasol, Hortico, Clayton,

Australia) was supplemented by a slow release fertilizer ('Osmocote', Scotts Australia Pty Ltd, Castle Hill). Young fully-expanded leaves were used for measurements.

**Measurements of $O_2$ evolution**

$O_2$ evolution from leaf discs was measured in a gas-phase oxygen electrode (Hansatech, King's Lynn, UK) chamber maintained at 25°C. The sample chamber contained 1% $CO_2$ supplied by fabric matting moistened with 1 M $NaHCO_3/Na_2CO_3$ (pH = 9). $O_2$ evolution was measured in continuous white light from a halogen lamp (400-740 nm, peak spectral irradiance 620 nm, with about 7% of the irradiance within the wavelength range 700-740 nm) over several minutes until steady state. The slope at approximately 40 s after cessation of illumination (when a cooling artefact had subsided) was subtracted algebraically from the steady-state net oxygen evolution rate to yield the *gross* oxygen evolution rate. The gross oxygen evolution rate was multiplied by 4 to obtain the linear electron flux $LEF_{O2}$. The actinic irradiance was varied by increasing it in steps from darkness to yield a light-response curve. For calibration of the oxygen signals, 1 mL of air at 25°C (taken to contain 8.05 μmol $O_2$) was injected into the gas-phase $O_2$ electrode chamber.

**Measurement of Chl *a* fluorescence**

A pulse amplitude modulation fluorometer PAM 101/103 (Walz, Effeltrich, Germany) was used to measure Y(II). The modulated excitation light (1.6 kHz, unless automatically switched to 100 kHz when the saturating pulse was applied) was either

144 blue (wavelength 462 nm, average irradiance = 0.05 µmol $m^{-2}$ $s^{-1}$), red (wavelength
145 665 nm, 0.05 µmol $m^{-2}$ $s^{-1}$) or green (wavelength 511 nm, up to 0.38 µmol $m^{-2}$ $s^{-1}$).
146 To obtain sufficient green excitation irradiance, several commercial green LEDs were
147 tested. The chosen green LED (Cree C503B-GAN 535 nm Green LED) was the
148 brightest, and was driven by an amplified (modulated) voltage from the Emitter
149 terminal of the PAM 101. A stainless-steel metal mesh, of the kind used in a
150 Hansatech oxygen electrode, was placed underneath a leaf disc or a leaf attached to a
151 plant, to reflect green excitation light back into the leaf tissue, while allowing gas
152 diffusion in and out of the abaxial side.

153 A saturating pulse of white light, of duration 0.8 s and irradiance 7,300 µmol $m^{-2}$
154 $s^{-1}$, was used to obtain the maximum Chl fluorescence yield in a dark-adapted sample
155 ($F_m$) or a light-adapted sample ($F_m'$). In retrospective tests of saturation by the pulse
156 of white light, we compared $LEF_{fl}$ obtained using a saturating pulse of irradiance
157 12,000 µmol $m^{-2}$ $s^{-1}$ or 7,300 µmol $m^{-2}$ $s^{-1}$. The increases in $LEF_{fl}$ (using an average
158 green excitation irradiance of 0.24 µmol $m^{-2}$ $s^{-1}$) that we obtained at 12,000 µmol $m^{-2}$
159 $s^{-1}$ compared with 7,300 µmol $m^{-2}$ $s^{-1}$ were at most (at the actinic irradiance 1,500 µmol
160 $m^{-2}$ $s^{-1}$) only slight for spinach (2%), poplar (4%), rice (4%) and cotton (5%). Below
161 1,000 µmol $m^{-2}$ $s^{-1}$, (spinach, poplar and rice) or 600 µmol $m^{-2}$ $s^{-1}$ (cotton), there was
162 no statistical difference between 12,000 µmol $m^{-2}$ $s^{-1}$ 7,300 µmol $m^{-2}$ $s^{-1}$. Thus, we
163 consider a pulse at 7,300 µmol $m^{-2}$ $s^{-1}$ to be saturating for the actinic irradiance range
164 relevant to this study.

The Chl fluorescence was measured via the Detector terminal of the PAM 101 (Walz, Effeltrich, Germany), using a filter which transmitted fluorescence of wavelength >710 nm. The rationale of selecting longer wavelengths is the expectation that re-absorption of long-wavelength fluorescence would be minimized, thereby allowing detection from greater depths in the tissue.

The photochemical yield of PSII, Y(II), was calculated as $1 - F_s/F_m'$, where $F_s$ is the steady-state fluorescence yield and $F_m'$ the maximum fluorescence yield in the light-adapted state (Genty *et al.* 1989). The fluorescence-based linear rate of electron transport through PSII (and PSI) was calculated as $LEF_{fl} = Y(II) \times I \times 0.85 \times 0.5$, where $I$ is the irradiance, 0.85 the leaf absorptance and 0.5 the assumed partitioning (also see experimental estimations in Table 1) of the absorbed light between the two photosystems.

The photochemical quenching parameter $qP$ was calculated as $(F_m' - F_s)/(F_m' - F_o')$, where $F_o'$ is the minimum Chl fluorescence yield of open PSII reaction centre traps in the light-adapted state. $F_o'$ was calculated according to Oxborough and Baker (1997).

**Measurement of the $P700^+$ signal from leaf segments**

Measurement of the photochemical yield of PSI, Y(I), is based on the technique of Klughammer and Schreiber (1994, 2007), slightly modified by introducing a strong far-red pulse shortly before the saturating pulse of white light, as described by Kou *et al.* (2013). The measurement employed a dual-wavelength (820/870 nm) P700 unit

(ED-P700DW) connected to a pulse amplitude modulation (PAM 101) fluorometer (Walz, Effeltrich, Germany) in the reflectance mode, with a metal mesh underneath the abaxial side of a leaf segment inside a gas-phase oxygen electrode in which the upper water jacket had a vertical port for accepting a multifurcated light guide. When a leaf attached to the plant was sampled, the same upper water jacket was placed on the leaf, with the metal mesh underneath the leaf. The metal mesh served to reflect the near-infra-red measuring beam or the fluorescence excitation light back to the leaf tissue, while allowing gas diffusion into the abaxial side of the leaf. Actinic illumination was provided to the adaxial side of the leaf disc. Timing of data acquisition and application of various lights was controlled by a pulse-delay generator (Model 555, Berkeley Nucleonics, San Rafael, CA, USA).

The measurement of Y(I) was conducted in two stages (Kou *et al*. 2013). First, a leaf sample was illuminated to steady state for about 10 minutes during which oxygen evolution or Chl fluorescence was monitored. Then the system was quickly (within < 1 min during which the leaf sample was in darkness) switched to $P700^+$ measurement. To re-establish steady state, the leaf sample was illuminated with a given actinic light for 8.8 s before data acquisition started, followed by a strong far-red pulse (600 µmol $m^{-2}$ $s^{-1}$, 100 ms duration), in the middle of which a saturating pulse (10,000 µmol $m^{-2}$ $s^{-1}$, 10 ms duration) was applied to reach the maximum permissible oxidation of P700 ($P_m'$) in the presence of actinic light. The duration of total actinic illumination was 9.0 s. The sequence was repeated every 9.3 s, so that the dark time (which helped to establish the baseline corresponding to fully reduced

P700) between repeats was about 3.2% of the total length of time. Twenty-five repeat signals were averaged.

The second stage of Y(I) measurement was to determine the maximum signal corresponding the maximum P700 photo-oxidation when the acceptor-side limitation was negligible. Weak far-red light (92 μmol $m^{-2}$ $s^{-1}$) was applied to attain a steady $P700^+$ concentration. Then a saturating pulse (0.5 ms duration, 10,000 μmol $m^{-2}$ $s^{-1}$) was applied to fully oxidize the remaining P700 ($P_m$). The values of $P_m$, $P_m'$, and the steady-state oxidation state of P700 in the presence of a given actinic light were used to calculate Y(I) according to Klughammer and Schreiber (2007).

## Results

### Light-response curves of photosynthetic electron transport determined via gross oxygen evolution and via the yield of Chl *a* fluorescence excited by blue, red or green modulated light

The gross rate of oxygen evolution, in white halogen light and in 1% $CO_2$, of spinach leaf discs cut from plants grown in a glasshouse showed typical responses. At the maximum irradiance of almost 1,700 μmol $m^{-2}$ $s^{-1}$ tested, the gross rate of oxygen evolution multiplied by 4 ($LEF_{O2}$) was nearly, but not yet fully, light-saturated (Fig. 1, closed circles). $LEF_{O2}$ was compared with the fluorescence-based $LEF_{fl}$. Fig. 1 shows that the extent of matching of $LEF_{fl}$ with $LEF_{O2}$ varied with irradiance and with wavelength of the modulated excitation light. At low actinic irradiance, below about

500 µmol $m^{-2}$ $s^{-1}$, the matching was good regardless of excitation wavelength. Above this irradiance, however, the matching was the poorest with blue, and then red excitation light. Matching was improved with green excitation light; at an average excitation irradiance of 0.38 µmol $m^{-2}$ $s^{-1}$, the matching was better than with 0.05 µmol $m^{-2}$ $s^{-1}$. However, above actinic irradiance 1,100 µmol $m^{-2}$ $s^{-1}$, the fluorescence-based $LEF_{fl}$ underestimated the linear electron flux obtained as $LEF_{O2}$.

Similarly, poplar leaf discs in 1% $CO_2$ showed poor matching of $LEF_{O2}$ and $LEF_{fl}$ obtained in blue excitation light, slightly better matching in red excitation light, and somewhat better matching with weak green excitation light (0.07 µmol $m^{-2}$ $s^{-1}$, Fig. 2a). In another batch of poplar plants, matching was good at an average green excitation irradiance of 0.38 µmol $m^{-2}$ $s^{-1}$, up to an actinic irradiance of ~1,000 µmol $m^{-2}$ $s^{-1}$ (Fig. 2b). At even higher green excitation irradiance, the matching deteriorated (data not shown).

In rice leaf segments measured in 1% $CO_2$, the matching was similarly improved as the green excitation light increased to 0.38 µmol $m^{-2}$ $s^{-1}$ (Fig. 3). However, when the actinic irradiance exceeded ~1,100 µmol $m^{-2}$ $s^{-1}$, the matching deteriorated, $LEF_{fl}$ being considerably smaller than $LEF_{O2}$.

In cotton leaf discs measured in 1% $CO_2$, the matching also improved as the excitation light was changed from blue to red to green. However, the matching was not good when the actinic irradiance exceeded ~700 µmol $m^{-2}$ $s^{-1}$, even with green excitation light. This maximum actinic irradiance for good matching is lower in

249 cotton than in the other three plant species.

250 **Estimation of cyclic electron flux around PSI as the difference (ΔFlux) between**
251 **ETR1 and $LEF_{fl}$**

252 Given the reasonably good matching between $LEF_{fl}$ and $LEF_{O2}$ when Chl *a*
253 fluorescence was excited by green light at 0.38 µmol $m^{-2}$ $s^{-1}$ and in white actinic light
254 of irradiance < 1,000 µmol $m^{-2}$ $s^{-1}$ (spinach, poplar and rice leaves), or < 700 µmol
255 $m^{-2}$ $s^{-1}$ (cotton leaves), we proceeded to estimate cyclic electron flux around PSI as
256 the difference (ΔFlux) between ETR1 and $LEF_{fl}$. These electron fluxes were
257 determined on intact leaves attached to the plant in air, or in leaf discs in air enriched
258 with 1% $CO_2$, the latter conditions used in oxygen measurements. In spinach leaves
259 attached to the plant in air, $LEF_{fl}$ was not yet saturated at 1,100 µmol $m^{-2}$ $s^{-1}$, and
260 ETR1 was even less light-saturated (Fig. 5a). ΔFlux, was very small (relative to
261 $LEF_{fl}$) at irradiance < 300µmol $m^{-2}$ $s^{-1}$; above 500 µmol $m^{-2}$ $s^{-1}$, however, it increased
262 steadily until, at 1,000 µmol $m^{-2}$ $s^{-1}$, it was about one-third of $LEF_{fl}$. Similar results
263 for ΔFlux were obtained with spinach leaf discs in 1% $CO_2$ (Fig. 5b).

264 Spinach leaf discs were vacuum infiltrated with water (control), 200 µM
265 antimycin A (AA, an inhibitor of the PGR5-dependent cyclic pathway) or 100 µM
266 methyl viologen (MV, a mediator of electron transfer to molecular oxygen). Excess
267 intercellular water was allowed to evaporate in darkness until the tissue was no longer
268 translucent, before measurements. Table 2 shows that ΔFlux was close to zero after
269 these treatments.

In an intact poplar leaf attached to a cut branch of a plant, ΔFlux was very small (relative to $LEF_{fl}$) in white actinic light below actinic irradiance 100 µmol $m^{-2}$ $s^{-1}$, above which it increased practically linearly (Fig. 6a). In cut leaf discs in 1% $CO_2$, ΔFlux increased in the same way as in the intact leaf, even though ETR1 was somewhat smaller at high actinic irradiance, and $LEF_{fl}$ showed a slight decline at high actinic irradiance (Fig. 6b).

In rice leaves attached to the plant, ΔFlux was negligibly small (relative to $LEF_{fl}$) below actinic irradiance 200 µmol $m^{-2}$ $s^{-1}$. It then increased linearly up to the maximum actinic irradiance 1,100 µmol $m^{-2}$ $s^{-1}$, at which it reached 62% of $LEF_{fl}$ (Fig. 7a). In cut rice leaf segments in 1% $CO_2$ (Fig. 7b), both ETR1 and $LEF_{fl}$ were smaller than in intact leaves. Nonetheless, ΔFlux behaved similarly as in intact leaves.

In cotton leaves, we had to restrict the actinic irradiance to below 800 µmol $m^{-2}$ $s^{-1}$ for matching of $LEF_{O2}$ and $LEF_{fl}$. The $LEF_{fl}$ so obtained was similar in an intact leaf attached to the plant in air to that of a leaf disc in 1% $CO_2$ (Fig. 8). In either case, ΔFlux (relative to $LEF_{fl}$) was negligibly small below 100 µmol $m^{-2}$ $s^{-1}$, but it increased linearly above that irradiance. ΔFlux was slightly larger in an intact cotton leaf than in a cut leaf disc.

**The decrease in $LEF_{fl}$ at high actinic irradiance**

At high actinic irradiance, $LEF_{fl}$ decreased in all four species, resulting in a deviation

290 from the $LEF_{O2}$ curve. To investigate the reason behind the decrease in $LEF_{fl}$, we
291 plotted $qP \times I$ (the product of the photochemical quenching parameter and irradiance)
292 against irradiance (Fig. 9a) in cotton leaves. It is seen that $qP \times I$ increased rapidly
293 with irradiance, then more slowly and even decreased slightly at irradiance >700
294 µmol $m^{-2}$ $s^{-1}$, implying that $qP$ decreased more drastically than irradiance increased.
295 By contrast, a plot of $(F_v'/F_m') \times I$ (the product of the light-adapted ratio of variable
296 fluorescence to maximum fluorescence and irradiance) against irradiance is a nearly
297 straight line, implying that $F_v'/F_m'$ was relatively constant.

298 **DISCUSSION**

299 One method of estimation of CEF involves determination of both the electron flux
300 through PSI (ETR1) and the linear electron flux ($LEF_{O2}$) through both photosystems
301 in series under identical conditions (Kou *et al.* 2013). Determination of $LEF_{O2}$ in an
302 oxygen electrode necessitates the use of a high $CO_2$ concentration to suppress
303 photorespiration and the cutting of a leaf segment or disc to be placed in the electrode
304 chamber. Further, oxygen measurements are inherently slow and, therefore, unable
305 to follow changes in CEF with sufficient time resolution, for example, during
306 photosynthetic induction. These shortcomings prompted us to seek an alternative
307 method of determining the linear electron flux.

308 Chlorophyll *a* fluorescence potentially offers such a convenient and
309 non-intrusive technique: it can be applied to an intact leaf attached to a plant, in air,
310 and with good resolution time. Various studies have attempted to correlate Chl *a*

311 fluorescence parameters with gas-exchange measurements. For instance, Weis and
312 Berry (1987) obtained a linear plot of $\phi_{O2}/qP$ against $qN$, where $\phi_{O2}$ is the gross rate of
313 oxygen evolution divided by the irradiance $I$, $qP$ is a photochemical quenching
314 parameter and $qN$ is a non-photochemical quenching parameter. In principle,
315 measurements of $qP$, $qN$ and $I$ should yield the gross rate of oxygen evolution from an
316 equation fitted to the linear plot. Unfortunately, the relationship is not universal
317 among leaves of plants; there are occasional exceptions to a single straight line
318 (Öquist and Chow 1992).

319 Genty *et al*. (1987) reported a near-linear relation between Y(II) and $\phi_{CO2}$, the
320 $CO_2$ assimilation rate per unit irradiance in barley and maize, as did Edwards and
321 Baker (1993) in maize. Seaton and Walker (1990) and Öquist and Chow (1992), on
322 the other hand, observed a curvilinear relation between Y(II) and $\phi_{O2}$, the gross $O_2$
323 evolution rate per unit irradiance. Further, there were exceptions to what appeared at
324 first sight to be a universal relation that can be fitted by a single curve (Öquist and
325 Chow 1992). The difficulty of obtaining a universal relation between Chl
326 fluorescence measurements and gas-exchange measurements is no doubt due to the
327 differential sampling of the leaf tissue by the two techniques: oxygen is evolved from
328 the whole tissue, but the fluorescence is predominantly detected from chloroplasts
329 near the surface of the leaf facing the detector. For this reason, fluorescence-derived
330 $LEF_{fl}$ depends on the spectral quality of the measuring beam, that of the actinic light,
331 and the wavelength band over which the fluorescence is detected (Evans *et al*. 2017).
332 By attempting to optimize the fluorescence measurement in this study, we hoped to

obtain a signal that is more representative of the whole leaf tissue and, therefore, better matched with gas exchange measurements, at least up to a certain actinic irradiance.

**Optimizing $LEF_{fl}$ to match $LEF_{O2}$**

We used green excitation light which is attenuated more slowly as it penetrates the leaf tissue (Terashima *et al*. 2009) and which, therefore, reports from greater depths in the leaf tissue. We also detected Chl fluorescence at wavelengths ≥710 nm; the long-wavelength emission, if occurring from deep tissue, is less likely to be re-absorbed by chlorophyll on its way to the detector. Using these measurement conditions, we obtained $LEF_{fl} = Y(II) \times I \times 0.85 \times 0.5$, as explained in Methods. The assumed partitioning of the absorbed, broad-spectrum halogen light between the two photosystems is 0.5 to each photosystem. For spinach grown in a glasshouse, this was indeed the case experimentally (Fan *et al*. 2016). Below, we experimentally obtain the partitioning of broad-spectrum halogen light for the four species, all grown in the same glasshouse. It is noted that at low actinic irradiance (≤ 500 for spinach and rice; ≤ 150 μmol $m^{-2}$ $s^{-1}$ for cotton and poplar), the variation of $LEF_{fl}$ with actinic irradiance was practically independent of the spectral quality and/or irradiance of the weak excitation light. This is probably because, at a relatively low actinic irradiance, only the chloroplasts in shallow leaf tissue were photosynthesizing to any great extent. Under such conditions, by simply equating $Y(II) \times I \times 0.85 \times f_{II}$ with $LEF_{O2}$ for irradiance ≤ 150 μmol $m^{-2}$ $s^{-1}$ (poplar and cotton) or ≤ 500 μmol $m^{-2}$ $s^{-1}$ (spinach and

354 rice), we derived values of $f_{II}$ as shown in Table 1. The average $f_{II}$ is very close to
355 0.5, justifying our use of the value of $f_{II}$ = 0.5 for the partitioning to PSII and $f_I$ = 0.5
356 for the partitioning to PSI in the four species, in broad-spectrum white light.

357 The linear electron flux detected by Chl fluorescence $LEF_{fl}$ flows through both
358 photosystems in series, to reduce $NADP^+$ to NADPH or, to a much lesser extent in
359 angiosperms, reduce $O_2$ to form superoxide in the Mehler reaction. The linear
360 electron flux detected as oxygen evolution $LEF_{O2}$, on the other hand, would be
361 underestimated if oxygen were simply consumed in the Mehler reaction. However,
362 the resulting superoxide is scavenged in the water-water cycle to release oxygen again
363 (Asada 2000, Miyake 2010); therefore, no net consumption of oxygen occurs despite
364 any Mehler reaction, and $LEF_{O2}$ correctly measures the linear electron flux through
365 both photosystems even when the Mehler reaction occurs, provided the water-water
366 cycle runs efficiently. Thus, neither $LEF_{fl}$ nor $LEF_{O2}$ is compromised by any Mehler
367 reaction.

368 At high actinic irradiance it is obvious that, to obtain good matching between
369 $LEF_{fl}$ and $LEF_{O2}$, excitation with blue light was not as good as with red light, which
370 was not as good as with green light; further, a higher average excitation irradiance of
371 green light, up to about 0.38 µmol $m^{-2}$ $s^{-1}$, seemed to be optimal. (Figs. 1-4). Thus,
372 using this green optimal irradiance for the measuring beam, there was good matching
373 of $LEF_{O2}$ and $LEF_{fl}$ up to a broad-spectrum actinic irradiance of ~1,000 µmol $m^{-2}$ $s^{-1}$
374 for spinach, rice and poplar, and about 700 µmol $m^{-2}$ $s^{-1}$ for cotton leaves.

375 Beyond the maximum actinic irradiance below which the matching was good,
376 $LEF_{fl}$ decreased slightly, even when $LEF_{O2}$ continued to increase with irradiance.
377 This discrepancy between $LEF_{fl}$ and $LEF_{O2}$ at high actinic irradiance prompted us to
378 re-measure $LEF_{fl}$ using two saturating pulses, one at irradiance 12,000 μmol $m^{-2}$ $s^{-1}$,
379 and the other at 7,300 μmol $m^{-2}$ $s^{-1}$ which was originally used. The increases in $LEF_{fl}$
380 (using an average green excitation irradiance of 0.24 μmol $m^{-2}$ $s^{-1}$) that we obtained at
381 12,000 μmol $m^{-2}$ $s^{-1}$ compared with 7,300 μmol $m^{-2}$ $s^{-1}$ were at most (at the actinic
382 irradiance 1,500 μmol $m^{-2}$ $s^{-1}$) only slight for spinach (2%), poplar (4%), rice (4%) and
383 cotton (5%) (Data not shown). These differences, while real, are not sufficient to
384 explain the discrepancy between $LEF_{fl}$ and 4 x the gross oxygen evolution rate at
385 actinic irradiance 1,500 μmol $m^{-2}$ $s^{-1}$ (13% for spinach, 28% for poplar, 17% for rice
386 and 28% for cotton). In all four plant species, no significant difference in $LEF_{fl}$
387 between the two saturating pulse intensities was obtained when the actinic irradiance
388 was below a certain value: 1,000 μmol $m^{-2}$ $s^{-1}$ for spinach, rice and poplar, and 660
389 μmol $m^{-2}$ $s^{-1}$ for cotton. That is, a pulse at 7,300 μmol $m^{-2}$ $s^{-1}$ was saturating at or
390 below these irradiances; these are also actinic irradiance values below which we
391 obtained a good empirical matching of $LEF_{fl}$ and $LEF_{O2}$.

392 The slight decrease in $LEF_{fl}$ at high actinic irradiance is almost certainly due to
393 detection of the fluorescence predominantly from chloroplasts in shallower depths of
394 the tissue. In shallow tissue the actinic irradiance is not yet attenuated substantially,
395 and $Q_A$ is in a more reduced state (*qP* lower) than is the case in deeper tissue.
396 Indeed, plotting the product of *qP* and *I* against *I* gave a curve that reached a

maximum at an irradiance at which $LEF_{fl}$ began to deviate from $LEF_{O2}$ (Fig. 9a). Above that maximum irradiance, $qP \times I$ even declined slightly with increase in irradiance. Since $LEF_{fl} = Y(II) \times I \times 0.85 \times f_{II}$, where $Y(II) = qP \times F_v'/F_m'$, there is an underestimation of $LEF_{fl}$ due to the use of a $qP$ that represents chloroplasts in shallower depths of the tissue rather than in the whole tissue. By contrast to $qP$, the photochemical yield of open PSII reaction centre traps ($F_v'/F_m'$) was rather constant, since a plot of the product of $F_v'/F_m'$ and $I$ against $I$ yielded a near-straight line (Fig. 9b).

**Estimation of CEF from the difference between ETR1 and $LEF_{fl}$**

Since Chl fluorescence can be measured from a leaf attached to a plant, and since $LEF_{fl}$ and $LEF_{O2}$ can be empirically matched, at least up to a certain maximum actinic irradiance, we are in a position to estimate CEF as the difference between ETR1 and $LEF_{fl}$ in intact spinach leaves in air. Fig. 5a shows ETR1 increasing with irradiance, even when $LEF_{fl}$ began to plateau. The difference (ΔFlux) is attributable mainly to CEF, since ΔFlux at 980 μmol $m^{-2}$ $s^{-1}$ in spinach leaf discs is almost completely inhibited by antimycin A (Kou *et al*. 2013) and completely abolished by antimycin A or MV as shown in Table 2. At 980 μmol $m^{-2}$ $s^{-1}$, ΔFlux (= ETR1 − $LEF_{fl}$) was approximately one-third of $LEF_{fl}$ in an intact spinach leaf (Fig. 5a), just as ΔFlux (in that case the difference between ETR1 and $LEF_{O2}$) was about one-third of $LEF_{O2}$ in leaf discs in 1% $CO_2$ (Kou *et al*. 2013). Further, at actinic irradiance <300 μmol $m^{-2}$ $s^{-1}$, ΔFlux was very small relative to $LEF_{fl}$, just there was little ΔFlux at low actinic

irradiance when compared with $LEF_{O2}$ (Kou et al. 2013). There was little difference between the ΔFlux of an intact spinach leaf in air in the laboratory environment and that of leaf discs in 1% $CO_2$.

ΔFlux in spinach leaf discs after vacuum infiltration with antimycin A (AA) was close to zero at both 500 and 1,000 µmol $m^{-2}$ $s^{-1}$ (Table 2). This suggests that AA inhibited the PGR5-dependent CEF pathway completely. Further, this result indicates that, in spinach, there was little or no charge recombination in PSI at these irradiances, for any charge recombination would have contributed to a residual ΔFlux. In *Arabidopsis thaliana*, the NDH-dependent pathway is minor compared with the PGR5-dependent CEF pathway and NDH may even aid the AA-sensitive, PGR5-dependent CEF pathway (Kou et al. 2015). In the presence of MV, ΔFlux was also close to zero. MV, by mediating electron transfer to molecular oxygen, should have minimized both CEF and charge recombination, so this result was expected. Unfortunately, we were not able to use vacuum infiltration on leaf tissues of the other three species without losing photosynthetic activity. Uptake through the cut petioles of poplar and cotton leaves overnight was attempted, as was floating leaf discs on solutions, but we were not confident that the inhibitors were reaching all the intended sites of action.

In intact poplar leaves attached to a branch (Fig. 6a) and in cotton leaves attached to the plant (Fig. 8a), ΔFlux increased with actinic irradiance with a smaller lag (< 100 µmol $m^{-2}$ $s^{-1}$) than in the other two species. The same was true of leaf

discs in 1% $CO_2$ (Fig. 6b). The decrease in $LEF_{fl}$ at high actinic irradiance was more pronounced in leaf discs than in an intact leaf. This could be due to some water loss which resulted in tissue contraction of leaf discs, thereby exacerbating the difficulty of detecting the fluorescence from the chloroplasts in deeper tissue.

ETR1 was greater in intact rice leaves compared with leaf segments, as was the case of $LEF_{fl}$. This observation confirms that measurements on intact leaves are superior to those on leaf discs of some plant species, and justifies the search for a method, such as Chl fluorescence in this study, that allows measurements on an intact system. Possibly, linear electron transport in rice segments was decreased by stomatal closure associated with water loss from the cut tissue (Fig. 7). In intact rice leaves in air as well as leaf segments in 1% $CO_2$, a lag (up to ~200 µmol $m^{-2}$ $s^{-1}$) was also apparent before ΔFlux increased steadily with irradiance (Fig. 7). At 1,000 µmol $m^{-2}$ $s^{-1}$, ΔFlux was comparable to $LEF_{fl}$ in cut leaf segments, whereas it was only about 60% of $LEF_{fl}$ in intact leaves. This could be due to water loss from leaf segments; water deficit increases CEF as estimated in various ways (Golding *et al.* 2004; Kohzuma *et al.* 2009; Kou *et al.* 2013), despite a decrease in linear electron transport.

Cotton leaf discs showed matching of $LEF_{O2}$ and $LEF_{fl}$ up to a lower maximum actinic irradiance than the other three species, so we measured ETR1 and $LEF_{fl}$ only up to 800 µmol $m^{-2}$ $s^{-1}$. Above ~100 µmol $m^{-2}$ $s^{-1}$, ΔFlux increased steadily in both intact cotton leaves attached to the plant and in leaf discs in 1% $CO_2$, reaching ≥ 50%

of $LEF_{fl}$ at the highest irradiance, 800 µmol $m^{-2}$ $s^{-1}$. Cyclic electron transport is a significant contributor to (1) the resistance of cotton to high-light stress, facilitating movement of cotton leaves to track the sun and intercept more radiation (Yao *et al*. 2018) and (2) improving the stability of the two photosystems under mild water deficit conditions (Yi *et al*. 2018).

In summary, we selected conditions of measurement so that $LEF_{fl}$ matched $LEF_{O2}$ quite closely, but only up a certain maximum irradiance of actinic light supplied from a halogen lamp. Up to this maximum actinic irradiance, it is possible to estimate CEF as the difference (ΔFlux) between ETR1 and $LEF_{fl}$ in leaves attached the plant.

**Acknowledgements**

The authors declare no conflicts of interest. This work was supported by a China Scholarship Council Fellowship (to M-M Z), and a grant from the Australian Research Council (to WSC, DP1093827). We thank Adam Pynt for modifying the PAM 101 to enable excitation with green light. We are grateful to Reviewer 4 for pointing out the need to test the effect of a much more intense saturating pulse.

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553 **Table 1. Estimate of the fraction of absorbed light partitioned to PS II ($f_{II}$) in leaf**
554 **discs in 1% $CO_2$.** The average irradiance of modulated excitation was 0.05 µmol $m^{-2}$
555 $s^{-1}$ for blue or red excitation, and varied for green excitation (average irradiance in
556 parentheses)

| Spinach | Excitation light | | | | | | |
|---|---|---|---|---|---|---|---|
| Actinic irradiance | blue | red | Green (0.05) | Green (0.07) | Green (0.18) | Green (0.38) | Average |
| 16 | 0.47 | 0.48 | 0.52 | - | - | 0.47 | |
| 38 | 0.46 | 0.47 | 0.52 | - | - | 0.51 | |
| 81 | 0.49 | 0.49 | 0.53 | - | - | 0.49 | |
| 163 | 0.53 | 0.50 | 0.54 | - | - | 0.50 | |
| 506 | 0.53 | 0.51 | 0.52 | - | - | 0.51 | 0.50±0.02 (sd) |

557

| Poplar | Excitation light | | | | | | |
|---|---|---|---|---|---|---|---|
| Actinic irradiance | blue | red | Green (0.05) | Green (0.07) | Green (0.18) | Green (0.38) | Average |
| 13 | 0.54 | 0.43 | - | 0.39 | - | 0.54 | |
| 33 | 0.47 | 0.43 | - | 0.37 | - | 0.53 | |
| 72 | 0.48 | 0.42 | - | 0.44 | - | 0.52 | |
| 145 | 0.50 | 0.52 | - | 0.47 | - | 0.52 | 0.47±0.05 (sd) |

558

| Rice | Excitation light | | | | | | |
|---|---|---|---|---|---|---|---|
| Actinic irradiance | blue | red | Green (0.05) | Green (0.07) | Green (0.18) | Green (0.38) | Average |
| 20 | 0.46 | 0.49 | - | 0.43 | 0.53 | 0.44 | |
| 41 | 0.54 | 0.51 | - | 0.49 | 0.47 | 0.53 | |
| 84 | 0.52 | 0.51 | - | 0.50 | 0.50 | 0.56 | |
| 169 | 0.51 | 0.49 | - | 0.50 | 0.48 | 0.52 | |
| 523 | 0.60 | 0.52 | - | 0.49 | 0.46 | 0.50 | 0.50±0.04 (sd) |

559

| Cotton | Excitation light | | | | | | |
|---|---|---|---|---|---|---|---|
| Actinic irradiance | blue | red | Green (0.05) | Green (0.07) | Green (0.18) | Green (0.38) | Average |
| 16 | 0.46 | 0.45 | - | 0.43 | - | 0.35 | |
| 36 | 0.49 | 0.46 | - | 0.47 | - | 0.43 | |
| 76 | 0.49 | 0.49 | - | 0.48 | - | 0.45 | |
| 152 | 0.51 | 0.51 | - | 0.50 | - | 0.49 | 0.47±0.04 (sd) |

560

**Table 2**

**The difference between ETR1 and $LEF_{fl}$ (ΔFlux) in spinach leaf discs, as affected by an inhibitor of cyclic electron transport or a mediator of oxygen reduction**

Leaf discs were vacuum infiltrated with water (control), 200 µM AA or 100 µM MV) and, after evaporation of excess intercellular water, were illuminated at two irradiances. Values are means for 3 to 4 leaf discs ± s.d.

| | Δflux | |
|---|---|---|
| Treatment | 500 µmol $m^{-2}$ $s^{-1}$ | 1000 µmol $m^{-2}$ $s^{-1}$ |
| water control | 25.6±4.6 | 77.9±23.5 |
| AA | 2.3±13.6 | −3.4±12.7 |
| MV | 0.3±5.8 | −10.4±5.5 |

579

580 **Figure legends**

581 **Fig. 1**. Response of the linear photosynthetic electron flux of spinach leaf discs in 1%
582 $CO_2$ to the irradiance of actinic light from a halogen lamp. The linear electron flux
583 was measured either as the gross rate of oxygen evolution multiplied by 4 (•) or Chl
584 fluorescence excited by modulated light from light-emitting diodes emitting at blue
585 (♦, 0.05 µmol $m^{-2}$ $s^{-1}$), red (■, 0.05 µmol $m^{-2}$ $s^{-1}$) or green wavelengths (Δ, 0.05
586 µmol $m^{-2}$ $s^{-1}$; ▲, 0.38 µmol $m^{-2}$ $s^{-1}$). Values are means of six to seven leaf discs (±
587 s.d.).

588 **Fig. 2**. Response of the linear photosynthetic electron flux of poplar leaf discs in 1%
589 $CO_2$ to the irradiance of actinic light from a halogen lamp. The linear electron flux
590 was measured either as the gross rate of oxygen evolution multiplied by 4 (•, first
591 batch of plants; ○, second batch of plants) or Chl fluorescence excited by modulated
592 light from light-emitting diodes emitting at blue (♦, 0.05 µmol $m^{-2}$ $s^{-1}$), red (■, 0.05
593 µmol $m^{-2}$ $s^{-1}$) or green wavelengths (Δ, 0.07 µmol $m^{-2}$ $s^{-1}$, first batch of plants; ▲,
594 0.38 µmol $m^{-2}$ $s^{-1}$, second batch of plants). Values are means of nine to ten leaf
595 discs (± s.d.).

596 **Fig. 3**. Response of the linear photosynthetic electron flux of rice leaf segments in 1%
597 $CO_2$ to the irradiance of actinic light from a halogen lamp. The linear electron flux
598 was measured either as the gross rate of oxygen evolution multiplied by 4 (•) or Chl
599 fluorescence excited by modulated light from light-emitting diodes emitting at green

600 wavelengths (Δ, 0.07 µmol $m^{-2}$ $s^{-1}$; ◊, 0.18 µmol $m^{-2}$ $s^{-1}$; ▲, 0.38 µmol $m^{-2}$ $s^{-1}$).
601 Values are means of five to six leaf discs (± s.d.).

602 **Fig. 4**. Response of the linear photosynthetic electron flux of cotton leaf discs in 1%
603 $CO_2$ to the irradiance of actinic light from a halogen lamp. The linear electron flux
604 was measured either as the gross rate of oxygen evolution multiplied by 4 (•) or Chl
605 fluorescence excited by modulated light from light-emitting diodes emitting at blue
606 (♦, 0.05 µmol $m^{-2}$ $s^{-1}$), red (■, 0.05 µmol $m^{-2}$ $s^{-1}$) or green wavelengths (Δ, 0.07
607 µmol $m^{-2}$ $s^{-1}$; ▲, 0.38 µmol $m^{-2}$ $s^{-1}$). Values are means of six leaf discs (± s.d.).

608 **Fig. 5**. Response of the total photosynthetic electron flux through PSI, ETR1 (■)
609 and the Chl fluorescence-based linear photosynthetic electron flux through both
610 photosystems, $LEF_{fl}$ (•) of spinach leaves attached to the plant in air (*a*) or leaf discs
611 in 1% $CO_2$ (b). The difference between ETR1 and $LEF_{fl}$ is ΔFlux (▲), used as an
612 estimate of the cyclic electron flux around PSI. Values are means of seven leaves (±
613 s.d.).

614 **Fig. 6**. Response of the total photosynthetic electron flux through PSI, ETR1 (■)
615 and the Chl fluorescence-based linear photosynthetic electron flux through both
616 photosystems, $LEF_{fl}$ (•) of poplar leaves attached to a branch of the plant in air (*a*) or
617 of leaf discs in 1% $CO_2$ (b). The difference between ETR1 and $LEF_{fl}$ is ΔFlux (▲),
618 used as an estimate of the cyclic electron flux around PSI. Values are means of
619 seven leaves (± s.d.).

620 **Fig. 7**. Response of the total photosynthetic electron flux through PSI, ETR1 (■)

and the Chl fluorescence-based linear photosynthetic electron flux through both photosystems, $LEF_{fl}$ (●) of rice leaves attached to the plant in air (*a*) or of leaf segments in 1% $CO_2$ (b). The difference between ETR1 and $LEF_{fl}$ is ΔFlux (▲), used as an estimate of the cyclic electron flux around PSI. Values are means of seven leaves (± s.d.).

**Fig. 8**. Response of the total photosynthetic electron flux through PSI, ETR1 (■) and the Chl fluorescence-based linear photosynthetic electron flux through both photosystems, $LEF_{fl}$ (●) of cotton leaves attached to the plant in air (*a*) or of leaf discs in 1% $CO_2$ (b). The difference between ETR1 and $LEF_{fl}$ is ΔFlux (▲), used as an estimate of the cyclic electron flux around PSI. Values are means of seven leaves (± s.d.).

**Fig. 9**. A plot of $qP \times I$ (a) and $F_v'/F_m' \times I$ (b) against irradiance $I$ for cotton leaf discs in 1% $CO_2$. Values are means of nine leaves (± s.d.).

635 **Fig. 1**
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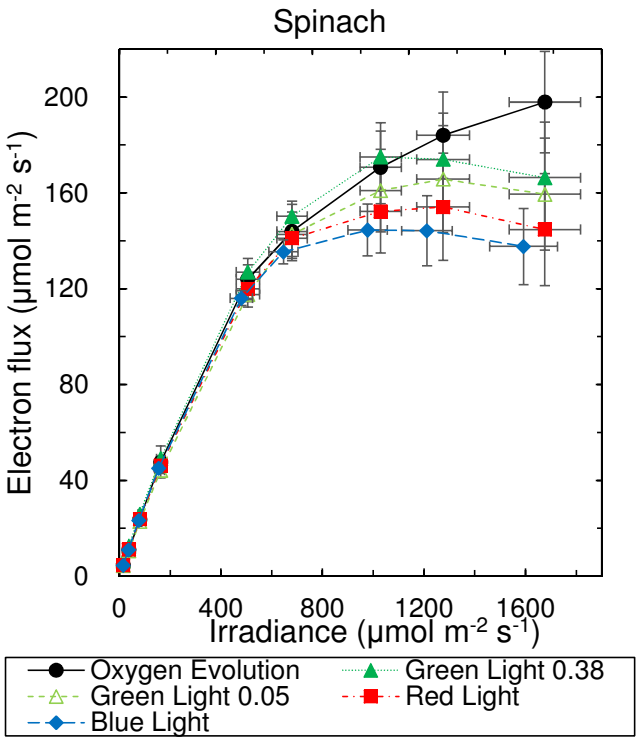


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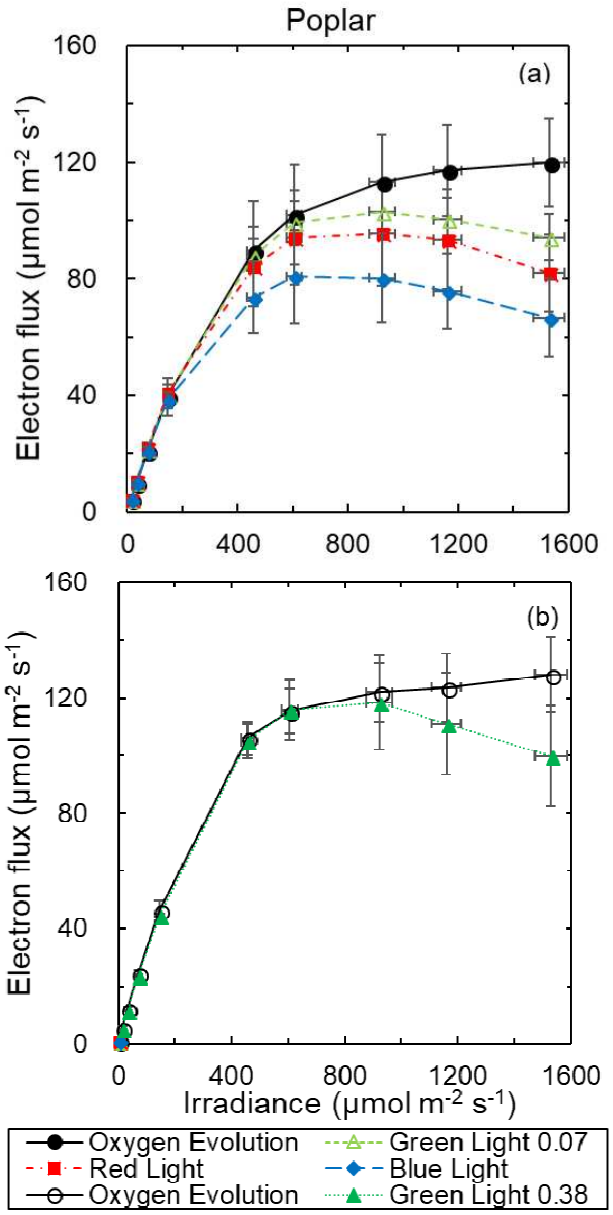
**Fig. 2**



662 **Fig. 3**
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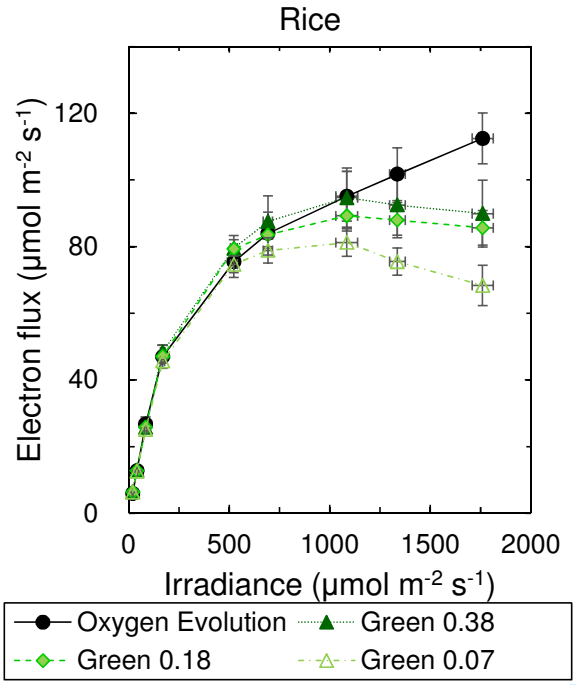


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**Fig. 4**

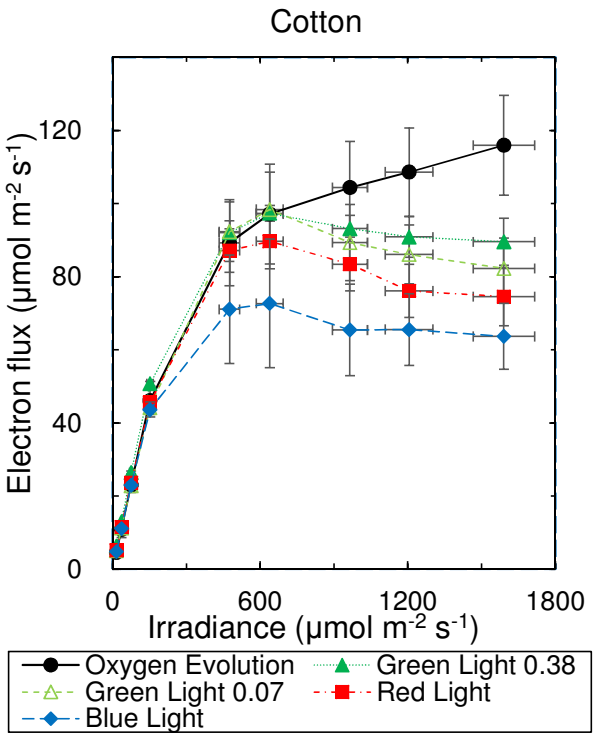

**Fig. 5**

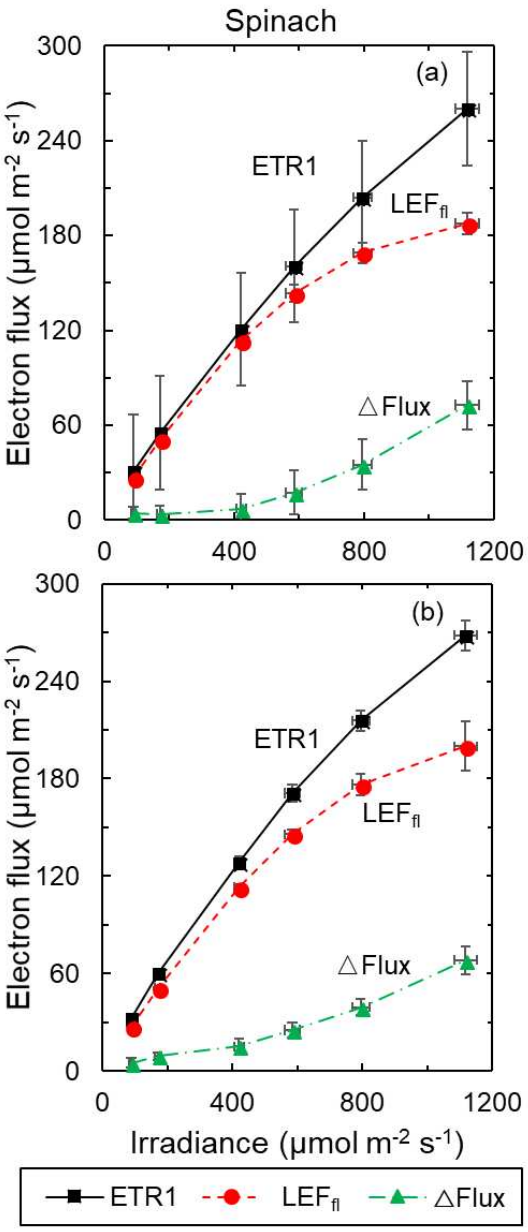

**Fig. 6**

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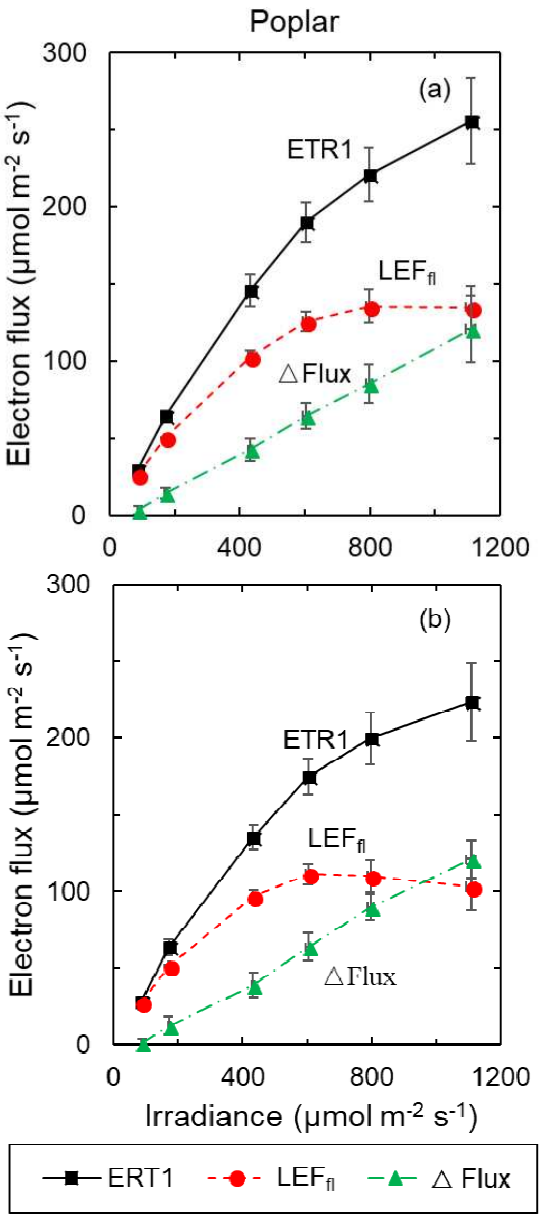

**Fig. 7**

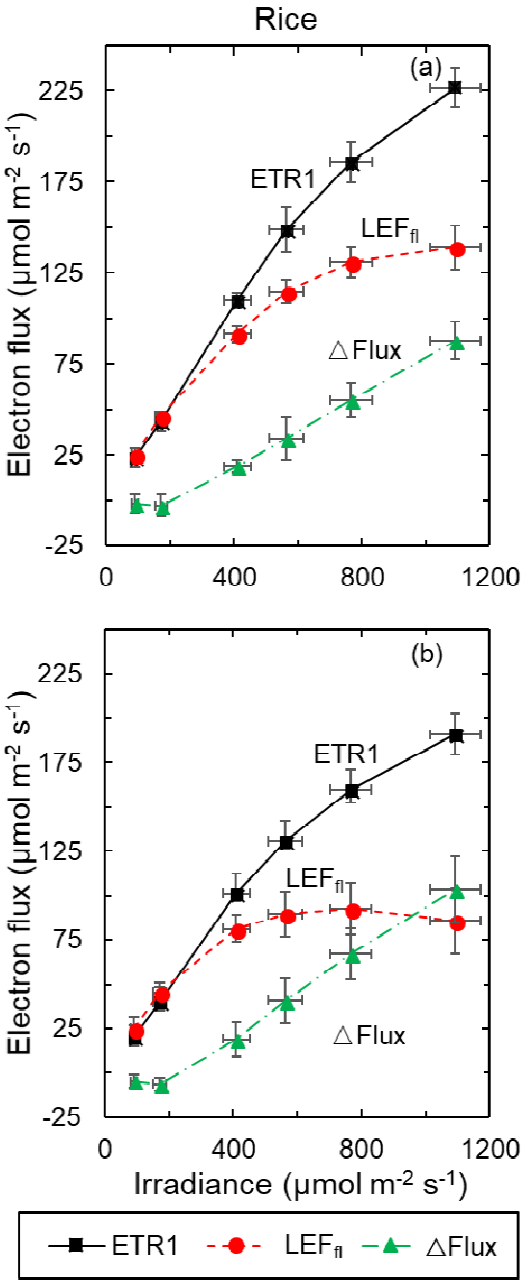

**Fig. 8**

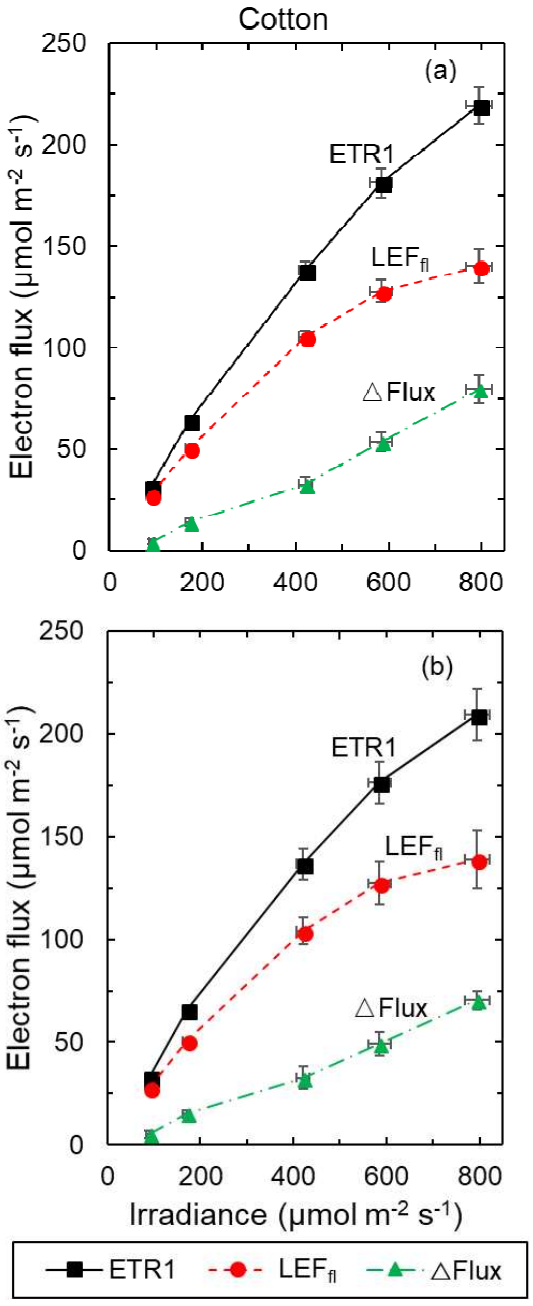

**Fig. 9**