

1 **Light Quality Affects Chloroplast Electron Transport Rates Estimated from Chlorophyll**
2 **Fluorescence Measurements.**

3 Running title: Light quality effects on chlorophyll fluorescence

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11

12 **Abstract**

13 Chlorophyll fluorescence has been used widely to calculate photosynthetic electron transport rates.
 14 Portable photosynthesis instruments allow for combined measurements of gas exchange and
 15 chlorophyll fluorescence. We analysed the influence of spectral quality of actinic light on chlorophyll
 16 fluorescence and the calculated electron transport rate and compared this to photosynthetic rates
 17 measured by gas exchange in the absence of photorespiration. In blue actinic light, electron-
 18 transport rate calculated from chlorophyll fluorescence overestimated the true rate by nearly a
 19 factor of two, whereas there was closer agreement under red light. This was consistent with
 20 prediction made with a multilayer leaf model using profiles of light absorption and photosynthetic
 21 capacity. Caution is needed when interpreting combined measurements of chlorophyll fluorescence
 22 and gas exchange, such as the calculation CO_2 partial pressure in leaf chloroplasts.

23

24 **Keywords**

25 Mesophyll conductance, Light profiles, *Spinacea oleracea*, *Flaveria bidentis*, *Setaria viridis*,

26 **Abbreviations**

27 α leaf absorptance of photosynthetically active radiation

28 β fraction of absorbed light captured by photosystem II

29 ϕ_{PSII} photochemical efficiency of photosystem II

30 ϕ_{CO_2} CO_2 fixed per photon absorbed

31 A rate of CO_2 assimilation

32 ATP adenosine triphosphate

33 C_c CO_2 partial pressure at the site of carboxylation

34 F_m' maximum fluorescence in the light

35 F_s steady state fluorescence under actinic light

36 g_m mesophyll conductance

37 I irradiance

- 38 J electron transport rate
- 39 J_f electron transport rate calculated from fluorescence
- 40 P_{max} maximum photosynthetic rate
- 41 PSII photosystem II
- 42 R_d dark respiration rate
- 43 RuBP ribulose 1,5-bisphosphate
- 44
- 45

46 Introduction

47 Fluorescence is a property of chlorophyll that provides a rich source of information about the light
48 reactions of photosynthesis. There are three fates for a photon of light absorbed by chlorophyll:
49 photochemistry, non-radiative dissipation as heat, and fluorescence. These three pathways compete
50 and the amount of fluorescence depends on the efficiency of the other two pathways. With the
51 development of instruments using modulated or pulsed light to measure chlorophyll fluorescence
52 (Schreiber et al. 1986), the separation of the light being used to measure fluorescence from actinic
53 light driving photosynthesis allowed great flexibility in the colour and intensity of light sources. The
54 discovery that the fluorescence parameter photochemical efficiency (ϕ_{PSII}) was directly proportion to
55 the quantum yield of photosynthesis (Genty et al. 1989) resulted in the development of new
56 instruments and the widespread use of chlorophyll fluorescence in photosynthesis research. The
57 attraction of ϕ_{PSII} is that being a ratio, it is a robust optical parameter that does not rely on calibration.

58 To convert ϕ_{PSII} into the rate of linear electron transport, ϕ_{PSII} is multiplied by the incident irradiance,
59 the leaf absorptance (α) and the fraction of absorbed light that is captured by PSII (β). Leaf
60 absorptance can be measured with an integrating sphere (McCree 1971) and a spectroradiometer and
61 is mainly determined by chlorophyll content per unit leaf area (Evans and Poorter 2001). Determining
62 the fraction of absorbed light that is captured by PSII is more problematic. It has been inferred from
63 comparisons between ϕ_{PSII} and photosynthetic quantum yield determined by gas exchange under
64 non-photorespiratory conditions (ϕ_{CO_2}), but is generally assumed to be 0.5 for C_3 plants and from 0.4
65 to 0.5 for C_4 plants (Edwards and Baker 1993; Krall and Edwards 1990; Laisk and Loreto 1996). An equal
66 split between PSII and PSI ($\beta = 0.5$) maximises linear electron transport in C_3 leaves. However, C_4 leaves
67 contain chloroplasts in mesophyll and bundle sheath cells and as the additional ATP required for the
68 CO_2 pump probably requires cyclic electron transport, this is reflected in a lower value for β .

69 The value of ϕ_{PSII} varies with irradiance and the response depends on the photosynthetic capacity.
70 Fluorescence detected from a leaf originates from the combination of fluorescence emitted by many

71 chloroplasts distributed through the leaf. The electron transport rate calculated from fluorescence
72 emitted by the leaf only equals the actual electron transport rate of the leaf if a special condition is
73 met. This occurs when the profile of absorbed actinic light exactly matches the profile of
74 photosynthetic capacity. Evidence for gradients in photosynthetic capacity through a leaf can be
75 inferred from the fact that light response curves of photosynthesis differ depending on whether light
76 is supplied to the upper or lower surface (Moss 1964; Ögren and Evans 1993; Schreiber et al. 1977).
77 Direct evidence for photosynthetic properties of chloroplasts differing through a leaf have been
78 obtained for *Camellia* and *Spinacea* (Terashima and Inoue 1984, 1985a, b). Gradients in light through
79 leaves have also been shown using paradermal sectioning (Terashima and Saeki 1983), inserted optical
80 fibres (Cui et al. 1991; Vogelmann and Björn 1984) or chlorophyll fluorescence imaging (Brodersen
81 and Vogelmann 2010; Koizumi et al. 1998; Oguchi et al. 2011; Slattery et al. 2016; Takahashi et al.
82 1994; Vogelmann and Evans 2002; Vogelmann and Han 2000). As the profile of light absorption varies
83 with the wavelength of light, it follows that the special condition when electron transport calculated
84 from fluorescence equals the true rate of electron transport will not always hold true.

85 The use of LEDs to supply the measuring and actinic light for fluorescence instruments has become
86 common practice. Depending on the instrument, the measuring light is typically either blue (475nm)
87 or red (625-650nm) and filters are chosen such that light at longer wavelengths (typically > 680 to 720
88 nm) reaches the detector. Fluorescence escaping from a leaf arises from emissions from chlorophyll
89 throughout the leaf minus any reabsorption within the leaf. Since chlorophyll strongly absorbs 680nm
90 light, fluorescence around this wavelength will mainly be detected from chloroplasts near the leaf
91 surface because of the higher probability of reabsorption of the fluorescence emitted deeper within
92 the leaf. Reabsorption by chlorophyll decreases at longer wavelengths, so detecting longer
93 wavelength (e.g. 730 nm) light means that fluorescence emitted deeper in the leaf contributes to the
94 overall signal (Evans and Vogelmann 2003).

95 Blue light is mainly absorbed near the leaf surface, whereas red light absorption spreads deeper into
96 the leaf (Vogelmann and Han 2000). Consequently, using blue light as the fluorescence measuring light
97 primarily samples chloroplasts near the leaf surface. A red measuring light will penetrate deeper
98 inside the leaf and increase the contribution of fluorescence from deeper chloroplasts. The
99 wavelength of actinic light also alters the profile of photosynthesis through the leaf. Blue actinic light
100 will saturate chloroplasts near the surface, yet if fluorescence is being detected with a red measuring
101 light, then fluorescence from chloroplasts deeper within the leaf confounds the simple interpretation
102 of ϕ_{PSII} .

103 One of the applications of chlorophyll fluorescence in combination with gas exchange measurements
104 has been to infer the CO_2 partial pressure at the site of carboxylation (C_c) and hence derive an estimate
105 of mesophyll conductance for CO_2 diffusion between the intercellular airspaces and the chloroplast
106 (g_m). This method proposed and validated by Harley and colleagues (Harley et al. 1992; Loreto et al.
107 1992) has been widely used (Pons et al. 2009). However, the method relies upon the electron
108 transport rate calculated from fluorescence (J_f) being correct in an absolute sense. As there is no
109 simple way to measure the value for the fraction of absorbed light captured by PSII (β), inevitably this
110 contributes uncertainty in J_f . An extreme example of this was observed by Loreto et al. (2009) who
111 found that the estimated value for g_m decreased as the proportion of blue light increased. Their
112 explanation was that β differs between red and blue actinic light and in addition, blue light has reduced
113 transfer efficiency.

114 Our objective was to investigate the consequence of the colour of light on the estimation of J_f . Some
115 portable photosynthesis instruments measure both chlorophyll fluorescence and gas exchange with
116 the capability to vary the proportion of red and blue in the actinic light. We measured C_3 leaves in the
117 absence of photorespiration (low O_2) and C_4 leaves and found that fluorescence overestimated J_f
118 under blue actinic light. To investigate the influence of the colour of the modulated measuring light,

119 additional experiments using a fluorometer with blue measuring light were undertaken. The
120 experimental results can be explained using a multilayer photosynthesis model (Evans 2009).

121 **Results**

122 *Irradiance response of gross CO₂ assimilation rate and chloroplast electron transport estimated from* 123 *chlorophyll fluorescence*

124 The irradiance response of gross CO₂ assimilation rate (A+R_d) and J_f were compared in *Setaria viridis*
125 under actinic light with a mix of 10 % blue and 90 % red (Figure 1 A, C) and 90 % blue and 10 % red
126 (Figure 1 B, D). The J_f was divided by 4 to facilitate comparison of the two rates (von Caemmerer 2000).
127 There was a close correlation between A+R_d and J_f/4 when the actinic light contained 90 % red light,
128 whereas the J_f/4 was significantly greater than A+R_d when the actinic light contained 90 % blue light.
129 This is contrary to the expectation of a good correlation during C₄ photosynthesis where the CO₂
130 concentrating mechanism suppresses photorespiration (Edwards and Baker 1993). Under 90 % blue
131 light, the discrepancy between A+R_d and J_f increased with increasing irradiance. In both scenarios,
132 maximum chlorophyll fluorescence in the light (F_m') decreased with increasing irradiance while
133 steady-state fluorescence (F_s) varied little (Figure 1C, D).

134 *Estimates of linear electron transport increase with an increase in the fraction of blue light.*

135 We explored the impact of actinic light composition at high irradiance in *S. viridis*, *Flaveria bidentis*
136 (both C₄ species) and *Spinacea oleracea* measured under low oxygen partial pressure (Figures 2 and 3
137 and Supplemental Figure 1). All experiments show an increase in J_f/4 with an increase in the fraction
138 of blue light, whereas there was little change in gross CO₂ assimilation rate resulting in a 2 to 3-fold
139 increase in the ratio of the two. There was good agreement between estimated of J_f/4 and A+R_d under
140 red light in *S. oleracea*, *F. bidentis*, but J_f/4 was slightly great than A+R_d in *S. viridis*.

141 *The effect measuring beam light colour on the estimates of chlorophyll fluorescence*

142 Gas exchange and fluorescence measurements were made with a portable photosynthesis instrument
143 (LI-6800) fitted with the multiphase flash fluorometer (6800-01) with a red measuring beam. The
144 measuring beam of a 6800-fluorometer was altered with blue LEDs (475 nm) replacing the standard
145 red (625nm) LEDs to explore the effect light colour of the measuring beam has on estimates of
146 chloroplast electron transport. Figure 4 compares the ratio of gross CO₂ assimilation rate to $J_f/4$
147 measured with blue (A) or the standard red (B) measuring beam at different irradiances and different
148 spectral composition of the actinic irradiance. There is better agreement between gross CO₂
149 assimilation rate and $J_f/4$ with a blue measuring beam and the difference is less sensitive to the
150 spectral composition of actinic light.

151 *Using a multilayer photosynthesis model to explain estimates of chloroplast electron transport*

152 To interpret the observed dependence of estimates of chloroplast electron transport on light quality,
153 we have used a multilayer photosynthesis model (Evans 2009). Profiles of the relative fraction of blue
154 and red light absorbed at different depths through a spinach leaf illuminated from the top are shown
155 together with the profile of photosynthetic capacity (Figure 5A; data are taken from Vogelmann and
156 Evans (2002)). The modelled fluorescence profile expected under actinic light containing 90 % blue or
157 90 % red light (Figure 5C) were used to calculate the profile of ϕ_{PSII} with depth in the leaf (Figure 5D).
158 Under 90 % red, ϕ_{PSII} remains similar deep into the leaf. As little blue light is absorbed deep within
159 the leaf, ϕ_{PSII} is greater in these layers with an actinic light containing 90% blue. The high F_m' relative
160 to F_s fluorescence contributed by these layers distorts the total F_m' signal emerging from the leaf. The
161 modelled irradiance dependence of ϕ_{PSII} at three depths within the leaf is shown for the two
162 scenarios: 90 % red light (Figure 6 A) or 90 % blue light (Figure 6 B), with the leaf illuminated from the
163 top in both cases. The decline of ϕ_{PSII} with increasing irradiance for the top layer of the leaf is similar
164 with red or blue actinic light (solid lines), but deep within the leaf, ϕ_{PSII} declines less under 90 % blue
165 light compared to 90 % red light (dotted lines).

166 Using this data, the model was used to calculate an actual chloroplast electron transport rate from
167 the photosynthetic profile, which could be compared to that estimated from the chlorophyll
168 fluorescence. The model mimics the increase in J_f as the fraction of blue light in the actinic light
169 increases (Figure 7 A) with either a red or a blue measuring beam. Agreement between true J and J_f is
170 closest when the colour of both the measuring beam and actinic light are the same (Figure 7 B). There
171 is good quantitative agreement between model prediction and empirical measurements made for *S.*
172 *oleracea* and *N. tabaccum* (Figure 7 B).

173 **Discussion**

174 Measurements of leaf chlorophyll fluorescence provide a rapid non-invasive tool for estimating
175 photosynthetic activity of a leaf and has proven to be useful in field studies (Earl and Tollenaar 1998a,
176 b). Combined measurements of gas exchange and chlorophyll fluorescence in the field are now also
177 feasible with many portable photosynthesis instruments available (Tomas et al. 2013; Tomeo and
178 Rosenthal 2017).

179 The LI-6800 photosynthesis instrument not only allows concurrent measurement of both chlorophyll
180 fluorescence and gas exchange, but also provides the opportunity to vary the proportion of red and
181 blue over the full range of actinic irradiance. This provided us with the opportunity to explore the
182 relationship between electron transport derived from both chlorophyll fluorescence and gas
183 exchange. We measured C_4 leaves and C_3 leaves in the absence of photorespiration and found that
184 electron transport was overestimated by fluorescence with the discrepancy growing as the fraction of
185 blue actinic light increased (Figure 1, 2, 3 and Sup Figure 1.). Similar results were observed by Loreto
186 et al. (2009) although as they were only able to make measurements at lower irradiance, their
187 differences were not as extreme. Here we discuss possible underlying mechanisms for this
188 phenomenon.

189 *Parameters influencing calculated electron transport capacity*

190 To calculate chloroplast electron transport rate from chlorophyll fluorescence, the fluorescence
191 parameter ϕ_{PSII} is multiplied by the incident irradiance, the leaf absorptance (α) and the fraction of
192 absorbed light that is captured by PSII (β) (equation 1, Edwards and Baker 1993; Genty et al. 1989).
193 Although there was generally good agreement between $J_f/4$ and $A+R_d$ under red light, we observed
194 up to a 3-fold difference between electron transport rates estimated from chlorophyll fluorescence
195 and gas exchange as the fraction of blue light increased. As leaf absorptance is expected to increase
196 by only 3.6 % when red is replaced with blue light (legend to Figure 2), changes in α cannot explain
197 the difference. Loreto et al. (2009) who used red light to measure fluorescence, suggested that a
198 decrease in β in blue actinic light contributed to part of the difference. In addition, they suggested 35
199 to 45 % of blue light was absorbed by carotenoids that did not transfer energy to photosystem II with
200 high efficiency, citing the work of Duysens (1952). Laisk et al. (2014) present evidence that 30% of blue
201 light is absorbed by photosynthetically inactive pigments. However, Siefermann –Harms and
202 Ninnemann (1982) reported that xanthophylls in the light harvesting chlorophyll a/b complex can
203 absorb 43% of light between 390 and 530nm and transfer this with 100% efficiency to photosystem
204 reaction centres. Similarly, Croce et al. (2001) showed rapid energy transfer from carotenoids to both
205 chlorophyll a and b, which argues against blue light being significantly less effective at driving
206 photosystem II photochemistry. To align $J_f/4$ and $(A + R_d)$ in our *N. tabacum*, we would have to
207 decrease β from 0.45 in 90 % red light to 0.3 in 90 % blue light (equation 1 and Supplemental Figure
208 3). We made all our calculations of J_f assuming $\beta=0.5$. While a case can be made that β may be less
209 than that, particularly in C_4 species, variation in β is unlikely to account for the large difference we
210 observed at high irradiance. Imposing a lower β value also does not lead to the superimposition of the
211 irradiance response curves of CO_2 assimilation rate measured under the two actinic light compositions
212 shown in Figure 1.

213 *What are we detecting with leaf measurements of chlorophyll fluorescence?*

214 The photosynthetic capacity, as measured by modulated light, varies with spectral composition
215 resulting in very different estimates of J_f . We suggest that the experimental results can be best
216 explained using a multilayer photosynthesis model shown in Figure 5 (Evans 2009). Fluorescence
217 detected from a leaf originates from the combination of many chloroplasts distributed through a leaf.
218 Electron transport calculated from fluorescence only equals the actual electron transport rate of the
219 leaf if the profile of absorption of actinic light exactly matches the profile of photosynthetic capacity.
220 Changing light quality influences the match between the profile of actinic light absorption and
221 photosynthetic capacity (Figure 5). Because blue light is absorbed primarily in the top layers of the
222 leaf, there is greater variation of ϕ_{PSII} with depth in the leaf, leading to an overall greater sum of ϕ_{PSII} ,
223 which in turn generates a greater J_f (Figure 5 and 6). Blue light is also sensed by phototropins which
224 lead to chloroplast movement and rearrangement within mesophyll cells (Jarillo 2001, Kagawa 1999,
225 Wada 2013). Change in irradiance also result in chloroplast movement (Williams et al 2003). Such
226 movements will alter the profile of light absorption through the leaf with respect to the profile in
227 photosynthetic capacity. Due to the absence of any data, we have not attempted to model the
228 consequence of chloroplast movements.

229 The measuring beam of the PAM fluorometer also affects the estimates of ϕ_{PSII} . The model
230 simulations on leaf depth assumed a red measuring beam (Figure 5 and 6). Where the red measuring
231 beam was replaced with a blue measuring beam (Figure 4), there was less difference between J_f and
232 $A+R_d$. In Figure 7 the model predictions of true electron transport J are compared with that estimated
233 from fluorescence elicited with a red or blue measuring beam. The model predictions of true electron
234 transport (J) compared favourably to our experimental results, suggesting an overestimation of J_f with
235 a red measuring beam and a slight under estimation with a blue measuring beam (Figure 7B). Our
236 results highlight the difficulty of obtaining tight quantitative comparisons between chloroplast
237 electron transport obtained from measurements of CO_2 assimilation rates and those of chlorophyll
238 fluorescence. Although the estimates of ϕ_{PSII} are dependent on the light colour of the measuring
239 beam, it is not possible or practical to specify a perfect colour for the measuring beam light

240 composition. The difference between the light absorption profile and the profile of photosynthetic
241 capacity is responsible for the variation in estimates of J_f with varying light quality. However, having
242 the same light quality for both the actinic and chlorophyll fluorescence measuring beam is better than
243 using different qualities for the two light sources. Chlorophyll fluorescence has been used to measure
244 light absorption profiles in leaves (Vogelmann and Evans 2002; Vogelmann and Han 2000) and new
245 developments in light sheet microscopy are making it easier (Slattery et al. 2016), but obtaining the
246 profile of photosynthetic capacity remains difficult.

247 *Using combined measurements of gas exchange and chloroplast electron transport to estimate*
248 *mesophyll conductance.*

249 A popular application of combined chlorophyll fluorescence and gas exchange measurements has
250 been to infer the CO_2 partial pressure at the site of carboxylation in the chloroplast and hence derive
251 an estimate of mesophyll conductance for CO_2 diffusion between the intercellular airspaces and the
252 chloroplast (g_m). J required to support CO_2 assimilation can be estimated from measurements of CO_2
253 assimilation rate by summing requirements for ATP and NADPH generation in the photosynthetic
254 carbon reduction (PCR) and the photorespiratory carbon oxidation (PCO) cycle (Farquhar et al. 1980;
255 von Caemmerer and Farquhar 1981). Various equations exist and the most straight forward assumes
256 that NADPH is the most limiting and that the demand for ATP is met (von Caemmerer 2000). To
257 estimate J , the partial pressure of CO_2 at the carboxylation sites within chloroplasts needs to be
258 known. Generally estimates of J_f are greater than J when estimated on the basis of intercellular CO_2
259 partial pressure and g_m is calculated by finding the chloroplast CO_2 partial pressure for which $J_f = J$. This
260 method was proposed and validated by Harley and colleagues (Harley et al. 1992; Loreto et al. 1992)
261 and has been widely used (Flexas et al. 2007a; Flexas et al. 2007b; Tomas et al. 2013; Tomeo and
262 Rosenthal 2017). There are several factors that can contribute to differences between estimates of J_f
263 and J , such as electron transport to other acceptors (e.g. oxygen, nitrate reduction) which are not
264 captured in the equations for J and/or uncertainty in the estimates for J_f associated with the values

265 assumed for α and β (equation 1). Although the possible error of different light absorption profiles is
266 well recognised (Pons et al. 2009), in practice it has been ignored when estimating g_m in different
267 species or crop genotypes.

268 Absorption profiles impact on estimates of J_f at different irradiances (Figure 1 and Supplemental Figure
269 2) with the result that estimating of g_m at different irradiances using the fluorescence technique may
270 be problematic (see also Th eroux-Rancourt and Gilbert 2017). In some instances, J_f is calibrated
271 against CO_2 assimilation rate measured under low O_2 concentrations (to suppress photorespiration),
272 over a range of CO_2 concentrations or irradiance (I) to create a range in J . Linear regressions are
273 determined from either the relationship between ϕ_{PSII} and J/I or J_f and J . These calibrations would
274 vary depending on the modulated-light wavelength (see Supplementary Figure 2) as well as differing
275 when CO_2 or I is used to vary J .

276 While exact quantitative rigour in calculating J_f may be difficult to achieve, chlorophyll fluorescence is
277 still very useful. Firstly, in C_3 species it provides an independent method of assessing the transition
278 from Rubisco to RuBP regeneration limitation as CO_2 concentration varies (von Caemmerer et al.
279 2009). Secondly, being an optical method, it can be used to measure leaves or plants remotely without
280 any need to enclose them in a cuvette. This has proved very effective for screening plants for
281 photosynthetic mutations (Badger et al. 2009; Murchie and Lawson 2013; Niyogi et al. 1998). Finally,
282 as long as people remain alert to the error that can arise due to any mismatch between the profile of
283 absorbed actinic light and photosynthetic capacity, chlorophyll fluorescence does provide a rich signal
284 that is useful when investigation photosynthetic properties of leaves.

285

286 **Material and Methods**

287 *Plant growth conditions*

288 *Setaria viridis* were grown in controlled environmental chambers, irradiance 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$,
289 ¹, 16-h photoperiod, 28°C day, 24°C night in 2L pots in garden soil mix fertilised with Osmocote (Scotts,
290 Australia). Pots were watered daily.

291 *Spinacea oleracea* and *Flaveria bidentis* were grown in a naturally lit glasshouse with day/night
292 temperatures set at 28/18°C. Plants were grown in well-draining 5 L pots filled with commercial
293 potting mix fertilised with Osmocote (Scotts, Australia). Pots were watered daily.

294 *Nicotiana tabacum* cv. Brightleaf was grown in a naturally lit glasshouse with supplemental lighting
295 ($\sim 300 \mu\text{mol m}^{-2} \text{s}^{-1}$) to control day length (16hrs) with day/night temperature of 22 °C Plants were
296 grown in well-draining 6.1 L pots filled with commercial potting mix with fertilization (Miracle Grow
297 Potting Mix, Scotts Miracle Grow LLC. , Marysville, OH, USA). Pots were bottom watered every other
298 day. Photosynthesis was measured on the 7 or 8th leaf which was fully expanded and mature.

299 *Gas exchange and fluorescence measurements*

300 Gas exchange and fluorescence measurements were made with a portable photosynthesis instrument
301 (LI-6800, LI-COR Inc, Lincoln, NE, USA) fitted with the multiphase flash fluorometer (6800-01). With
302 this PAM fluorometer, actinic light composition can be varied between 100 % blue (475 nm) and 100
303 % red (625 nm). For experiments with blue modulated light, the measuring beam of a 6800-01 was
304 altered with blue LEDs (475 nm) replacing the standard red (625nm) LEDs. Gas exchange
305 measurements were made at various irradiances and light colour composition (as indicated in the
306 figure legends) in air containing 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ and 2 % O_2 for *S. oleracea* or 0.5 % O_2 for *N.*
307 *tabacum* and a leaf temperature of 25 °C, or 21 % O_2 and 28 °C for *S. viridis* and *F. bidentis*.

308 For light response curves, leaves were acclimated in the leaf chamber for 20 - 40 minutes at the highest
309 irradiance until steady-state gas-exchange and chlorophyll fluorescence were achieved. Then

310 irradiance was reduced at 3 minute intervals for *S. oleracea*, *S. viridis* and *F. bidentis* or 2 - 5 minute
311 for *N. tabacum*. In experiments where the red:blue ratio of irradiance varied, leaves were acclimated
312 in the leaf chamber for 20 minutes until steady-state was reached with a light composition of 10 %
313 blue and 90 % red. Then irradiance was changed to 100% red and subsequent measurements were
314 made at 3 minute intervals with decreasing red light content. For the *N. tabacum* light responses with
315 the blue and red modulated fluorometers, the irradiance spectral composition was changed to a
316 randomly selected colour balance. Two light responses for *N. tabacum* were measured
317 simultaneously on the same leaf (one instrument on each side of the mid-vein) as described above
318 with the addition that leaf vapour pressure difference was held constant at 1.2 kPa. Dark respiration,
319 R_d , was measured at the end of the light response curve for each leaf and gross CO_2 assimilation, was
320 approximated as $A + R_d$.

321 All fluorescence measurements (*S. oleracea*, *S. viridis*, *N. tabacum* and *F. bidentis*) were made using
322 the multiphase flash fluorescence protocol (MPF) with a saturating intensity of $10,000 \mu\text{mol m}^{-2} \text{s}^{-1}$
323 and a ramp depth of 25 %. Steady-state chlorophyll fluorescence (F_s) was measured at 100 kHz (*S.*
324 *oleracea*, *S. viridis*, and *F. bidentis*) and 50 kHz (*N. tabacum*). Maximum chlorophyll fluorescence (F_m')
325 was measured at 250 kHz during the saturating pulse.

326 *Leaf absorptance measurement.*

327 Absorptance of light by *N. tabacum* leaves was measured after each light response on the same
328 portion of the leaf using a LI-1800 spectroradiometer and integrating sphere (LI-COR, Inc). The
329 absorptance for the upper surface of the leaf was calculated from the absorptance at each wavelength
330 weighted by the spectral output of the actinic LEDs.

331 *Calculation of chloroplast electron transport.*

332 Chloroplast electron transport (J_r) was calculated using the software of the LI-6800 according to the
333 equation

$$334 \quad J_f = I\alpha\beta(\phi PSII) = I\alpha\beta(1 - F_s/F_m') \quad (1)$$

335 where I is the incident irradiance, α is the absorptance of the leaf and β is the fraction of absorbed
 336 irradiance that reaches PSII (assumed to be 0.5) (equation 3.1 von Caemmerer 2000).

337 *Multilayer model*

338 The profiles of light absorption (Vogelmann and Evans 2002) and P_{\max} (Evans and Vogelmann 2003)
 339 were applied in a multilayer model of photosynthesis to estimate the rates of electron transport and
 340 fluorescence arising from each layer. The default value for $P_{\max} = 200 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ was assumed for
 341 modelling *S. oleracea*. Fluorescence was calculated according to Evans (2009). We assume that F_s is
 342 directly proportional to the absorbed irradiance of the measuring light for each layer. F_m' is calculated
 343 for each layer, using the electron transport rate for each layer from the multilayer model and inverting
 344 the equation for photochemical efficiency:

$$345 \quad F_m' = F_s / (1 - \phi PSII)$$

346 where $\phi PSII$ is calculated from $J_i / (I_i \cdot \beta)$, and J_i is the electron transport rate for layer i , I_i is the irradiance
 347 absorbed by layer i and β was assumed to equal 0.5. The apparent $\phi PSII$ for the leaf is then calculated
 348 by summing the values for F_s and F_m' from each layer which can then be used to calculate J_f .

349 **Supplemental Data**

350 **Funding**

351 The research was supported by the Australian Research Council Centre of Excellence for Translational
 352 Photosynthesis (CE140100015).

353 **Acknowledgments**

354 We thank Soumi Bala for technical assistance with plant growth and gas exchange measurements. We
 355 are grateful to Jesse Griggs for modifying the 6800-01 fluorometer.

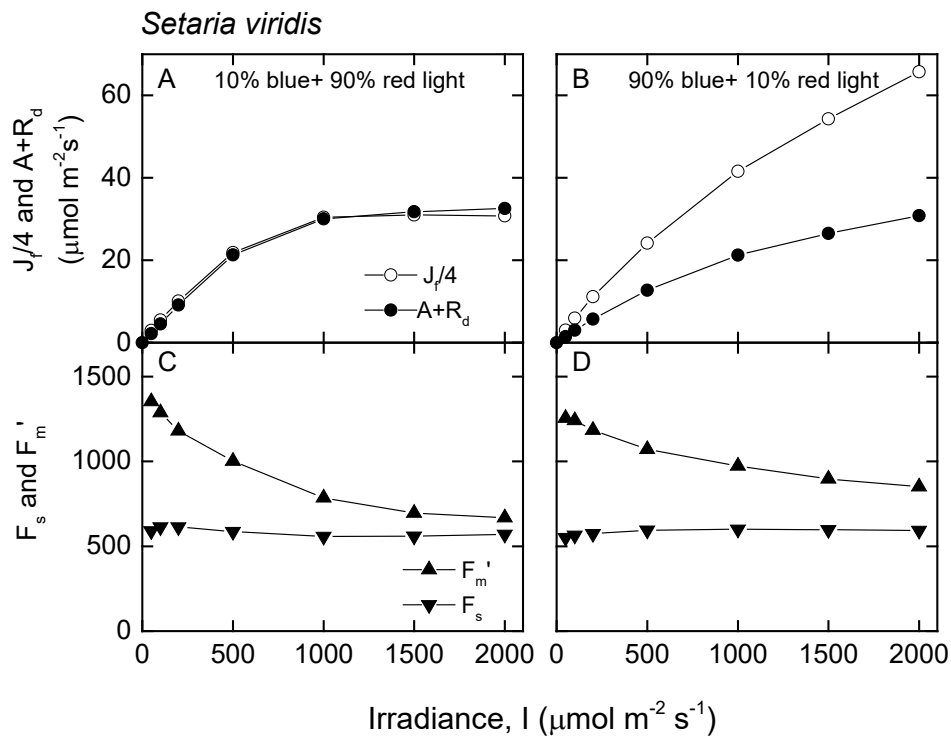
356 **Disclosures**

357 PBM is an employee of LI-COR Inc. and all references to instrumentation are what was used should
358 not be construed as an endorsement by all authors.

359

360 Figures

361



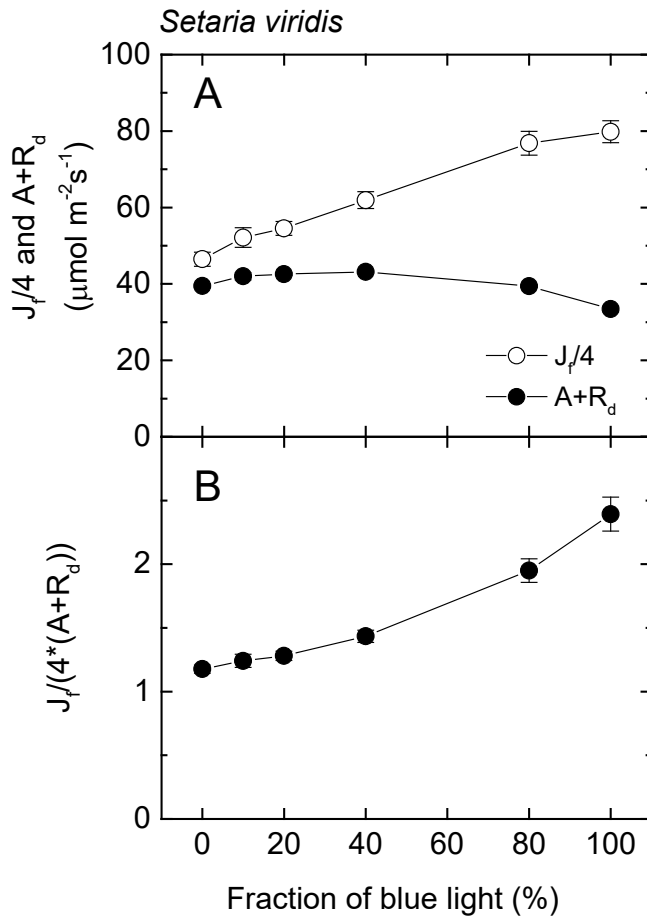
362

363 Figure 1

364 Chloroplast electron transport rate estimated from chlorophyll fluorescence, J_f , and gross CO_2
 365 assimilation rate (the sum of CO_2 assimilation rate, A , and mitochondrial respiration R_d) as a function
 366 of irradiance in *Setaria viridis*, a C_4 monocot. As 4 electrons are required per CO_2 assimilated in the
 367 absence of photorespiration, J_f is presented as $J_f/4$. Irradiance was a mix of 10% blue and 90% red
 368 light (panels A, C) or 90% blue and 10% red light (panels B, D). The responses to irradiance of
 369 fluorescence parameters F_s and F_m' used to calculate J_f (panels C, D). Measurements were made with
 370 an LI-6800 using the multiphase flash mode of the fluorometer. $J_f = 0.5 \cdot \alpha \cdot I \cdot (1 - F_s/F_m')$ where $\alpha =$
 371 0.843 in 10% blue and 90% red light 0.867 in 90% blue and 10% red light. Other conditions were: 400
 372 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ and 21% O_2 and a leaf temperature of 28 °C. R_d was taken to be the rate of CO_2 efflux
 373 in the dark.

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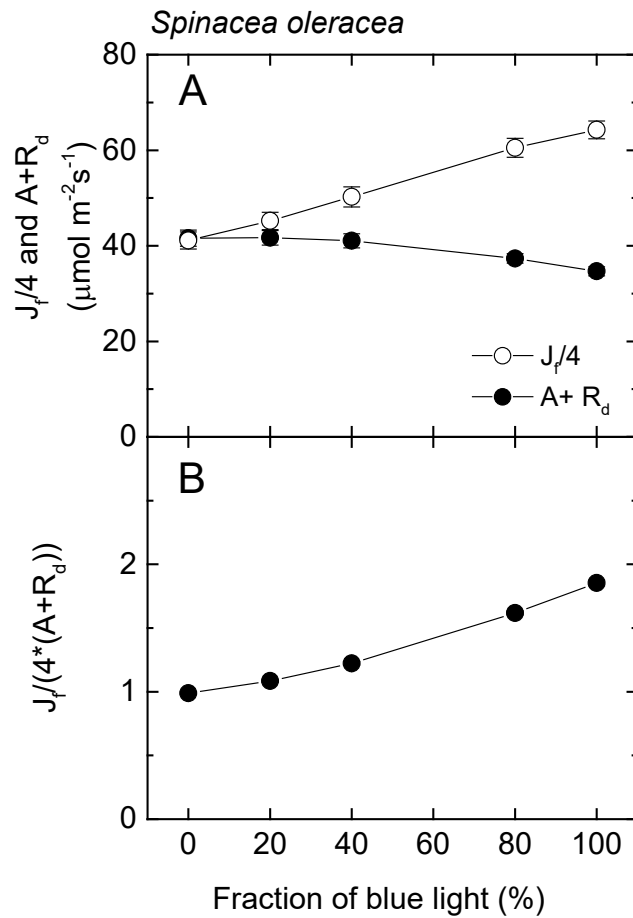


376

377 Figure 2

378 A) Chloroplast electron transport rate estimated from chlorophyll fluorescence ($J_f/4$) and gross
 379 CO_2 assimilation rate ($A + R_d$) as a function of the fraction of blue light in a constant
 380 irradiance of $2000 \mu\text{mol m}^{-2}\text{s}^{-1}$ for *Setaria viridis*. Measurements were made with an LI-6800
 381 using the multiphase flash protocol of the fluorometer. Other conditions were: $800 \mu\text{mol}$
 382 $\text{CO}_2 \text{ mol}^{-1}$ with 21% O_2 and a leaf temperature of 28°C . Leaf absorbance was assumed to
 383 vary in a linear fashion from 0.84 in red to 0.87 in blue light. Error bars are standard errors
 384 of the means ($n=4$).

385 B) The ratio $J_f / (4*(A+R_d))$ as a function of the fraction of blue light.

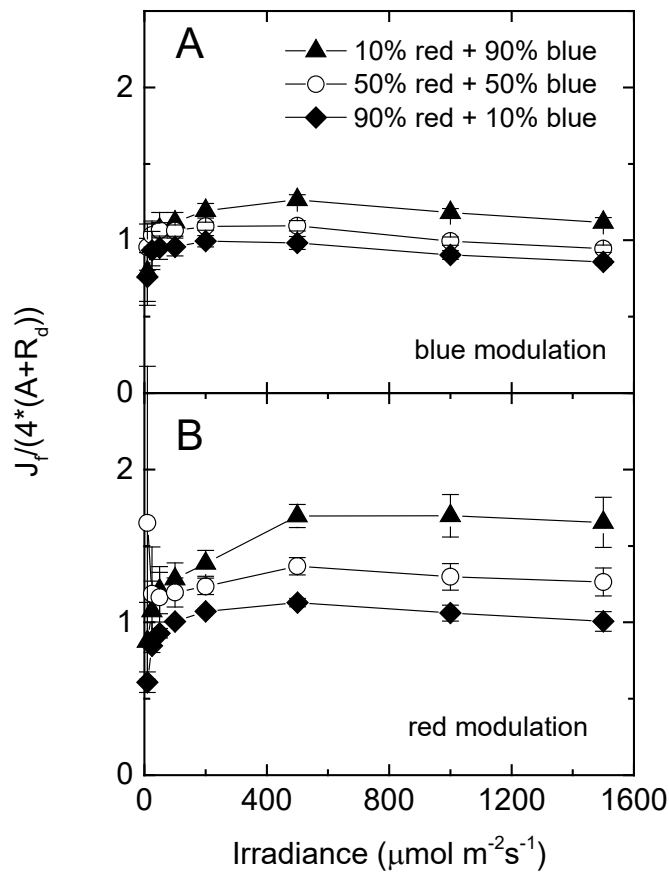


386

387

388 Figure 3

- 389 A) Chloroplast electron transport rate estimated from chlorophyll fluorescence ($J_f/4$) and gross
 390 CO_2 assimilation rate ($A + R_d$) as a function of the fraction of blue light in a constant
 391 irradiance of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ in *Spinacea oleracea*, a C_3 dicot. Measurements were made
 392 with an LI-6800 using the multiphase flash mode of the fluorometer. Other conditions were:
 393 $800 \mu\text{mol CO}_2 \text{ mol}^{-1}$ with 2% O_2 and a leaf temperature of 25°C . Leaf absorptance was
 394 assumed to vary in a linear fashion from 0.84 in red to 0.87 in blue light. Error bars are
 395 standard errors of the means ($n=4$).
- 396 B) The ratio $J_f/(4*(A+R_d))$ as a function of the fraction of blue light.



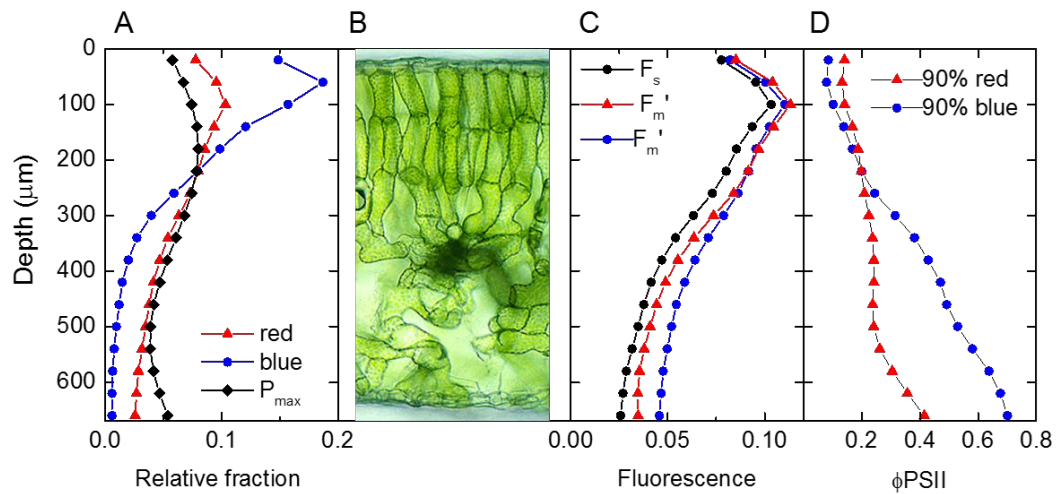
397

398 Figure 4

399 Ratio of the electron transport rate estimated from chlorophyll fluorescence (J_f) to gross CO_2
 400 assimilation rate ($4^*(A + R_d)$) as a function of actinic irradiances from 0 to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. The
 401 spectral balance of red and blue in the actinic light were altered between 90 % and 10 % for
 402 each. The J_f was calculated from chlorophyll fluorescence measurements taken with a modified,
 403 blue pulse-amplitude modulated (PAM) 6800-01 fluorometer (panel A) or a standard, red-
 404 modulated 6800-01 PAM fluorometer (panel B). Measurements were made using the multflash
 405 fluorescence protocol with a saturating pulse of $10,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 25% ramp on the
 406 fluorometer. Other conditions were: $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ with 0.5 % O_2 , leaf vapour pressure
 407 difference of 1.2 kPa and a leaf temperature of $25 \text{ }^\circ\text{C}$. Error bars are the standard error of $n = 4$
 408 (A) or $n = 3$ (B) replicate pair measurements of *Nicotiana tabacum*, cv. Brightleaf.

409

410



411

412

413 Figure 5

414 A) Multilayer model profiles of red and blue light absorption and P_{max} , B) *Spinacea oleracea* leaf cross415 section, C) predicted fluorescence profiles for F_s and F_m' under actinic light of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ with

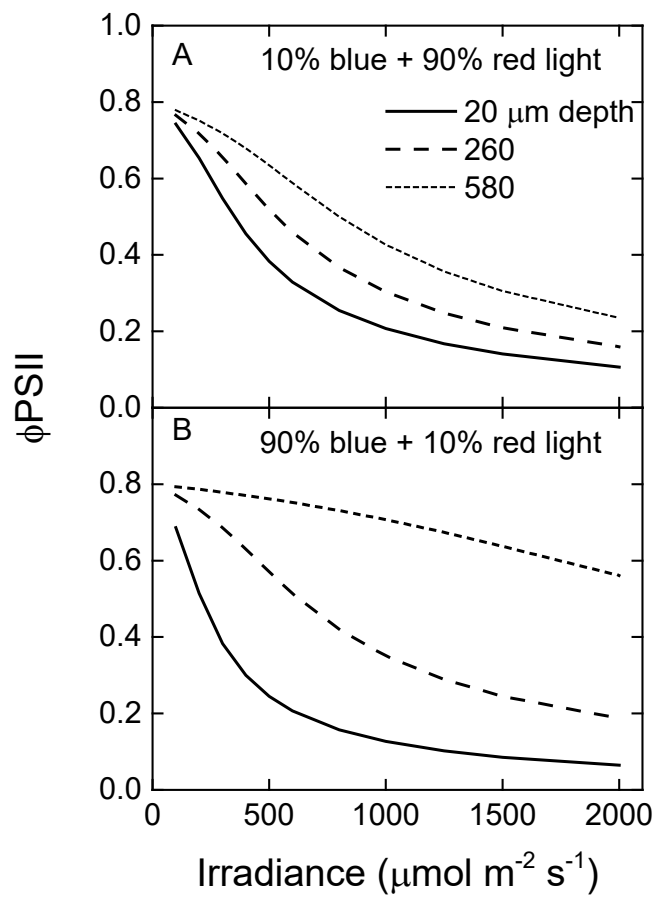
416 either 90 % blue + 10 % red (blue line) or 10 % blue + 90 % red (red line) actinic light composition

417 and D) profiles of photochemical efficiency, ϕPSII , under actinic light of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ with either

418 90 % blue + 10 % red (blue line) or 10 % blue + 90 % red (red line). Data are taken from Vogelmann

419 and Evans (2002).

420



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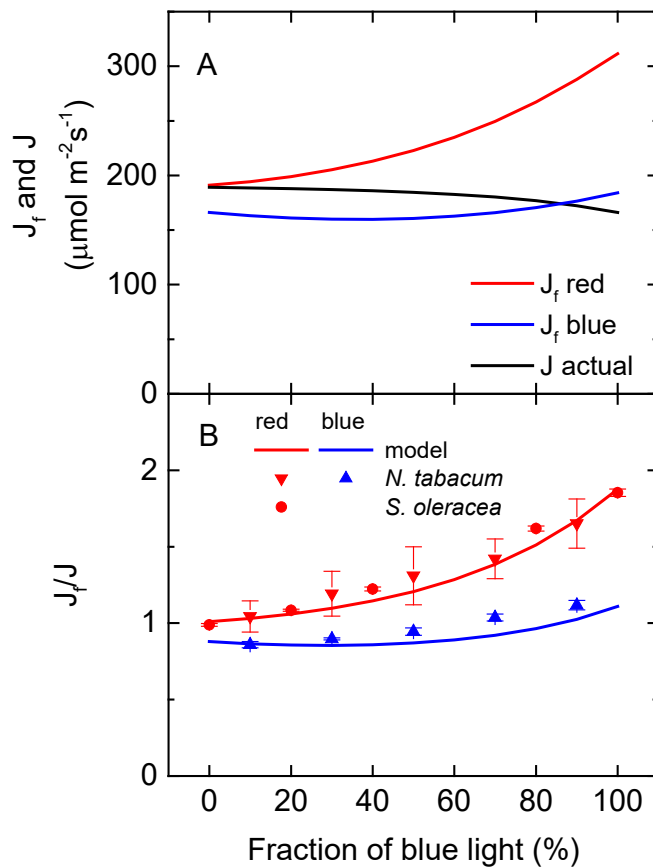
423 Figure 6

424 Modelled photochemical efficiency (ϕPSII) as a function of irradiance at the leaf surface for different
 425 layers within a *S. oleracea* leaf

426 A) Irradiance composed of 10 % blue and 90 % red light.

427 B) Irradiance composed of 90 % blue and 10 % red light.

428

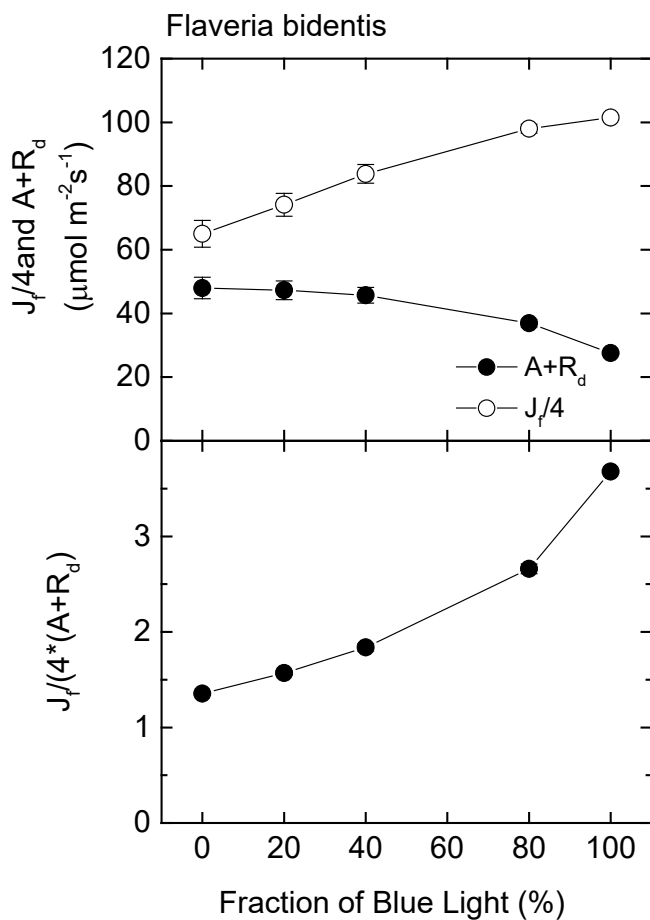


429

430 Figure 7

- 431 A) The influence of spectral composition of the actinic light on modelled actual electron
 432 transport rate (J , black line) and modelled apparent electron transport rate from
 433 fluorescence measured with a red modulated (J_f red) or blue modulated (J_f blue) light.
- 434 B) Ratio of apparent electron transport rate calculated from fluorescence to actual electron
 435 transport rate, modelled with actinic irradiance ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) containing
 436 various fractions of blue and red light. The red and blue lines are modelled using a red or
 437 blue modulated light. Red circles represent actual measurements for spinach leaves (from
 438 Figure 3B), red triangles are measurements from tobacco leaves (from Figure 4B) using red
 439 modulated light. Blue triangles are measurements from tobacco leaves (from Figure 4A)
 440 using blue modulated light. If the P_{max} profile matched the actinic light absorption profile, J_f/J
 441 J would be constant at 1.

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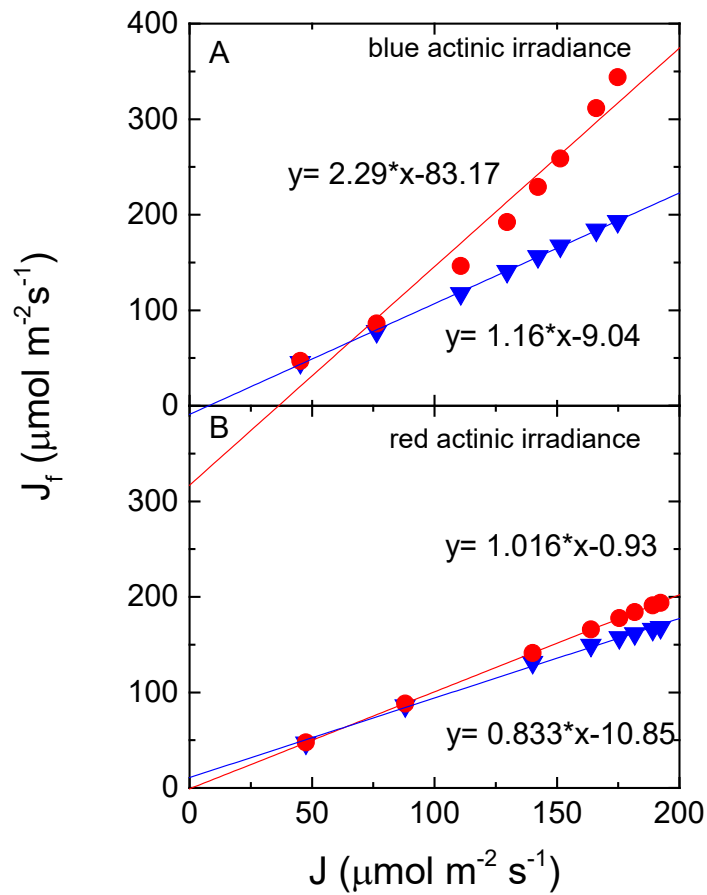
445 Supplemental Figure 1

446 A) Chloroplast electron transport rate estimated from chlorophyll fluorescence ($J_f/4$) and gross
 447 CO_2 assimilation rate ($A+R_d$) measured as a function of the fraction of blue light in a constant
 448 irradiance of $2000 \mu\text{mol m}^{-2}\text{s}^{-1}$ for *Flaveria bidentis*, a C_4 dicot. Measurements were made
 449 with an LI-6800 using the multiphase flash mode of the fluorometer. Other conditions were:
 450 $800 \mu\text{mol CO}_2 \text{ mol}^{-1}$ with 21% O_2 and a leaf temperature of 28°C . Leaf absorbance was
 451 assumed to vary in a linear fashion from 0.84 in red to 0.87 in blue light. Error bars are
 452 standard errors of the means ($n=4$).

453 B) The ratio $J_f/(4*(A+R_d))$ as a function of the fraction of blue irradiance.

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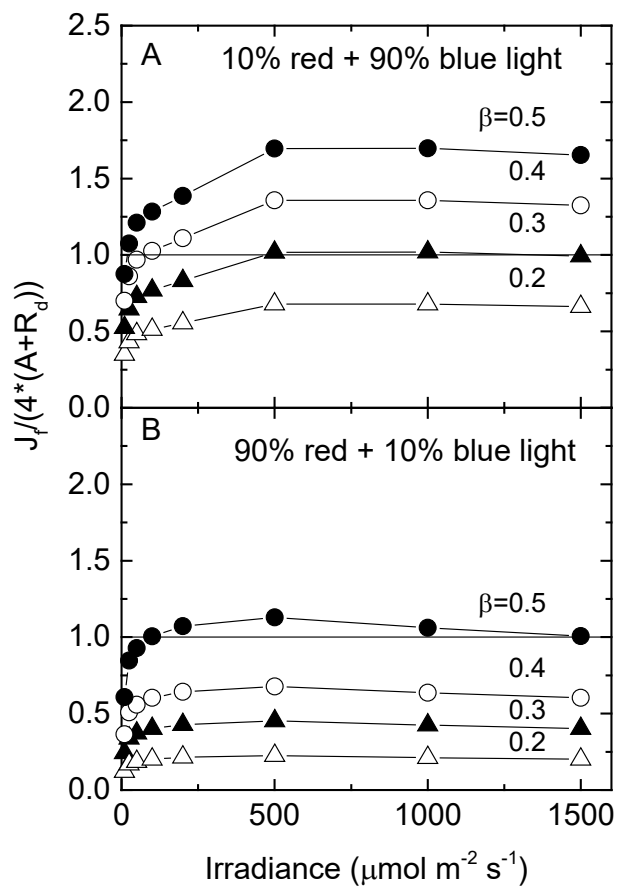
455



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457 Supplemental Figure 2

458 Relationship between modelled actual electron transport rate, J , and modelled apparent
 459 electron transport rate from fluorescence, J_f , measured with a red modulated (red circles) or
 460 blue modulated (blue triangles) light with variation in blue actinic irradiance (A) or red
 461 actinic irradiance (B).



462

463 Supplemental Figure 3

464 Sensitivity of the electron transport rate (J_f) to the fraction of absorbed light that is captured by
 465 PSII (β) when compared to gross CO_2 assimilation rate ($4^*(A + R_d)$) as a function of actinic
 466 irradiances. The impact of proportion of light absorption (α) was not tested since α was
 467 measured for each replicate sample. The spectral balance of red and blue in the actinic light
 468 were altered between 90 % and 10 %. Data are computed from that presented in Figure 4 of *N.*
 469 *tabacum*, cv. Brightleaf.

470

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