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2 **The operation of two decarboxylases (NADPME and**
3 **PEPCK), transamination and partitioning of C₄ metabolic**
4 **processes between mesophyll and bundle sheath cells allows**
5 **light capture to be balanced for the maize C₄ pathway**

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10 **Abstract:**

11 The C₄ photosynthesis carbon concentrating mechanism (CCM) in maize has two CO₂
12 delivery pathways to the Bundle Sheath (BS) (respectively via malate, MAL or aspartate, ASP),
13 and rates of PGA reduction, starch synthesis and PEP regeneration also vary between BS and
14 Mesophyll (M) cells. The theoretical partitioning of ATP supply between M and BS cells was
15 derived for these metabolic activities from simulated profiles of light penetration across a leaf,
16 with a potential 3-fold difference in the fraction of ATP produced in the BS relative to M, (from
17 0.29 to 0.96). A steady-state metabolic model was tested using varying light quality to
18 differentially stimulate M or BS photosystems. CO₂ uptake, ATP production rate (J_{ATP} derived
19 with a low O₂ / chlorophyll fluorescence method) and carbon isotope discrimination were
20 measured on plants under a low light intensity, which is considered to affect C₄ operating
21 efficiency. The light quality treatments did not change the empirical ATP cost of gross
22 assimilation (J_{ATP} / GA). Using the metabolic model, measured J_{ATP} / GA was compared to the
23 predicted ATP demand as metabolic functions were varied between M and BS. Transamination
24 and the two decarboxylase systems (NADPME and PEPCK) were critical for matching ATP and
25 NADPH demand in BS and M when light capture was varied under contrasting light qualities.

26 **Keywords**

27 Bundle sheath, C₄, isotopic discrimination, leakiness, mesophyll, light penetration profiles,
28 $\Delta^{13}C$.

29 **Introduction**

30 Interest in the C₄ pathway has been increased by the potential for enhancing crop productivity
31 and maintaining yield stability in the face of global warming and population pressure (Friso et
32 al., 2010; Zhu et al., 2010; Covshoff and Hibberd, 2012). Maize (*Zea mays*, L.), a C₄ plant of the
33 NADP-ME subtype, is a leading grain production cereal (FAO, 2012). C₄ photosynthesis is a
34 shared activity between mesophyll (M, abbreviations listed in Table 1) and bundle sheath (BS)
35 cells, coupled to allow the operation of a biochemical carbon concentrating mechanism (CCM).
36 The CCM effectively minimizes photorespiration by increasing the CO₂ concentration in BS
37 (C_{BS}), where Rubisco is exclusively expressed. Since BS and M are connected by
38 plasmodesmata, some CO₂ retrodiffuses. The refixation of that escaping CO₂ by the CCM
39 increases the activity of the CCM and the total ATP demand ($ATP_{BS} + ATP_M$) for gross CO₂
40 assimilation (GA), $(ATP_{BS} + ATP_M) / GA$, from a theoretical minimum of 5 ATP (Furbank et al.,
41 1990). Leakiness (Φ), the amount of CO₂ retrodiffusing relative to PEP carboxylation rate, is
42 therefore a proxy for the coordination between the CCM and assimilatory activity (Henderson et
43 al., 1992; Tazoe et al., 2006; Tazoe et al., 2008; Kromdijk et al., 2010; Ubierna et al., 2011;
44 Bellasio and Griffiths, 2013).

45 Recently, the maize C₄ subgroup has been shown to be complicated by the presence of two
46 BS decarboxylation enzyme systems (NADP-ME and PEP carboxykinase, PEPCK), presumably
47 both acting as CO₂ delivery pathways (respectively *via* malate, MAL or aspartate, ASP)
48 (Furumoto et al., 1999; Winkler et al., 1999; Furumoto et al., 2000; Eprintsev et al., 2011;
49 Furbank, 2011; Pick et al., 2011). There is also an extensive overlap between BS and M
50 functions since both cell types can synthesize starch (Spilatro and Preiss, 1987; Kanai and
51 Edwards, 1999) and reduce phosphoglyceric acid, PGA (Majeran and van Wijk, 2009) (see the
52 overall scheme in Fig. 1). Additionally, energetic partitioning can also vary between cell types,
53 since the total ATP produced (J_{ATP}) per CO₂ fixed in GA (J_{ATP} / GA) may be produced in BS
54 (mainly through cyclic electron flow around PSI) or in M (mainly through linear electron flow)
55 depending on the light *locally* available in BS or M (Kramer and Evans, 2011; Yin and Struik,
56 2012). Furthermore, although all NADPH is produced in M, the only compartment operating
57 linear electron transport and oxidising water, some NADPH is exported to BS through MAL
58 diffusion, to meet the reducing power demand therein ($NADPH_{BS}$). To capture the complex C₄
59 physiology, several models of C₄ photosynthesis have been developed (Berry and Farquhar,
60 1978; Laisk and Edwards, 2000; von Caemmerer, 2000; Laisk and Edwards, 2009). The earlier
61 approaches were developed into the von Caemmerer (2000) C₄ model. In particular the
62 associated light limited equations (referred subsequently as the ‘C₄ model’), are used to estimate
63 the parameters needed to resolve the isotopic discrimination model, widely employed to study
64 leakiness under low light conditions [for review see (Ubierna et al., 2011)]. The C₄ model
65 partitions J_{ATP} into two fractions: i) the ATP consumed by PEP carboxylase (PEPC) and ii) the
66 ATP consumed by the C₃ activity (glyoxylate recycling, PGA reduction, and RuBP
67 regeneration). These activities are located in M, BS or in both compartments (see the overall

68 scheme in Figure 1). However, the C_4 model simplifies the spatial compartmentalization between
69 BS and M, and in this paper we now develop the energetic implications of the differential
70 contribution of M and BS to C_4 photosynthesis under different light regimes.

71 Because of these anatomical, metabolic and energetic complexities, C_4 metabolism is highly
72 sensitive to limiting light intensity (Bellasio and Griffiths, 2013), and potentially light quality
73 (Evans et al., 2007). Light quality has a greater influence on C_4 photosynthesis than on C_3 . Leaf
74 pigments preferentially absorb the blue and red region of the spectra and some wavelengths
75 penetrate deeper into leaves. It was shown in C_3 leaves that exposure to different wavelengths
76 results in characteristic light penetrations profiles, which translated into different gradients in
77 photosystem II yield, rates of ATP production and assimilation (A) within the leaf (Terashima et
78 al., 2009). In C_4 leaves, because of the concentric anatomy, light reaches M cells before the
79 deeper BS (Evans et al., 2007), and could alter the balance between light harvesting and
80 energetic partitioning between BS and M.

81 In this paper, we model the likely profiles of light penetration for specific wavelengths
82 associated with Red, Green and Blue (R, G, B) light within a maize M and BS leaf cross section,
83 and calculate the impact on potential ATP production for each cell type. We calculate the
84 proportion of absorbed light (AB) for each wavelength, expressed as $AB_{BS/M}$, the fraction of
85 photons absorbed in BS relative to the photons absorbed in M, from which we derive $J_{ATPBS} /$
86 J_{ATPM} , the fraction of ATP produced in BS relative to the ATP produced in M. Secondly, we
87 developed a steady-state metabolic model (Fig. 1, Table 2), which augments the conventional C_4
88 model (von Caemmerer 2000), to capture the spatial separation between BS and M and partitions
89 the ATP demand between BS and M cells in terms of PGA reduction (PR), starch synthesis (SS)
90 and PEP regeneration, so as to meet the ATP availability in each cell type (Evans et al., 2007).
91 Thirdly, photosynthetic characteristics (leaf level ATP production rate, CO_2 assimilation,
92 stomatal conductance and Φ derived from on-line carbon isotope discrimination) were measured
93 under R, G, B, and RGB in combination, using a decreasing photon flux density (from 500 to 50
94 $\mu E m^{-2} s^{-1}$) to investigate the importance of metabolic plasticity under limiting light intensities.

95 For instance, $AB_{BS/M}$ and J_{ATPBS} / J_{ATPM} , were both lower under the blue wavelengths (460
96 nm), which are rapidly extinguished within the M leaf profile, than under white light, confirming
97 that light quality perturbs C_4 energetics. In spite of this shift, when maize plants were exposed to
98 different light qualities there was no change in Φ indicating that, *at steady state*, the co-
99 ordination between CCM activity and Rubisco assimilation was retained (Sun et al., 2011;
100 Ubierna et al., 2011). The modelled metabolic plasticity projected a window for ATP demand
101 partitioning, ATP_{BS} / ATP_M , which matched the values for J_{ATPBS} / J_{ATPM} supply estimated under
102 B, G and R wavelengths. We show that the plasticity of C_4 metabolism, and in particular the
103 possibility of shifting between malate and aspartate as primary carboxylase product, were of
104 pivotal importance in allowing plasticity of ATP and NADPH demand. In conclusion, our study
105 explains the extensive overlap between BS and M functions and the requirement for at least two

106 decarboxylase systems in NADP-ME subtype plants such as maize, providing an explanation for
107 empirical observations on diversity of decarboxylase activities and PEP regeneration pathways
108 (Rathnam, 1978; Chapman and Hatch, 1981; Wingler et al., 1999; Eprintsev et al., 2011;
109 Furbank, 2011; Pick et al., 2011).

110 **Results**

111 *Metabolic modelling of partitioning between BS and M.*

112 The complexity of C₄ biochemistry (Furbank, 2011) was first integrated in a comprehensive
113 steady-state model (Fig. 1), with key processes described by rate equations (Fig. 1, Box; Table 2)
114 and associated ATP demand (Eqn. 11-13 in Table 2). This model captures the spatial separation
115 between BS and M, the different pathways of the CCM (through ASP and MAL), the different
116 carboxylating enzymes and the process of starch synthesis, as a means to develop the traditional
117 C₄ model (von Caemmerer 2000). The metabolic model (Fig. 1) was generated on the
118 assumption that ATP does not freely diffuse between BS and M and any light-induced $J_{ATPBS} /$
119 J_{ATPM} fluctuations have to be countered by changing the partitioning of ATP demand (for a list of
120 abbreviations see Table 1). Fig. 1 depicts the reactions which are localized in BS, in M or in both
121 compartments. RuP phosphorylation is uniquely localized in BS to supply RuBP directly in
122 proximity to Rubisco, and facilitate the substrate saturation of the Enzyme. Glyoxylate recycling
123 is also a BS exclusive reaction (Fig. 1) (Yoshimura et al., 2004). This feature contributes to the
124 CCM (the so called C₂ cycle) and, in an evolutionary perspective, it was acquired at an early
125 stage (Sage et al., 2012; Schulze et al., 2013). PEP regeneration through PEP carboxykinase
126 (PEPCK) is located uniquely in BS (Wingler et al., 1999), while PEP regeneration through
127 pyruvate phosphate dikinase (PPDK), is located primarily in M (Fig. 1) (Friso et al., 2010;
128 Majeran et al., 2010), and any PPDK activity in BS is generally neglected (von Caemmerer,
129 2000) (see also discussion). PGA reduction (PR), respiration, and starch synthesis (SS) are
130 processes located both in BS and M (Spilatro and Preiss, 1987; Kanai and Edwards, 1999;
131 Majeran and van Wijk, 2009; Friso et al., 2010).

132 These processes are described in detail below, after an initial comparison of modelled light
133 profiles and measured photosynthetic characteristics under R, G and B wavelengths, to give a
134 quantitative description of the biochemical mechanisms underpinning acclimation, fluxes and
135 reaction rates, the dynamics of Φ and the effects on the total and relative ATP demand for
136 assimilation.

137 *Effect of light quality on ATP production in BS and M.*

138 To study the influence of light quality on ATP production partitioning, J_{ATPBS} / J_{ATPM} , we first
139 modelled C₄ anatomy (Fig. 2 left). Light penetration was modelled in two characteristic profiles

140 using the absorption-scattering theory (Fig. 2 right). Profiles were calibrated with leaf
 141 transmittance and reflectance at different wavelengths. Blue light was strongly absorbed (steep
 142 profile), green light was weakly absorbed (gradual profile) while red light had an intermediate
 143 profile of light penetration. These three profiles were integrated to estimate the contribution of
 144 absorbance within abaxial + abaxial mesophyll, interveinal mesophyll and bundle sheath (Fig.2),
 145 and calculated the partitioning of absorbed light (AB) in BS and M (AB_{BS}/M) under five
 146 relevant conditions (Table 3). AB_{BS}/M was used in turn to estimate the partitioning of ATP
 147 production, J_{ATPBS}/J_{ATPM} (Table 3) under the assumption that photochemical yield did not vary
 148 through the leaf profile (see ‘Estimated ATP production partitioning’ in Material and Methods).
 149 At 400 nm, AB_{BS}/M was lowest, representing J_{ATPBS}/J_{ATPM} of 0.29; at 540 nm, AB_{BS}/M was
 150 highest together with J_{ATPBS}/J_{ATPM} (0.96). Under blue light, J_{ATPBS}/J_{ATPM} was close to the
 151 lowest value (0.31), increasing under red (0.68), natural white light (0.76) and green light (0.80).
 152 These values were derived independently of light intensity so they can be considered to reflect
 153 the actual ATP availability in BS and M under a wide range of light intensities. Since ATP does
 154 not diffuse between BS and M, and has a relatively small pool, at steady state, J_{ATPBS}/J_{ATPM} can
 155 be directly compared to the ATP demand partitioning, ATP_{BS}/ATP_M . For this comparison, the
 156 values for J_{ATPBS}/J_{ATPM} under blue, red, white and green light were used subsequently to plot
 157 Fig. 4 B. The model predicts that light quality will unbalance the partitioning of ATP
 158 production, and a comprehensive ecophysiological investigation was therefore used to compare
 159 gross assimilation and ATP supply and partitioning between B and M cells.

160 *Effect of light quality on assimilatory traits.*

161 Figure 3 shows the responses of maize to different light qualities (Red, R; Green, G; Blue, B;
 162 or RGB combined) measured under decreasing irradiance. Net assimilation (A), measured
 163 through gas exchange (Fig. 3 A), and J_{ATP} (Fig. 3 B) measured with the low O_2 - ETR method
 164 using a saturating light pulse, were significantly higher under R light and decreased under RGB,
 165 G and B. Light quality had no significant effect on stomatal conductance (g_s , Supplementary Fig.
 166 S 1 A), but C_i/C_a was lower under R light (Supplementary Fig. S 1 B) as a consequence of the
 167 higher A . The CO_2 concentration in BS (C_{BS}), estimated by fitting a C_4 photosynthesis model to
 168 J_{ATP} , was higher under R and G light (Supplementary Fig. S 1 C), because of the higher A . The
 169 light compensation point (LCP), bundle sheath conductance (g_{BS}) and respiration in the light
 170 (R_{LIGHT}) were not significantly influenced by light quality (Table 4).

171 With the precise estimate of R_{LIGHT} and J_{ATP} , we calculated J_{ATP}/GA , which represents the
 172 experimentally-determined ATP cost for gross assimilation. J_{ATP}/GA was not influenced by
 173 light intensity but varied between light qualities from 5.37 to 5.73, under B, R, RGB and G light.
 174 J_{ATP}/GA was then used in Fig. 4 B, plotted against the ATP production partitioning $J_{ATPBS}/$
 175 J_{ATPM} , found above. The relatively minor increase in J_{ATP}/GA (c 0.3 ATP / CO_2) observed

176 experimentally contrasts with the metabolic disruption theoretically predicted under these
177 conditions (Evans et al., 2007). Our data was supported by real-time isotopic discrimination, Δ
178 (Fig. 3 C) and leakiness, Φ (Fig. 3 D), which were not influenced by light quality, showing that
179 the plant coped with a 2.5x shift in J_{ATPBS} / J_{ATPM} without any imbalance in the co-ordination of
180 CCM activity and assimilation. Obtaining Φ data under such low light intensities represents a
181 technical challenge and the experimental protocol was carefully optimised for low light intensity
182 (Bellasio and Griffiths, 2013). Data obtained under low light are subject to a potential error
183 magnification proportional to ξ [supporting figure S3, (Evans et al., 1986)], as clearly shown by
184 the large error bars in Fig. 3D, and therefore should be interpreted with care. Leakiness, $J_{ATP} /$
185 GA and the window of J_{ATPBS} / J_{ATPM} formed the basis of a metabolic model used to describe
186 these biochemical responses.

187 *Influence of BS activity on assimilatory metabolism and ATP demand (total and relative).*

188 The comprehensive metabolic model (Fig. 1) was developed to describe the biochemical
189 reactions directly and indirectly involved in C_4 assimilation by rate equations (Table 2). The
190 proportions of three assimilatory ATP-consuming processes [starch synthesis (SS),
191 phosphoglyceric acid reduction (PR) and PEP carboxykinase (PEPCK)] were manipulated to
192 increase within BS (allocated, Fig. 4 A). These processes overlap *between* BS and M and could
193 be allocated to BS without influencing the overall assimilation rate. By means of this progressive
194 allocation, we predicted: i) the minimum and maximum ATP demand partitioning, ATP_{BS} / ATP_M
195 (Eqn. 12 / Eqn. 13, Table 2); ii) the reaction rates and metabolite fluxes at a given ATP_{BS} / ATP_M ,
196 including the rate PEPCK and PPK, the relative CO_2 flux through ASP and MAL and the
197 partitioning of PGA reduction between BS and M; iii) the dynamics of total ATP demand for
198 gross assimilation $(ATP_{BS} + ATP_M) / GA$ at variable ATP_{BS} / ATP_M .

199 When the BS allocation rate of PR, PEPCK and SS was zero (referred as condition 1, number
200 1 in Fig. 1 and Fig. 4 B), the predicted ATP_{BS} / ATP_M was lowest (0.27). This value was
201 comparable to J_{ATPBS} / J_{ATPM} resolved from the optical model under blue wavelengths (400 nm
202 and 460 nm, Table 3), showing that metabolism could reduce ATP demand to match even the
203 lowest ATP supply in BS. In this condition the ATP demand in BS was brought about by RuP
204 phosphorylation and glyoxylate recycling, two processes that are known to be exclusive to BS.
205 The predicted $(ATP_{BS} + ATP_M) / GA$ was 5.74 (Fig. 4 B, dashed line), in agreement with $J_{ATP} /$
206 GA measured under blue light (5.73, Fig. 4 B, blue square). Since photosynthetic PGA
207 production is exclusive to BS (primarily from Rubisco carboxylase, or oxygenase activity and
208 glyoxylate recycling), when there is no ATP available for PGA reduction, (e.g. in condition 1)
209 the PGA diffuses to M and is reduced therein. After reduction, DHAP could supply starch
210 synthesis in M or diffuse back to BS to regenerate RuBP. The predicted NADPH demand in BS
211 ($NADPH_{BS}$) was therefore the lowest (Eqn. 14, Table 2; Fig. 4 A, dotted line), corresponding to

212 the NADPH demand for glyoxylate recycling in BS. The activity of malate dehydrogenase in M
213 (MDH_M), process responsible for exporting NADPH, was reduced by diverting the substrate
214 oxaloacetate (OAA) to transamination to aspartate (ASP). Hence, in condition 1, MDH_M had the
215 lowest activity (Fig.1, Eqn. 16 in Table 2) while transamination had the highest rate (Fig. 1, Fig.
216 4 A, dashed line, Eqn 17 in Table 2). Once ASP diffused to BS, it underwent a futile reduction-
217 oxidative decarboxylation that resulted in net CO_2 flux without a conjoint NADPH translocation
218 (Fig. 1).

219 When PR and SS were progressively allocated in BS, the predicted ATP demand $ATP_{BS} /$
220 ATP_M (Fig. 1, Fig. 4) progressively increased. The activation of PEPCK in BS, not only
221 contributed to the predicted increasing ATP demand in BS, but also lowered the predicted ATP
222 cost of assimilation $(ATP_{BS} + ATP_M) / GA$ because PEPCK regenerates PEP with half the ATP
223 demand of PPK. Condition 2 (number 2 in Fig. 1 and Fig 4) represents a state where $ATP_{BS} /$
224 ATP_M equals $J_{ATP_{BS}} / J_{ATP_M}$ resolved from the optical model under red light (0.68, Table 3). The
225 predicted $(ATP_{BS} + ATP_M) / GA$ (5.45, Fig. 4 B, dashed line) agreed with J_{ATP} / GA measured
226 under red light (5.47, Fig. 4 B, red square).

227 When the projected allocation of PR, PEPCK and SS to BS was highest (referred as condition
228 3, number 3 in Fig. 1, Fig. 4 A), the predicted ATP_{BS} / ATP_M was 0.8. This partitioning equals
229 $J_{ATP_{BS}} / J_{ATP_M}$ estimated by the optical model under green light, and it is similar to $J_{ATP_{BS}} / J_{ATP_M}$
230 estimated under natural white light (0.76, Table 3). Because PEPCK was activated at a highest
231 rate, only 70 % of PEP was regenerated through PPK and $(ATP_{BS} + ATP_M) / GA$ was lowest
232 (5.39, Fig. 4 B, dashed line), predicting well J_{ATP} / GA values measured under RGB (5.38, Fig. 4
233 B, RGB square) and green light (5.37, Fig. 4 B, green square). In condition 3, PGA reduction in
234 BS was highest, determining the highest $NADPH_{BS}$ (Fig. 1; Fig. 4 A dotted line; Eqn. 14, Table
235 2) and the highest MDH_M activity (Fig. 1, Eqn. 16 in Table 2). Because most of the OAA
236 produced by PEP carboxylase was reduced by MDH_M , transamination rate was lowest, just
237 enough to supply PEPCK activity in BS.

238 *Estimate of actual reaction and diffusion rates.*

239 Since all C_4 reactions were described by rate equations (Table 2) we could estimate actual
240 reaction rates. Although the predictions shown so far (including the optical part) are largely
241 independent of light intensity, in this step rates had to be calculated at a specific light intensity.
242 However, in order to compare these values in a wider range of light intensities, rates were
243 expressed as relative to GA . This was also functional to avoid any computational distortion
244 caused by respiration in the vicinity of the light compensation point. The irradiance of $125 \mu E m^{-2}$
245 s^{-1} , characteristic of illumination in the shade, was chosen because ongoing studies on low light
246 and light quality are relevant to the physiology of shading (see also discussion). Furthermore,
247 since low light provides a mean to directly manipulate C_4 metabolism, a great deal of

248 comparable work has been undertaken both in this lab (Kromdijk et al., 2008; Kromdijk et al.,
249 2010; Bellasio and Griffiths, 2013) and by other investigators (Ubierna et al., 2011; Ubierna et
250 al., 2013) under low irradiances. Reaction rates, shown in the boxes within Fig. 1 were obtained
251 by parameterizing the model with the data obtained during the experiment and with the output of
252 the C₄ model [all equations are reported in Suppl. Table S 2 but see also (Bellasio and Griffiths,
253 2013), and references therein]. Rates were calculated for the three relevant conditions mentioned
254 above, i.e. condition 1 corresponds to the lowest ATP demand in BS, condition 2 corresponds to
255 an intermediate ATP demand (matching ATP supply under red light) while condition 3
256 correspond to the highest ATP demand in BS (matching ATP supply under green light).
257 Condition 1, 2 and 3 were numbered 1, 2 and 3 in Fig. 1 and Fig. 4.

258 Discussion

259 The implications of the metabolic model for partitioning ATP demand are firstly considered
260 in terms of previous studies of C₄ decarboxylases in NADP-ME systems. The resultant ATP
261 partitioning and metabolic plasticity provided by these processes is then considered in terms of
262 overall C₄ energetic limitations (Evans et al., 2007). Finally, we go on to consider the
263 implications for multiple decarboxylase origins and function in terms of C₄ pathway evolution,
264 as well as light use and energy partitioning within a C₄ crop canopy.

265 *Modelling ATP demand: decarboxylase diversity in C₄ systems*

266 Recent developments in C₄ research have highlighted the complexity of C₄ metabolism, in
267 terms of extensive overlapping of BS and M functions (Friso et al., 2010; Majeran et al., 2010),
268 the presence of two distinct decarboxylating pathways (Meister et al., 1996; Wingler et al., 1999;
269 Pick et al., 2011), and plasticity in malate metabolism [(Eprintsev et al., 2011) and references
270 therein]. Although an involvement in balancing the energetic capacity in responses to
271 environmental conditions has been proposed (Eprintsev et al., 2011; Furbank, 2011), empirical
272 evidence and an associated metabolic model were needed to validate this suggestion. In this
273 study, we tested the capability of metabolism to respond to different ATP allocation between BS
274 and M by means of a comprehensive metabolic model. To overcome uncertainties in the causal
275 relationship between ATP availability and enzyme kinetics, we deduced the highest and lowest
276 possible BS reaction rates from physiological considerations and studied how the predicted ATP
277 demand partitioning ATP_{BS} / ATP_M would vary in response to incremental activation. We found
278 that ATP_{BS} / ATP_M could vary between 0.27 to 0.80 if starch synthesis, PGA reduction (PR) and
279 PEP regeneration were freely allocated between BS and M. In addition, we suggest that higher
280 ATP consumption in BS than the predicted maximum, could result from the transient activation
281 of PPDK in BS (Aoyagi and Nakamoto, 1985; Friso et al., 2010) or in a long-term response,
282 from the de-novo synthesis of PR enzymes. This shows the importance of the presence of PPDK

283 in BS and the possibility of metabolism to regulate the maximum BS rate of PGA reduction in
284 response to contrasting environmental conditions. These processes could take advantage of the
285 increased ATP availability in BS under 540 nm green light (Table 3) or under high irradiances
286 when ATP production in M is reduced (because of PSII yield quenching; Suppl. Fig. S 2).

287 The extensive overlap between BS and M functions was important for preserving the overall
288 assimilation rate, and for any process activated in BS, a complementary decrease in M could
289 rebalance overall metabolism so as the total rate of assimilation and ATP demand to remain
290 constant in spite of contrasting BS / M engagement. In addition, we showed the importance of
291 transamination (T) in balancing the reducing power needs in BS. When the ATP availability in
292 BS was low (e.g. condition 1), PGA reduction in BS was down-regulated, and therefore there
293 was a reduced NADPH demand in BS (Fig. 4 A, dotted line). Under these conditions, CO₂ was
294 delivered to BS through ASP, a pathway that bypasses malate reduction in M and, hence,
295 NADPH export to BS. This shift between ASP and MAL-mediated CCM indicates the
296 importance of maintaining both pathways in NADP-ME subtype C₄ plants. The predicted T rate
297 varied in response to environmental conditions from a minimum of 0.35 V_P to the entirety of the
298 CO₂ delivered to BS, in line with the observation that ASP can support physiological rates of
299 photosynthesis (Rathnam, 1978; Chapman and Hatch, 1981; Meister et al., 1996; Pick et al.,
300 2011). Under white light, the model predicted a 33 % T / V_P, which is in line with radiolabelling
301 and biochemical observations (Downton, 1970; Hatch, 1971; Chapman and Hatch, 1981). These
302 predictions are not influenced by whether transamination is mediated by the ‘conventional’
303 glutamate aminotransferases (Fig. 1), or by the more recently documented aspartate
304 aminotransferases (Pick et al., 2011), because, in the model, transamination is simply assumed to
305 be a fast, passively regulated process at equilibrium.

306 Although a mechanistic explanation goes beyond the scope of this study, it is worth noting
307 that the fine tuning between contrasting scenarios may be relatively straightforward at metabolic
308 level. In fact, both the CCM and the RPP pathway share diffusible metabolites between BS and
309 M cells and are mediated by fast reactions, which, in physiological conditions, are close to the
310 thermodynamic equilibrium (e.g. transamination reactions or sugar phosphate conversions). The
311 regulation of the fluxes may therefore be regulated in just a few key steps, for instance at the
312 level of PGA reduction, or malate decarboxylation. These have long been known to be regulated
313 by the stromal pH, by feedback from metabolite pools and by feed forward from light reactions
314 (Johnson and Hatch, 1970; Drincovich and Andreo, 1994; Detarsio et al., 2003; Murmu et al.,
315 2003; Trost et al., 2006; Eprintsev et al., 2011). The adjustment of the other reaction and
316 diffusion rates may then follow passively, mediated by the feedback provided by changing
317 relative metabolite concentrations in one or the other compartment.

318 *Modelling ATP supply as a function of light quality*

319 Previously, fluorescence microimaging had shown that, because of the characteristic C₄
320 concentric leaf anatomy, strongly absorbed blue wavelengths would result in preferential
321 absorption in M cells as compared to wavelengths of light which could penetrate deeper into the
322 leaf profile (Evans et al., 2007). However, difficulties in the interpretation of fluorescence
323 imaging, which are dependent on the different fluorescence yield of PSI-rich-BS and PSII-rich-
324 M, have prevented investigators from predicting the relative light harvesting in BS and M (Evans
325 et al., 2007). To overcome these difficulties, we characterized the profiles of light penetration in
326 a maize leaf by means of an absorption-scattering model, which represents a first attempt to
327 calculate the extent of light absorption imbalance caused by light quality. Because both BS and
328 M produce ATP in light reactions, light harvesting imbalances would alter ATP partitioning.
329 These ATP production imbalances were estimated using the relative stoichiometry of the
330 electron transport operating in BS and M. The effect was very different from the response to
331 changing light intensity, in fact, light quality only marginally influenced the total ATP available
332 at leaf level but resulted in a 3-fold difference in the fraction of ATP produced in the BS, from
333 0.29 to 0.96 (Table 3). These imbalances are potentially independent of the light intensity,
334 however photochemical yield (i.e. quenching) was assumed independent of the position within
335 the leaf (see also description of Eqn 3, below), an assumption which may not hold under high
336 light intensity. Regulation of photosystem yield through the leaf profile (Terashima et al., 2009)
337 might also occur under changing light quality. Differential quenching would represent an
338 additional point of plasticity in response to changing light quality, which would have the effect
339 of narrowing the window of possible ATP_{BS} / ATP_M . Since we were interested in biochemical
340 plasticity in response to ATP imbalance rather than in detailing the mechanics of electron
341 transport processes, we did not include the possible plasticity at thylakoid level, and hence
342 maximised the operating range of possible ATP_{BS} / ATP_M variation.

343 This *spatial* partitioning of ATP production J_{ATPBS} / J_{ATPM} is different from the *functional*
344 partitioning of ATP consumption of the C₄ model (von Caemmerer, 2000; Ubierna et al., 2011;
345 Bellasio and Griffiths, 2013) and from the theoretical partitioning of ATP demand ATP_{BS} / ATP_M
346 of the metabolic model presented here. In the C₄ model, the total ATP production, J_{ATP} , is simply
347 assumed to be produced by an undivided electron transport chain (Yin and Struik, 2012), then
348 partitioned to PEP regeneration activity or C₃ activity by a parameter known as x (Suppl. Table S
349 2). This operation does not involve spatial separations between BS and M. In this study we
350 followed this conventional approach, which has been widely validated, *then* captured the
351 partitioning between BS and M with the equations of the metabolic model (Table 2). On the
352 basis of this division of work we calculated the theoretical partitioning of ATP demand $ATP_{BS} /$
353 ATP_M . Values for ATP_{BS} / ATP_M were therefore derived independently from J_{ATPBS} / J_{ATPM} (J_{ATPBS}
354 $/ J_{ATPM}$ was not used in model parameterization). These independently derived J_{ATPBS} / J_{ATPM} and
355 ATP_{BS} / ATP_M were compared in Figure 4.

356 *Implications for electron transport processes*

357 To allow these ATP partitioning rearrangements there must be a high degree of flexibility at
358 the electron transport chain level. In fact, although linear electron flow (LEF) activity in BS is
359 often neglected [because of negligible expression of the O₂ evolving complex (Majeran and van
360 Wijk, 2009; Friso et al., 2010) and non-appreciable O₂ evolving activity (Meierhoff and
361 Westhoff, 1993)] evidence that appreciable linear flow can be supported by stromal reductants as
362 glutathione and ascorbate has been presented (Walker and Izawa, 1979; Ivanov et al., 2001;
363 Ivanov et al., 2005; Ivanov et al., 2007). These reductants are likely to be produced from
364 NADPH, supplied by malate imported from M (Kanai and Edwards, 1999; Laisk and Edwards,
365 2000). These processes couple reductant pools at thylakoid and stromal level, and are likely to
366 function as plasticity mechanisms, playing a pivotal role in acclimation to changing light
367 conditions.

368 Most of the LEF activity is localized in M chloroplasts, which evolve O₂ and supply all
369 reducing power requirements. For this reason, many reactions requiring NADPH (such as
370 nitrogen reduction) are localised in M, to benefit from NADPH availability (Majeran et al.,
371 2005). Even if M chloroplasts are specialized in NADPH production, the ratio of ATP versus
372 NADPH demand is highly sensitive to BS / M assimilation partitioning (Figure 1). Meeting this
373 variable requirement may involve differential engagement of LEF versus cyclic electron flow
374 (CEF). The particular features of the CEF operating in maize (Ivanov et al., 2007; Laisk et al.,
375 2010; Munekage et al., 2010; Hertle et al., 2013), may reflect, beyond the heterogeneity between
376 BS and M specialization, this characteristic need for plasticity in CEF / LEF engagement.

377 Regardless of this electron transport plasticity, the ATP production deficits induced by
378 changing light quality cannot be rebalanced within the individual BS chloroplast. In fact,
379 increasing the ATP production at the electron transport chain level would require light
380 (Takabayashi et al., 2005; Kramer and Evans, 2011), whose availability is not under metabolic
381 control. At the same time, the electron transport mediated dark production of ATP (Morstadt et
382 al., 2002; Bukhov and Carpentier, 2004; Egorova and Bukhov, 2004; Kuntz, 2004), has low
383 conversion efficiency (Kramer and Evans, 2011), hence an engagement of ATP chemiosynthesis
384 would be incompatible with the observed pattern of J_{ATP} / GA . ATP itself is not a suitable shuttle
385 to rebalance ATP deficits because the ATP molecule is relatively big, it has a relatively small
386 pool and homeostasis is critical, therefore every chloroplast has an independent ATP pool.
387 Maintaining balanced ATP consumption in spite of local ATP deficits requires rearranging the
388 localization of ATP demand, the fluxes, and the partitioning of metabolic work between the
389 mutually interdependent BS and M cells.

390 *Metabolic plasticity is effective in maintaining overall assimilation efficiency*

391 Previously, on the basis of theoretical considerations, it had been predicted that an unbalanced
392 ATP supply would result in a disruption of the delicate equilibrium between BS and M functions
393 with consequent loss in assimilatory efficiency (Henderson et al., 1992; Evans et al., 2007; Tazoe
394 et al., 2008). This prediction arose because metabolic rigidity would be expected under some of
395 the common simplifications used for C₄ biochemistry, whereby transamination is neglected,
396 PGA is reduced at a fixed rate in BS and NADPH delivery to BS is equimolar to CO₂ delivery
397 (Laisk and Edwards, 2000; Laisk and Edwards, 2009). In the updated description of C₄
398 metabolism provided in this paper, reaction rates are variable and tuneable. When the ATP
399 availability in BS was low (e.g. condition 1), PGA reduction in BS was downregulated, leaving
400 all available ATP for RuBP regeneration, resulting in unaltered Rubisco efficiency. Because the
401 ASP-mediated CCM delivers solely CO₂, while the MAL mediated CCM delivers both NADPH
402 and CO₂, the variable engagement of the two pathways allows the activity of the CCM to be
403 regulated independently of NADPH demand in BS, hence the optimal C_{BS} could be maintained
404 under all light qualities.

405 These predictions are supported by further model outputs, where we found no significant
406 effect of light quality on Φ and on C_{BS}, confirming the response found in a similar experiment
407 where plants were acclimated under high light (Sun et al., 2011). This observation, together with
408 the relatively minor change in J_{ATP} / GA (Fig. 4 B) show that C₄ metabolic balance was adjusting
409 to the shifts in ATP supply without the potential major disruptions mentioned above.

410 *Implications for light use at leaf and canopy level*

411 Photosynthesis in shaded conditions has critical importance in C₄ canopies as it may represent
412 up to 50 % of CO₂ uptake (Baker and Long, 1988; Long, 1993). Shade light has a reduced
413 intensity [typically 1 / 20 of full sunlight (Shirley, 1929)], and differs in spectral quality from
414 red-rich sunlight: diffuse sky radiation is enriched in blue, whereas canopy filtered light is
415 enriched in green (Smith, 1982). Under low light conditions it has been shown that Φ may
416 increase both at leaf e.g. (Kromdijk et al., 2010; Bellasio and Griffiths, 2013) and at canopy level
417 (Kromdijk et al., 2008). Theoretical considerations have associated this Φ increase with
418 decreased C₄ efficiency and a potential loss of photosynthetic carbon uptake (Furbank et al.,
419 1990; von Caemmerer, 2000; Kromdijk et al., 2008; Tazoe et al., 2008). Although other studies
420 have compared the effect of light quality on Φ under low irradiance or under different light
421 qualities (Kromdijk et al., 2010; Sun et al., 2011; Bellasio and Griffiths, 2013; Ubierna et al.,
422 2013), the novelty the approach presented here has been to couple the measured and predicted
423 ATP supply during assimilation under these conditions. In this experiment, which was
424 specifically optimized to acquire data under low light (Bellasio and Griffiths, 2013), we showed
425 that the total ATP cost of gross assimilation was not significantly influenced by light intensity,
426 and underwent a little variation under different light quality (Fig. 4 B). This showed that

427 metabolism *at steady state under low light intensities*, maintained efficiency in spite of changes
428 in light quality or intensity. This implies that the hyperbolic increase of Φ observed under
429 decreasing light intensities (Fig. 3 D), which underpins the predicted photosynthetic efficiency
430 loss, actually did not cost additional ATP, but resulted instead from mitochondrial
431 decarboxylation in BS (Bellasio and Griffiths, 2013). Similar conclusions were highlighted by
432 (Ubierna et al., 2013). This observation is consistent with V_P / V_C and the optimal 'x' being
433 largely independent of light intensity (von Caemmerer, 2000; Kromdijk et al., 2010), indicating a
434 constant degree of engagement of the CCM even under an apparent leakiness increase. Care
435 should therefore be taken when the ATP cost (and quantum yield) of C_4 photosynthesis is
436 derived from Φ , measured either at leaf or canopy scale, particularly in the vicinity of the light
437 compensation point (Furbank et al., 1990; von Caemmerer, 2000; Tazoe et al., 2008). In these
438 conditions we propose that the ATP cost should be calculated by summing the ATP cost of all
439 the active biochemical processes (e.g. Eqn 11, Table 2), instead of using leakiness as a proxy for
440 C_4 biochemical efficiency. The actual impact of Φ on canopy-level carbon uptake may depend
441 upon the extent of steady-state photosynthesis under low light or altered light quality conditions
442 (e.g. green enriched), and shorter-term, more transient conditions, when Φ may be more variable.

443 **Conclusion**

444 In this study we set out to investigate whether the maize C_4 system could respond to changing
445 environmental conditions by adjusting the C_4 (amino)acid (MAL or ASP) delivered from M to
446 BS, as well as the proportions of other metabolic reactions shared between both cell types, such
447 as starch synthesis, PGA reduction and PEP regeneration (Walker et al., 1986; Spilatro and
448 Preiss, 1987; Winkler et al., 1999; Friso et al., 2010; Majeran et al., 2010; Furbank, 2011). Using
449 contrasting light qualities and their projected extinction within the leaf profile, we could then
450 estimate the rate of ATP synthesis in M and BS compartments, as compared to the overall leaf-
451 level operating efficiency measured by gas exchange and real-time carbon isotope
452 discrimination. We depicted a scenario whereby metabolism, although subject to the general
453 constraints imposed by C_4 physiology, was able to take the maximum advantage of
454 environmental conditions by changing the relative engagement of BS and M functions, which
455 were ultimately under environmental control. The outputs, based on metabolic modelling and
456 empirical measurements, provide definitive evidence for the role of complementarity between
457 BS and M functions, allowing ATP demand to be regulated in response to contrasting
458 environmental conditions. The two decarboxylase systems in BS of maize, with a variable rate of
459 transamination, allow the regulation of NADPH supply to match demand in BS independently of
460 the delivery of CO_2 .

461 The findings of this study highlight the importance of C_4 metabolic models in helping to
462 explain acclimation and adaptation to changing light intensity for all C_4 subgroups. The

463 emerging complexity of the NADP-ME / PEPCK interactions certainly demands some
464 refinement to the widespread simplifications used to describe C₄ systems and to the assumptions
465 regarding the relatively fixed energetic partitioning in maize. Furthermore, we have clearly
466 linked metabolic plasticity to the capacity to maintain high photosynthetic efficiency under
467 changing environmental conditions, which could well be related to original functions of bundle
468 sheath decarboxylases and evolution of the C₄ syndrome (Hibberd and Quick, 2002; Griffiths et
469 al., 2013). Finally, the extent that such steady state conditions of low light and altered light
470 quality affect carbon uptake within an intact crop canopy remain to be determined, as compared
471 to more transient responses which may well increase leakiness and reduce carbon assimilation
472 under low light conditions.

473 **Materials and Methods**

474 *Metabolic model*

475 The processes contributing to assimilatory metabolism in maize (Furbank, 2011) were
476 integrated in a comprehensive steady-state model (Fig. 1). Some functional simplifications were
477 made. Cells were decompartmentalized. NADPH and NADH were considered equivalent or
478 convertible. Starch was assumed to be the only final product of photosynthesis (starch synthesis
479 has the same ATP cost per hexose than phloem-loaded sucrose, considering the stoichiometry of
480 1 ATP / H⁺ of the membrane H⁺ - ATPase and 1 H⁺ / sucrose of the sucrose symporter). The ATP
481 + NADH produced during respiration (assumed supplied by PGA) were neglected in calculations
482 because they are likely to be consumed by basal metabolism. Transamination (T) was assumed to
483 be passively regulated by substrate availability (all OAA not reduced by MDH was
484 transaminated), as T reactions are rapid conversions at equilibrium.

485 The specialization of BS and M was captured by assigning processes to BS, M, or to the
486 conjoint work of both compartments (allocatable processes). PEPCK (Walker et al., 1986;
487 Furumoto et al., 1999; Wingler et al., 1999; Furumoto et al., 2000) and glyoxylate recycling
488 (Yoshimura et al., 2004) were allocated to BS; linear electron flow (Meierhoff and Westhoff,
489 1993; Romanowska et al., 2006; Majeran and van Wijk, 2009; Friso et al., 2010; Kramer and
490 Evans, 2011) and PPDK (see discussion) were allocated to M; R_{LIGHT} was split equally between
491 M and BS (von Caemmerer, 2000; Kromdijk et al., 2010; Ubierna et al., 2011); PGA reduction
492 (PR) and starch synthesis (SS) were variably allocated. T rate was equal in BS and M as the pool
493 of ASP and ALA is shared. The model was described by steady state rate equations (Table 2).

494 *Minimum and maximum BS allocation*

495 The rate of variably allocated processes ranged between a minimum and maximum rate,
496 deduced from physiological considerations (but see discussion for the environmental influence

497 on maximum rates). Both BS and M express SS enzymes (Spilatro and Preiss, 1987; Majeran
498 and van Wijk, 2009) and synthesize starch e.g. (Rascio et al., 1980; Kanai and Edwards, 1999),
499 so SS was allocated to BS between 0 and SS_{TOT} . PR is not essential to BS so PR_{MIN} was set at 0.
500 PR is mainly a M process (Majeran et al., 2005; Majeran and van Wijk, 2009), so PR_{MAX} was
501 limited at $0.35 \cdot PR_{TOT}$. PEPCK is not essential to BS so $PEPCK_{MIN}$ was set at 0. $PEPCK_{MAX}$ was
502 set at $0.3 \cdot V_P$, identified by fitting the total ATP demand of assimilation to J_{ATP} / GA (Fig. 4 B).

503 *Parameterization*

504 Equations describing overall assimilation (Eqn. 4 to 10, in Table 2), were parameterized with
505 the measured data A , R_{LIGHT} , the output of the von Caemmerer C_4 model (V_O , V_P and V_C , Suppl.
506 Table S 2) (Bellasio and Griffiths, 2013) and Φ calculated from Δ under $PAR = 125 \mu E m^{-2} s^{-1}$
507 (see below). Then, reaction rates (Eqn. 4 to 21 in Table 2) were calculated under the minimum
508 (condition 1) and the maximum (condition 3) BS allocation (Fig. 1 and Fig. 4 B). Finally,
509 intermediate states (e.g. condition 2) were calculated by allocating reactions to BS in linear
510 increments (see continuous lines in Fig. 4 A).

511 The ATP demand in BS (ATP_{BS}) was calculated by adding the ATP demand of BS processes
512 (Table 2, Eqn. 12; Suppl. Table S 1). Analogously, ATP_M was calculated by summing the ATP
513 demand of M processes (Table 2, Eqn. 13; Suppl. Table S 1), and the partitioning of ATP demand
514 (ATP_{BS} / ATP_M) was calculated by dividing Eqn. 12 by Eqn. 13. Similarly the NADPH demand
515 in BS ($NADPH_{BS}$) was calculated by summing the NADPH demand of BS processes (Table 2,
516 Eqn. 14; Suppl. Table S 1). Rate equations for other processes are listed in Table 2.

517 *Estimated light harvesting in BS and M, AB BS / M*

518 A maize leaf cross section was simulated by rectangular units, enclosing a square BS (left
519 panel of Fig. 2). Inter-veinal distance (IVD, $106 \mu m$); M thickness ($100 \mu m$), BS / M area (0.26)
520 and the resulting BS side ($46 \mu m$) were averaged from (Hattersley, 1984; Usuda, 1985;
521 BongardPierce et al., 1996; Moreno-Sotomayor et al., 2002; Kromdijk et al., 2010). Because of
522 the square anatomy, the leaf light environment could be described by two light profiles: P1 and
523 P2. These were calculated applying the Kubelka-Munk absorption – scattering theory with the
524 method of Allen and Richardson (Allen and Richardson, 1968; Gates, 1980; Terashima et al.,
525 2009), modified to describe the simulated C_4 anatomy. Briefly, each profile was considered to be
526 made of a number n of light absorbing and scattering elements, the total number of elements in
527 the profile was N . The element $n=0$ was the illuminated, or adaxial point of the profile, which
528 included the upper epidermis, approximated to a single reflecting element. The element N was
529 the abaxial point of the profile, which included the lower epidermis, approximated to a single
530 reflecting element. Radiant flux directed downward was I , and that upward was J . Incident flux
531 was I_0 , and was taken to be the unity. For each profile, the flux reflected by the first element was

equivalent to the point reflectance and the flux transmitted by the last element was equivalent to the point transmittance: $J(0) = \text{Point refl.}$ and $I(N) = \text{Point transm.}$

Incremental absorption and scattering were calculated as (Gates, 1980):

$$dI = -(k + s)I dn + sJ dn \quad (1)$$

$$dJ = (k + s)J dn - sI dn \quad (2)$$

Where k is an absorption coefficient and s is a scattering coefficient. In P1, k was constant throughout the profile. In P2 k was three times higher in the elements corresponding to BS, because chlorophyll concentration is three times higher than in the surrounding M [BS / M chl content, 0.74 (Kanai and Edwards, 1973), multiplied by M / BS area, 4; Fig. 2, left]. I and J were solved for all elements of P1 and P2 according to the integration of Eqn. 1 and 2 proposed by (Gates, 1980). Modelled leaf reflectance resulted from averaging $J(0)$ contributions to the total leaf reflectance from P1 and P2 (56 % P1 and 44 % P2). Similarly, modelled leaf transmittance was calculated as a weighted average of $I(N)$ from P1 and P2. Modelled leaf reflectance and transmittance were calibrated with measured leaf reflectance and transmittance at different wavelengths [Table 3, (Woolley, 1971)] by varying k and s . This procedure allowed calculating P1 and P2 at different wavelengths (Figure 2 right). Absorbed light (AB) in BS resulted from integrating P2 over BS area. AB in M resulted from integrating P2 over adaxial and abaxial mesophyll (MAD + MAB) area plus the integral of P1 over the two interveinal mesophyll (MI) areas.

Estimated ATP production partitioning, J_{ATPBS} / J_{ATPM}

M chloroplasts are engaged in NADPH and ATP production, therefore, for a given number of light quanta they produce c. half the ATP produced in BS chloroplast. J_{ATPBS} / J_{ATPM} was calculated as:

$$\frac{J_{ATPBS}}{J_{ATPM}} = 2 \cdot AB \frac{BS}{M} \quad (3)$$

558
559 The coefficient 2 (in Eqn. 3) is based on widely accepted assumption that light is equally
560 shared between photosystems and on the simplification that the photochemical yield is
561 independent of the position within the leaf profile. In detail we assumed: i) exclusive linear
562 electron transport in M with equal PSI / PSII absorption partitioning (Meierhoff and Westhoff,
563 1993; Kramer and Evans, 2011); ii) exclusive cyclic electron transport in BS with no PSII
564 absorption (Romanowska et al., 2006; Majeran and van Wijk, 2009); iii) equal yield of PSI and
565 PSII (Miyake et al., 2005) (Supp. Fig. S 2 C); iv) equal H^+ / ATP stoichiometry of the ATP
566 synthase in BS and M: the enzyme complex is the same (Majeran and van Wijk, 2009; Friso et
567 al., 2010); v) twice the H^+ per photochemical event pumped in BS versus M: in M 1.5 H^+ are
568 extruded per photochemical event (Kramer and Evans, 2011), while in BS we assumed 3 H^+
569 extruded per photochemical event [average between 4 H^+ of the NDH-mediated (Kramer and
570 Evans, 2011; Peng et al., 2011) and 2 H^+ of the PGR5 / PGRL1-mediated electron flow (Kramer
571 and Evans, 2011; Hertle et al., 2013) both operating in maize BS (Ivanov et al., 2007)].

572 *Plants*

573 *Zea mays* L. (F1 Hybrid PR31N27, Pioneer Hi-bred, Italy) plants were grown in 1.5 L pots
574 filled with Levington pro M3 (Scotts, UK) in growth rooms (Conviroon Ltd, Winnipeg, Canada)
575 set at 16 h day length, temperature of 25 °C / 23 °C (day/night), 40 % relative humidity and PAR
576 = 600 $\mu E m^{-2} s^{-1}$ and manually watered daily with particular care to avoid overwatering. After
577 three weeks plants were measured once and then discarded.

578 *Gas exchange measurements with concurrent PSI / PSII Yield and on-line carbon isotopic* 579 *discrimination (Δ)*

580 The experimental setup was previously described (Bellasio and Griffiths, 2013). Briefly, an
581 infra-red gas analyser (IRGA, a LI6400XT, Li-cor, USA), was fitted with a 6400-06 PAM2000
582 adapter, and with a Li-cor 6400-18 RGB light source. The IRGA was fed with CO_2 ($\delta^{13}C = -8.3$
583 ‰, Isi, A) and either a mixture of 2 % O_2 / N_2 or ambient air. PS I yield and PS II yield, $Y(II)$,
584 were measured with a Dual Pam-F (Heinz Walz GmbH, Effeltrich, D). The IRGA was connected
585 to a cryogenic H_2O and CO_2 trapping-purification line. To determine the relationship between
586 $Y(II)$ and J_{ATP} , a light response curve was measured at 2 % O_2 and $C_a = 600 \mu mol / mol$. Under
587 the same light quality, 21 % O_2 and reference CO_2 set at 400 $\mu mol / mol$, a second light response
588 curve was measured, during which $Y(II)$ was determined and exhaust gas was trapped to
589 determine Δ . In one day a total of 12 CO_2 samples and 6 CO_2 references from each individual
590 plant were analysed directly with a VG SIRA dual inlet isotope ratio mass spectrometer
591 (modified and maintained by Pro-Vac Services Ltd, Crewe, UK). Δ was calculated as reported in
592 Supp. Fig. S 3, using Eqn. 22. R_{LIGHT} was calculated as the y-intercept of the linear regression of

593 A against PAR $\cdot Y(II) / 3$. J_{ATP} was calculated from chlorophyll fluorescence data, by calibrating
594 the relationship between ETR and $Y(II)$ under low O_2 , and then using such a calibration to
595 determine the small fraction of ATP consumed by photorespiration. The correction was minimal
596 because of the low O_2 sensitivity of maize [Suppl. Table S 2 (Bellasio and Griffiths, 2013)],
597 therefore the potential errors caused by using fluorescence measurements under contrasting light
598 qualities were negligible. Light responses were treated with dedicated software (Photosyn
599 assistant 1.2, Dundee Scientific, Dundee, UK), to calculate the light compensation point and by
600 repeated measures anova (Genstat), point estimates were subject to anova and Tukey multiple
601 comparison (Genstat).

602 *Leakiness Φ from isotopic discrimination Δ*

603 Leakiness was resolved from isotopic discrimination by use of the full Farquhar model
604 (Farquhar, 1983; Farquhar and Cernusak, 2012), parameterized with a C_4 photosynthesis model
605 (von Caemmerer, 2000), using equations and a fitting approach that were previously described
606 [Suppl. Table S 2 (Bellasio and Griffiths, 2013)]. Briefly, leakiness, Φ was resolved from Δ by
607 calculating the weighted individual fractionations of the discriminating processes operating in C_4
608 photosynthesis. The CO_2 concentration in the cellular compartments was calculated by means of
609 a C_4 model, parameterized with the light response data (A , C_i , C_a , J_{ATP}) and respiration in the
610 light (R_{LIGHT}). The C_4 model was rearranged to express a modelled J_{MOD} and fit to the total ATP
611 production rate J_{ATP} (Suppl. Table S 2), to yield a value for BS conductance for each individual
612 plant, independently from Δ .

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Tables.

Table 1. Abbreviations, definitions and units for variables and acronyms described in the text.

A	Net assimilation	$\mu\text{mol m}^{-2} \text{s}^{-1}$
AB	Absorbed light	
AB BS / M	Partitioning of absorbed light	dimensionless
ALA	Alanine	
ASP	Aspartate	
ATP	Adenosine 5' triphosphate	
ATP_{BS}	ATP demand in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
ATP_M	ATP demand in M	$\mu\text{mol m}^{-2} \text{s}^{-1}$
B	Blue	
BS	Bundle sheath	
C_{BS}	CO ₂ concentration in BS	$\mu\text{mol mol}^{-1}$
CCM	Carbon concentrating mechanism	
CEF	Cyclic electron flow	
DHAP	Dihydroxyacetone phosphate	
ETR	Electron transport rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
G	Green	
GA	Gross assimilation ($A + R_{LIGHT}$)	$\mu\text{mol m}^{-2} \text{s}^{-1}$
g_{BS}	Bundle sheath conductance to CO ₂ , calculated by fitting J_{MOD} to J_{ATP}	$\text{mol m}^{-2} \text{s}^{-1}$
GLA	Glycolic acid	
IRGA	Infra-red gas analyzer	
IVD	Inter vein distance	μm
J_{ATP}	Total ATP production rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
J_{ATPBS}	ATP production rate in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
J_{ATPM}	ATP production rate in M	$\mu\text{mol m}^{-2} \text{s}^{-1}$
J_{MOD}	Modelled ATP production rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
LEF	Linear electron flow	
LCP	Light compensation point	
M	Mesophyll	
MAL	Malic acid	
MDH	Malate dehydrogenase	
MDH_{BS}	Malate dehydrogenase reaction rate in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
MDH_M	Malate dehydrogenase reaction rate in M	$\mu\text{mol m}^{-2} \text{s}^{-1}$
ME	Malic enzyme	
ME	Malic enzyme reaction rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
NADPH	Nicotinamide adenine dinucleotide phosphate	
$NADPH_{BS}$	NADPH demand in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
$NADPH_{TOT}$	Total NADPH demand	$\mu\text{mol m}^{-2} \text{s}^{-1}$
OAA	Oxaloacetic acid	
PAR	Photosynthetically active radiation	$\mu\text{E m}^{-2} \text{s}^{-1}$
PEP	Phosphoenolpyruvate	
PEPCK	Phosphoenolpyruvate carboxykinase	
$PEPCK$	PEPCK reaction rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
PGA	3-phosphoglyceric acid	
PGLA	2-phosphoglycolic acid	
PPDK	Pyruvate phosphate dikinase	
$PPDK$	PPDK reaction rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
PR	PGA reduction	
PR_{BS}	PGA reduction rate in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
PR_M	PGA reduction rate in M	$\mu\text{mol m}^{-2} \text{s}^{-1}$
PSI	Photosystem I	
PSII	Photosystem II	
PYR	Pyruvic acid	
R	Red	
R_{BS}	Respiration in the light in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
R_{LIGHT}	Respiration in the light	$\mu\text{mol m}^{-2} \text{s}^{-1}$
R_M	Respiration in the light in M	$\mu\text{mol m}^{-2} \text{s}^{-1}$
RPP	Reductive pentose phosphate	
Rubisco	Ribulose biphosphate carboxylase oxygenase	
RuBP	Ribulose-1,5-bisphosphate	
RuP	Ribulose-5-phosphate	
SS	Starch synthesis	
SS_{BS}	Starch synthesis rate in BS	$\mu\text{mol m}^{-2} \text{s}^{-1}$
SS_M	Starch synthesis rate in M	$\mu\text{mol m}^{-2} \text{s}^{-1}$
SS_{TOT}	Total starch synthesis rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
T	Transamination	
T	Transamination rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
V_C	Rubisco carboxylation rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
V_O	Rubisco oxygenation rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
V_P	PEP carboxylation rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
Y(II)	Yield of photosystem II	
Δ	¹³ C isotopic discrimination	‰
$\delta^{13}\text{C}$	¹³ C isotopic composition relative to Pee dee belemnite	‰
ϕ	Leakiness	dimensionless

Table 2. Steady state equations for the metabolic model of C_4 assimilation. Processes described by Eqn. 4 to 10 can be calculated directly from the measured data A , R_{LIGHT} and the output of the von Caemmerer C_4 model (V_O , V_P and V_C), while Eqn. from 11 to 21 require prior allocation of starch synthesis (SS), PGA reduction (PR) and PEPCK. For simplicity, enzyme names in italics represent the enzyme reaction rate. For stoichiometric consistency, reaction rates are calculated as rates of substrate transformation.

Process	Symbol	Reaction rate	Eqn.	Localization	Notes
Gross assimilation	GA	$A + R_{LIGHT}$	(4)		GA and R_{LIGHT} rates are expressed per CO_2
RuP phosphorylation	-	$V_C + V_O$	(5)	BS	RuP phosphorylation supplies Rubisco carboxylating activity (V_C), together with oxygenating activity (V_O).
PGA reduction tot	PR_{TOT}	$2V_C + \frac{3}{2}V_O - \frac{R_{LIGHT}}{3}$	(6)	BS and M	This equation calculates the total rate of PGA reduction on the basis of the PGA produced by Rubisco carboxylation ($2V_C$), Rubisco oxygenation (V_O) and glyoxylate recycling ($0.5V_O$) and considers the PGA consumed by respiration. 1/3 is the stoichiometric conversion between respiration (expressed per CO_2) and PR (expressed per triose).
NADPH demand tot	$NADPH_{TOT}$	$PR_{TOT} + \frac{1}{2}V_O$	(7)	BS and M	PGA reduction consumes 1 NADPH per PGA. The total rate of PGA reduction is PR_{TOT} (see note to Eqn 6). In glyoxylate regeneration (per glyoxylate) 0.5 NADH is produced by glycine decarboxylase, 0.5 NADH is consumed by hydroxypyruvate reductase and one ferredoxin (equivalent to 0.5 NADPH) is consumed by glutamine synthetase. In total 0.5 NADPH is consumed per glyoxylate formed (equivalent to V_O rate, see Table S 2) (Yoshimura et al., 2004).
DHAP entering RPP	-	$PR_{TOT} - SS_{TOT}$	(8)	BS	The DHAP entering the RPP pathway corresponds to the total PGA reduction rate minus the DHAP used for starch synthesis, which in this work is expressed per triose.
Starch synthesis tot	SS_{TOT}	$\frac{A}{3}$	(9)	BS and M	In this model all net assimilation is converted to starch. This assumption does not influence energetics as starch synthesis has the same ATP demand as phloem-loaded sucrose. In Eqn 9, 1/3 converts the stoichiometry of A (expressed per CO_2) to the stoichiometry of SS (expressed per triose).
PEP regeneration tot	-	V_P	(10)	BS and M	PEP regeneration rate equals PEP consumption rate V_P at steady state. PEP can be regenerated either by PPK (mainly in M, but active also in BS) or by PEPCK in BS. In this study PPK activity was assumed to be zero in BS
ATP demand tot	$ATP_{BS} + ATP_M$	$PR_{TOT} + V_C + 2V_O + \frac{1}{2}SS_{TOT} + PEPCK + 2PPDK$	(11)	BS and M	Eqn 11 calculates the total ATP demand as the sum of ATP demand for PGA reduction (1 ATP per PGA corresponding to PR), RuBP regeneration (1 ATP per RuBP corresponding to $V_C + V_O$), glyoxylate recycling (1 ATP per glyoxylate, corresponding to V_O), starch synthesis (0.5 ATP per triose, corresponding to SS) and PEP regeneration (1 ATP per PEPCK catalytic event or 2 ATP per PPK catalytic event). Compared to the original formulation of the C_4 model, Eqn 11 separates the ATP demand for PEPCK and PPK, it includes the ATP demand for SS, and considers the PGA utilised by respiration which does not need to be reduced (see Eqn 6).
ATP demand in BS	ATP_{BS}	$PR_{BS} + V_C + 2V_O + \frac{1}{2}SS_{BS} + PEPCK$	(12)	BS	The ATP demand in BS is brought about by PGA reduction (at the rate of PR_{BS}), RuBP regeneration (at the rate of $V_C + V_O$), glyoxylate recycling (at the rate of V_O), starch synthesis (0.5 ATP per triose) and PEPCK activity (1 ATP per OAA) (see note to Eqn 11).
ATP demand in M	ATP_M	$2PPDK + \frac{1}{2}SS_M + PR_M$	(13)	M	The ATP demand in M is brought about by PGA reduction (at the rate of PR_M), starch synthesis and PPK (2 ATP per PYR) (see note to Eqn 11).
NADPH demand in BS	$NADPH_{BS}$	$PR_{BS} + \frac{1}{2}V_O$	(14)	BS	The NADPH demand in BS is brought about by PGA reduction (1 NADPH per PGA) and glyoxylate recycling, which consumes 0.5 NADPH per glyoxylate (corresponding to V_O , see also Suppl. Table S 1).
NADPH supply to BS	-	MDH_M	(15)	BS	All NADPH available in BS is produced in M and exported through the malate shuttle because we have assumed that no LET (i.e. water oxidation) occurred in BS. For this reason, the NADPH supply to BS corresponds to the NADPH consumed to reduce OAA to MAL in M, the process responsible for NADPH export, and not to the rate of MAL decarboxylation in BS, which depends on T , PEPCK and MDH_{BS} (Eqn. 19).
MDH activity in M	MDH_M	$PR_{BS} + \frac{1}{2}V_O$	(16)	M	MDH activity supplies the NADPH demand in BS. Eqn 16 was derived from Eqn 14 and 15.
Transamination	T	$V_P - MDH_M$	(17)	BS and M	Eqn 17 expresses that at steady state all OAA is either transaminated or reduced. Since T bypasses the MDH_M reaction, which is the reaction responsible for NADPH export to BS (see note to Eqn 15), T has the function of balancing NADPH supply and demand, this becomes apparent when Eqn 15 and 17 are combined.
Malate dehydrogenase	MDH_{BS}	$T - PEPCK$	(18)	BS	MDH is assumed to operate a fast conversion at equilibrium therefore it is passively regulated by the substrate availability: the OAA that is not used by PEPCK is reduced to MAL by MDH. MDH may use NADH, since no NADPH dependent reduction of OAA has been observed in maize (Kanai and Edwards, 1999) and it is likely mitochondrial (Rathnam, 1978; Chapman and Hatch, 1981). The NADH regeneration may be carried out by chloroplastic ME which is reported to react both with NADP and NAD (Chapman and Hatch, 1981). However the process may be more complicated [(Eprintsev et al., 2011) and references therein]. Note that in this study we assumed that cells are compartmentalized while PEPCK rate was manipulated to increase between 0 and a maximum rate in response to ATP availability (see minimum and maximum BS allocation for details).
Malic enzyme	ME	$MDH_M + MDH_{BS}$	(19)	BS	Eqn 19 expresses that the rate of MAL oxidation by ME corresponds to the rate of MAL produced by MDH activity in M plus the rate of MAL produced by MDH activity in BS.
Pyruvate phosphate dikinase	$PPDK$	$V_P - PEPCK$	(20)	M	The PEP regenerated by PEPCK in BS diffuses to M and reduces the requirement of PEP regenerated by PPK in M.
PGA reduction M	PR_M	$PR_{TOT} - PR_{BS}$	(21)	M	PGA reduction is a shared process between BS and M.

Table 3. Energy partitioning between BS and M at different wavelengths. Measured leaf reflectance and transmittance (Woolley, 1971) was used to parameterize the optical model (Fig. 2) to calculate the likely profiles of light penetration at different wavelengths. Absorbed light partitioning (AB BS / M) was calculated integrating such light absorption profiles (Fig. 2, right panel) over the corresponding BS and M areas (Fig. 2 left) and used to calculate ATP production partitioning J_{ATPBS} / J_{ATPM} .

Wavelength	Description	Refl. %	Transm. %	$AB \frac{BS}{M}$	$\frac{J_{ATPBS}}{J_{ATPM}}$
400 nm	Lowest $AB \frac{BS}{M}$	4	0.1	0.15	0.29
460 nm	Blue LED used	5	1	0.16	0.31
635 nm	Red LED used	6	7	0.34	0.68
400 – 700 nm	Natural white light	8	9	0.38	0.76
522 nm	Green LED used	8	11	0.40	0.80
540 nm	Highest $AB \frac{BS}{M}$	13	23	0.48	0.96

Table 4. Physiological responses of maize plants to different light qualities. The light compensation point (LCP) was determined by fitting light curves with dedicated software; Respiration in the light (R_{LIGHT}) was determined by linear regression of A against $PAR \cdot Y(II) / 3$; bundle sheath conductance (g_{BS}) was determined by fitting a modelled J_{ATP} to the measured J_{ATP} (Fig. 3). Differences were not significant for $P < 0.05$. Mean values \pm SE. $n = 4$

	Unit	Mean	RGB	R	G	B
LCP	$\mu E m^{-2} s^{-1}$	29.04	28.05 (± 1.8)	21.24 (± 3.1)	33.10 (± 4.1)	33.75 (± 5.7)
R_{LIGHT}	$\mu mol O_2 m^{-2} s^{-1}$	1.169	1.202 (± 0.090)	1.231 (± 0.11)	1.148 (± 0.11)	1.095 (± 0.12)
g_{BS}	$mol m^{-2} s^{-1}$	0.00104	0.00127 ($\pm 3.1 \cdot 10^{-4}$)	0.00117 ($\pm 3.1 \cdot 10^{-4}$)	0.00075 ($\pm 3.1 \cdot 10^{-4}$)	0.00247 ($\pm 3.1 \cdot 10^{-4}$)

Figures

Fig. 1. Metabolic model of C_4 assimilation, rates of reaction, and net fluxes between BS and M. The overall scheme reports the reactions of the CCM (Furbank, 2011), Rubisco carboxylation, the reactions of the RPP pathway, the synthesis of starch, respiration and glyoxylate recycling reactions. The tables, with the corresponding enzyme name, show the actual reaction rates, expressed as relative to GA ($5.13 \mu\text{mol}/\text{m}^2 \text{s}^{-1}$), per unit of substrate transformed. Rates were estimated by parameterizing the model equations (Table 2) with data measured under $\text{PAR} = 125 \mu\text{E m}^{-2} \text{s}^{-1}$ ($A = 3.96 \mu\text{mol m}^{-2} \text{s}^{-1}$; $R_{\text{LIGHT}} = 1.17 \mu\text{mol m}^{-2} \text{s}^{-1}$; $J_{\text{ATP}} = 28.6 \mu\text{mol m}^{-2} \text{s}^{-1}$), the output of the C_4 model ($V_C = 5.35 \mu\text{mol m}^{-2} \text{s}^{-1}$; $V_P = 5.89 \mu\text{mol m}^{-2} \text{s}^{-1}$; $V_O = 0.44 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the output of the isotopic discrimination model ($\Phi = 0.23$), under three characteristic ratios of ATP partitionings. These were numbered 1, 2 and 3. Condition 1 corresponds to the lowest ATP available in BS (ATP partitioning similar to that under blue light, Fig. 4 B), condition 2 correspond to an intermediate ATP availability in BS (ATP partitioning equal to that under red light, Fig. 4 B), condition 3 corresponds to the highest ATP available in BS (ATP partitioning equal to that under green light, Fig. 4 B). The inset shows net metabolite fluxes between M and BS in multiples of GA . The ATP demand in BS (ATP_{BS}) and M (ATP_{M}), the total NADPH demand ($NADPH_{\text{TOT}}$) and the NADPH demand in BS ($NADPH_{\text{BS}}$) were also calculated in the same three relevant conditions.

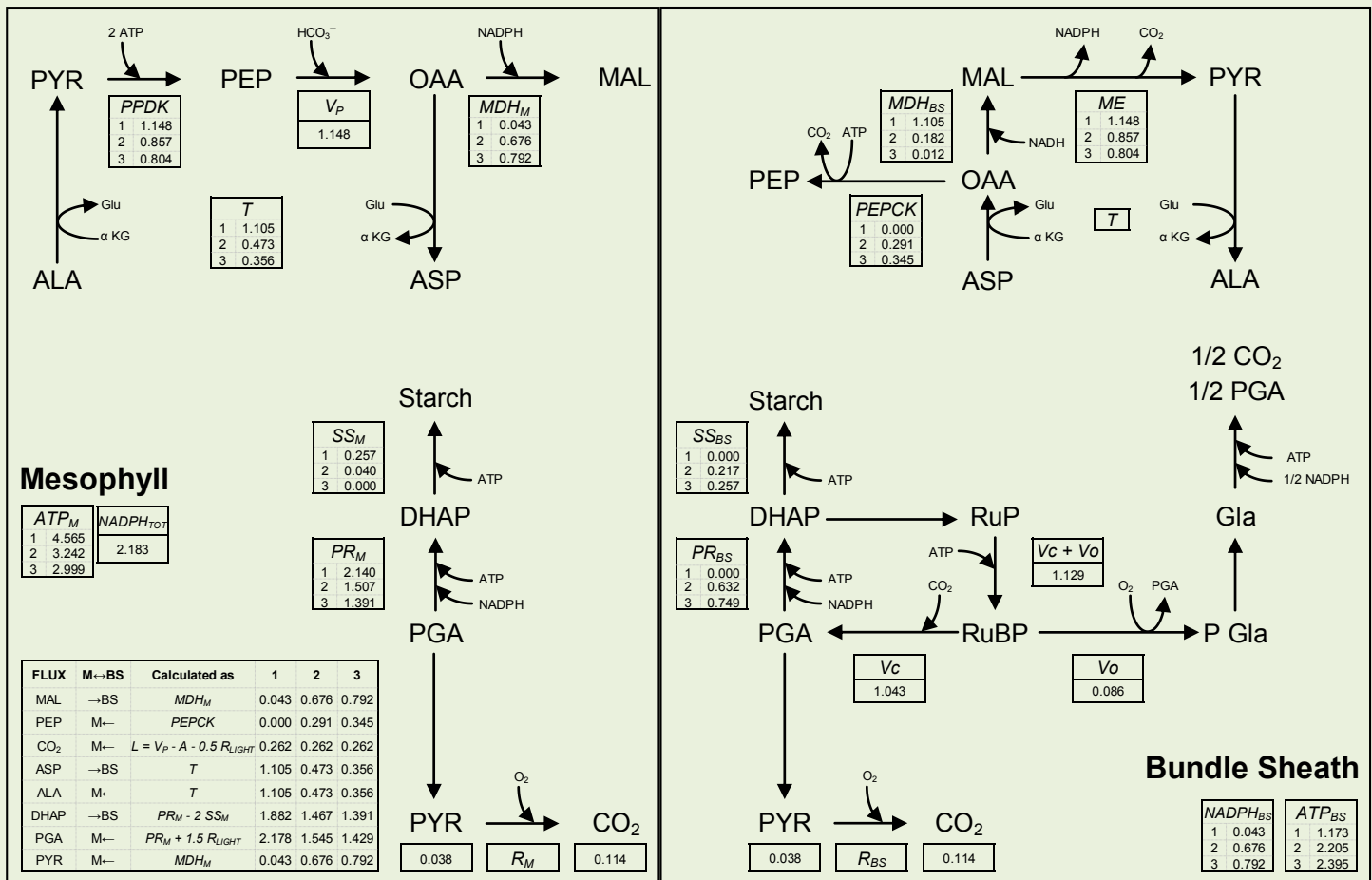


Fig. 2. Light penetration in a maize leaf. Left panel shows the modelled maize anatomy. A square BS is surrounded by three portions of mesophyll: interveinal mesophyll (MI), adaxial mesophyll (MAD) and abaxial mesophyll (MAB). Epidermis was approximated as a flat reflecting surface. Light penetration was studied through profiles P1 and P2. Right panel shows P1-light profiles (bold lines) and P2-light profiles (thin lines) calculated with the Kubelka-Munk (absorption + scattering) theory and calibrated with spectroscopic data (Table 3). Radiation is expressed as the sum of downward + upward photon flux, as a fraction of incident photon flux (dimensionless), and plotted against the depth in the absorbing path of the leaf.

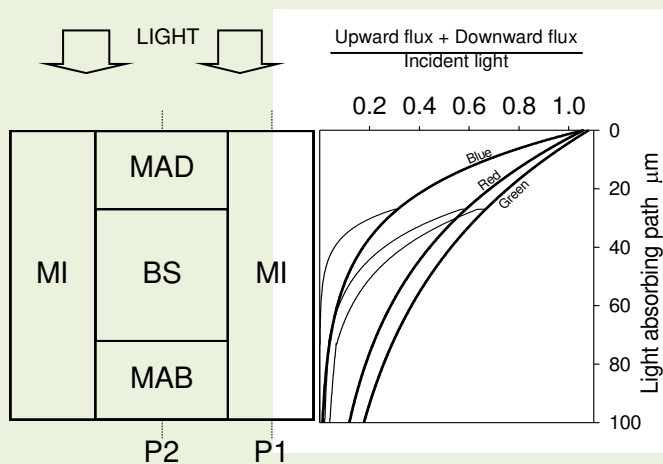


Fig. 3. Maize responses to decreasing light intensity under different light qualities. **(A)** Net assimilation (A). The curves were fitted in order to calculate the light compensation point (Table 4). The inset shows a magnification. **(B)** Total ATP production rate (J_{ATP}), measured with the low O_2 - ETR method. **(C)** On-line isotopic discrimination during photosynthesis (Δ). **(D)** Leakiness (Φ) resolved from Δ . Error bars represent standard error. $n=4$.

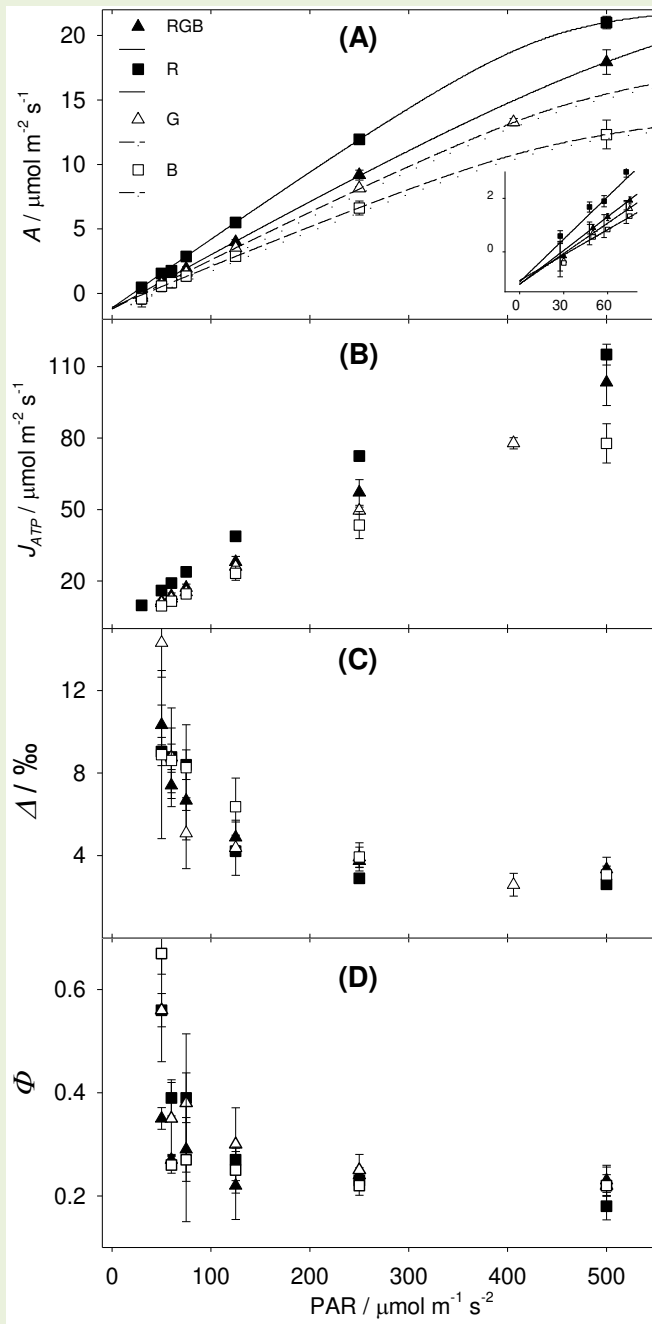
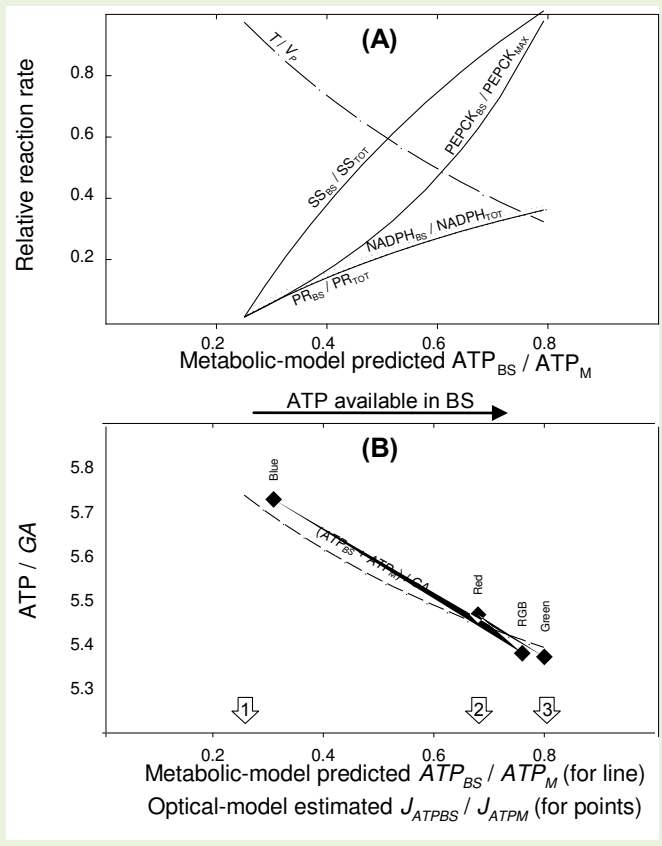


Fig. 4. Partitioning of metabolic activities in BS cells and associated shifts in ATP and NADPH demand. Panel (A), the output of the metabolic model, shows as a function of increasing theoretical ATP demand partitioning (ATP_{BS} / ATP_M), i) the increasing contribution of BS (solid lines) to the total PGA reduction (PR, relative to the total), starch synthesis (SS, relative to the total) and PEPCK (relative to the highest rate); ii) the predicted NADPH demand in BS, relative to the total ($NADPH_{BS} / NADPH_{TOT}$, dotted line), and iii) the predicted transamination rate, relative to V_P (T / V_P , dashed line). In panel (B) the output of the metabolic model is compared with the empirical data. Model output is shown by a dashed line: the predicted ATP demand for gross assimilation $(ATP_{BS} + ATP_M) / GA$ is plotted as a function of predicted ATP demand partitioning ATP_{BS} / ATP_M . Empirical data are shown as diamonds: the measured J_{ATP} / GA , under blue, red, RGB and green light (Table 1), is plotted against the estimated ATP production partitioning $J_{ATP_{BS}} / J_{ATP_M}$ at 460 nm, 635 nm, white light and 522 nm (estimated through the optical model, Table 3). The lowest ATP_{BS} / ATP_M was named condition 1 (left arrow), the partitioning corresponding to red light was named condition 2 (central arrow) while the highest ATP_{BS} / ATP_M was named condition 3 (right arrow).



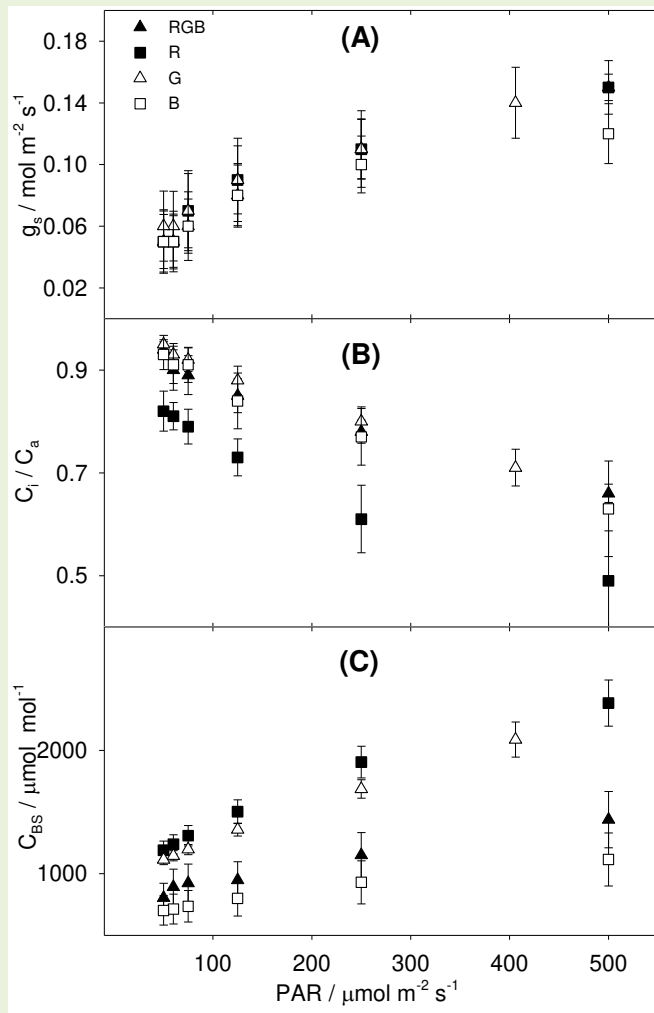
Supplementary Table S 1. ATP and NADPH demand for key C₄ processes.

Process	Localization	Per	ATP	NADPH
PGA reduction to DHAP	M+BS	PGA	1	1
RuP phosphorylation	BS	pentose	1	
Glyoxylate regeneration	BS	Glyoxylate	1	0.5
Starch synthesis	M+BS	triose	0.5	
PEPCK		OAA	1	
PPDK	M	PYR	2	

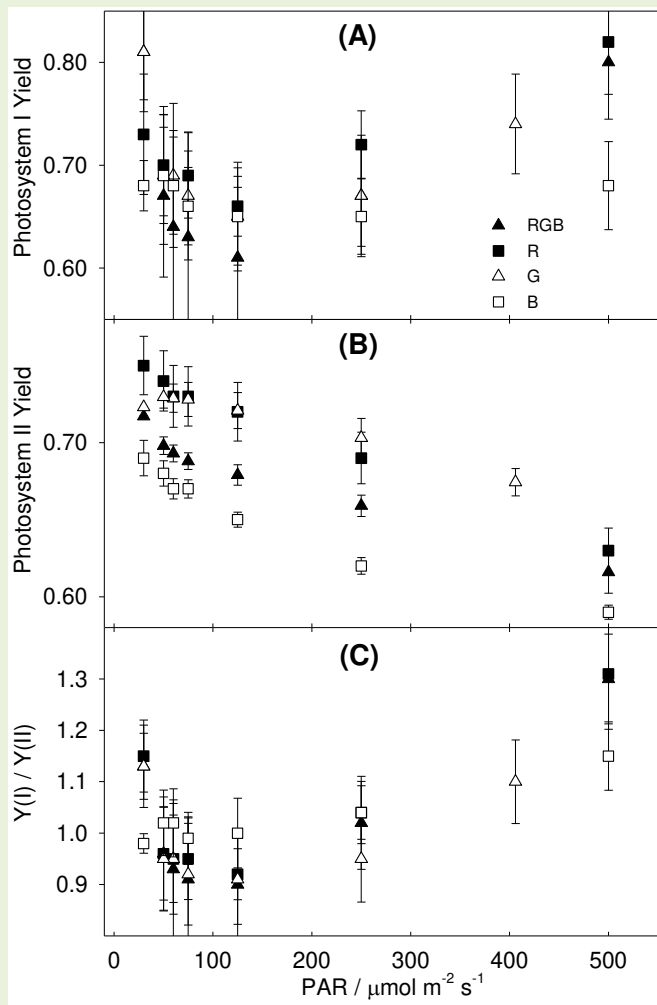
Supplementary Table S 2. Definitions, equations, and variables used in the models.

Symbol	Definition	Values / Units / References
a	^{13}C fractionation due to diffusion of CO_2 in air. Because of vigorous ventilation we neglected the fractionation of the boundary layer (Kromdijk <i>et al.</i> , 2010).	4.4 ‰ (Craig, 1953)
a_d	^{13}C fractionation due to diffusion of CO_2 in water	0.7 ‰ (O'Leary, 1984)
b_3	^{13}C fractionation during carboxylation by Rubisco including respiration and photorespiration fractionation $b_3 = b'_3 - \frac{e' R_{\text{LIGHT}} + f \cdot F}{V_c}$ (Farquhar, 1983). Where F is the photorespiration rate and was calculated as $F=0.5 \cdot V_o$ (von Caemmerer, 2013), Nerea Ubierna, personal communication]	‰
b_3'	^{13}C fractionation during carboxylation by Rubisco	30 ‰ (Roeske and O'Leary, 1984)
b_4	Net fractionation by CO_2 dissolution, hydration and PEPC carboxylation including respiratory fractionation $b_4 = b'_4 - \frac{e' R_M}{V_P}$ (Farquhar, 1983; Henderson <i>et al.</i> , 1992).	‰
b_4'	Net fractionation by CO_2 dissolution, hydration and PEPC carboxylation.	-5.7 ‰ at 25 °C but variable with temperature (Farquhar, 1983; Henderson <i>et al.</i> , 1992; Kromdijk <i>et al.</i> , 2010).
C_{BS}	CO_2 concentration in the bundle sheath $C_{BS} = \frac{x J_{ATP} R_{\text{LIGHT}} - A}{2 g_{BS}} + C_M$	$\mu\text{mol mol}^{-1}$
C_M	CO_2 concentration in the mesophyll $C_M = C_i - \frac{A}{g_M}$	$\mu\text{mol mol}^{-1}$
C_i	CO_2 concentration in the intercellular spaces as calculated by the IRGA (Li-cor manual Eqn 1-18).	$\mu\text{mol mol}^{-1}$
e	^{13}C fractionation during decarboxylation	0 ‰ to -10 ‰ (Gillon and Griffiths, 1997; Ghashghaie <i>et al.</i> , 2001; Igamberdiev <i>et al.</i> , 2004; Hymus <i>et al.</i> , 2005; Barbour <i>et al.</i> , 2007; Sun <i>et al.</i> , 2012), -6 ‰ in this study (Kromdijk <i>et al.</i> , 2010).
e'	^{13}C fractionation during decarboxylation, including the correction for measurement artefacts: $e' = e + \delta^{13}\text{C}_{\text{measurements}} - \delta^{13}\text{C}_{\text{growth chamber}}$ In this study $\delta^{13}\text{C}_{\text{measurements}} = -6.38$ ‰; $\delta^{13}\text{C}_{\text{growth chamber}} = -8$ ‰ (Wingate <i>et al.</i> , 2007)	‰
e_s	^{13}C fractionation during internal CO_2 dissolution	1.1 ‰ (Vogel <i>et al.</i> , 1970; Mook <i>et al.</i> , 1974; Vogel, 1980).
f	^{13}C fractionation during photorespiration.	-11.6 ‰ (Lanigan <i>et al.</i> , 2008).
g_{BS}	Bundle sheath conductance to CO_2 , calculated by curve fitting	$\text{mol m}^{-2} \text{s}^{-1}$
g_M	Mesophyll conductance to CO_2	$1 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$ (Kromdijk <i>et al.</i> , 2010)
g_s	Stomata conductance to CO_2	$\text{mol m}^{-2} \text{s}^{-1}$
J_{ATP}	ATP production rate $J_{ATP} = \frac{3 G A_{\text{Low } O_2} Y(II)}{0.59 Y(II)_{\text{Low } O_2}}$	$\mu\text{mol m}^{-2} \text{s}^{-1}$ (Bellasio and Griffiths, 2013)
J_{MOD}	Modelled ATP production rate $J_{MOD} = \frac{-y + \sqrt{y^2 - 4wz}}{2w}$ $w = \frac{x-x^2}{6A}$; $y = \frac{1-x}{3} \left[\frac{g_{BS}}{A} + \left(C_M - \frac{R_M}{g_{BS}} - \gamma^* O_M \right) - 1 - \frac{\alpha \gamma^*}{0.047} \right] - \frac{x}{z} \left(1 + \frac{R_{\text{LIGHT}}}{A} \right)$; $z = \left(1 + \frac{R_{\text{LIGHT}}}{A} \right) \left(R_M - g_{BS} C_M - \frac{7 g_{BS} \gamma^* O_M}{3} \right) + (R_{\text{LIGHT}} + A) \left(1 - \frac{7 \alpha \gamma^*}{3 \cdot 0.047} \right)$	$\mu\text{E m}^{-2} \text{s}^{-1} \mu\text{m}$ (von Caemmerer, 2000; Bellasio and Griffiths, 2013; Ubierna <i>et al.</i> , 2013)
O_M	O_2 mol fraction in the mesophyll cells (in air at equilibrium)	210000 $\mu\text{mol mol}^{-1}$
O_{BS}	O_2 mol fraction in the bundle sheath cells (in air at equilibrium) $O_{BS} = O_M + \frac{\alpha A}{0.047 g_{BS}}$ (von Caemmerer, 2000)	$\mu\text{mol mol}^{-1}$
R_M	Mesophyll non photorespiratory CO_2 production in the light $R_M = 0.5 R_{\text{LIGHT}}$ (von Caemmerer, 2000; Kromdijk <i>et al.</i> , 2010; Ubierna <i>et al.</i> , 2011)	$\mu\text{mol m}^{-2} \text{s}^{-1}$
s	Fractionation during leakage of CO_2 out of the bundle sheath cells	1.8 ‰ (Henderson <i>et al.</i> , 1992).
t	Ternary effects $t = \frac{(1+\alpha)E}{2000 g_{ac}}$ where $E / \text{mmol m}^{-2} \text{s}^{-1}$ is the transpiration rate (calculated by the IRGA software, parameter Trmmol), $g_{ac} / \text{mol m}^{-2} \text{s}^{-1}$ is the conductance to diffusion of CO_2 in air (calculated by the IRGA software, parameter CndCO2), α is the isotopic fractionation during diffusion in air.	‰ (Farquhar and Cernusak, 2012)
V_c	Rubisco carboxylation rate $V_c = \frac{(A+R_{\text{LIGHT}})}{1 - \frac{\gamma^* O_{BS}}{C_{BS}}}$ (Ubierna <i>et al.</i> , 2011)	$\mu\text{mol m}^{-2} \text{s}^{-1}$
V_o	Rubisco oxygenation rate $V_o = \frac{V_c - A - R_{\text{LIGHT}}}{0.5}$ (Ubierna <i>et al.</i> , 2011)	$\mu\text{mol m}^{-2} \text{s}^{-1}$
V_P	PEP Carboxylation rate $V_P = \frac{x J_{ATP}}{2}$	
ϕ	Leakiness estimated with the isotope method including respiratory and photorespiratory fractionation, ternary effects and estimating CBS with the C_4 model $\phi = \frac{C_{BS} - C_M}{C_M} \frac{b_4 C_M (1+t) + \alpha (C_a - C_i) - C_a \Delta O_{BS} (1-t)}{(1+t)[C_a \Delta O_{BS} (1-t) - \alpha (C_a - C_i)] - b_3 C_{BS} + s(C_{BS} - C_M)}$	dimensionless (Farquhar and Cernusak, 2012)
x	Partitioning factor of J_{ATP} between C_4 activity V_P and C_3 activity (reductive pentose phosphate pathway, RuBP regeneration and photorespiratory cycle)	0.4 (von Caemmerer, 2000; Kromdijk <i>et al.</i> , 2010; Ubierna <i>et al.</i> , 2011; Ubierna <i>et al.</i> , 2013)
α	Fraction of PSII active in BS cells	0.15 (Edwards and Baker, 1993; von Caemmerer, 2000; Kromdijk <i>et al.</i> , 2010).
γ^*	Half of the reciprocal of the Rubisco specificity	0.000193 (von Caemmerer, 2000).
$Y(II)$	Yield of photosystem II $Y(II) = \frac{F_m - F_s}{F_m}$ (Genty <i>et al.</i> , 1989)	dimensionless

Supplementary Fig. S 1. (A) Stomatal conductance and (B) C_i / C_a responses measured by gas exchange under decreasing light intensity, and under different light qualities. (C): response of C_{BS} to decreasing light intensity, under different light qualities, estimated by the C_4 model. Error bars represent standard error. $n=4$.



Supplementary Fig. S 2. Maize photochemical responses to decreasing light intensity under different light qualities. **(A)** Yield of photosystem I, determined with the low O_2 -Electron Transport Rate method (Bellasio and Griffiths, 2013) **(B)** Yield of photosystem II, $Y(II)$, determined by chlorophyll fluorescence. **(C)** $Y(I) / Y(II)$. Error bars represent standard error. $n=4$.



Supplementary Fig. S 3. values for the calculation of Δ .

Δ was calculated as (Evans et al., 1986):

$$\Delta_{OBS} = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \quad (22)$$

Where: $\xi = \frac{C_e}{C_e - C_o}$; δ_e is the isotopic composition of the reference gas. δ_o is the isotopic composition of the gas leaving the cuvette. C_e and C_o represent the CO₂ mole fraction respectively entering and leaving the cuvette. These were corrected for differences in water content according to (von Caemmerer and Farquhar, 1981).

