Reduction of bundle sheath size boosts cyclic electron flow in C$_4$ *Setaria viridis* acclimated to low light

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SUMMARY

When C$_4$ leaves are exposed to low light, the CO$_2$ concentration in the bundle sheath (BS) cells decreases, causing an increase in photorespiration relative to assimilation, and a consequent reduction in biochemical efficiency. These effects can be mitigated by complex acclimation syndromes, which are of primary importance for crop productivity but are not well studied. We unveil an acclimation strategy involving the coordination of electron transport processes. First, we characterize the anatomy, gas exchange and electron transport of C$_4$ *Setaria viridis* grown under low light. Through a purposely developed biochemical model, we resolve the photon fluxes and reaction rates to explain how the concerted acclimation strategies sustain photosynthetic efficiency. Our results show that a smaller BS in low-light-grown plants limited leakiness (the ratio of CO$_2$ leak rate out of the BS over the rate of supply via C$_4$ acid decarboxylation) but sacrificed light harvesting and ATP production. To counter ATP shortage and maintain high assimilation rates, plants facilitated light penetration through the mesophyll and upregulated cyclic electron flow in the BS. This shade tolerance mechanism, based on the optimization of light reactions, is possibly more efficient than the known mechanisms involving the rearrangement of carbon metabolism, and could potentially lead to innovative strategies for crop improvement.

Keywords: C$_4$ photosynthesis, Kranz anatomy, light harvesting, bundle sheath, light reactions, carbon reactions, NADP-ME, gas exchange, modelling.

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INTRODUCTION

Leaves of the majority of C$_4$ plants are organized in concentric cylinders: an external tube of mesophyll (M) cells surrounds a tube of bundle sheath (BS) cells, which wrap around the innermost vasculature (Figure 1). This spatial arrangement compels light and CO$_2$ entering C$_4$ leaves to pass through M cells before reaching the BS. CO$_2$ is hydrated to HCO$_3^-$ by carbonic anhydrase in the M cytosol and then fixed by phosphoenolpyruvate carboxylase (PEPC) into C$_4$ acids, which may be transaminated or chemically reduced. C$_4$ acids diffuse to BS cells where they are decarboxylated, primarily by NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), PEP carboxykinase (PEPCK), or by a combination of these. Decarboxylation provides higher CO$_2$ partial pressure at the site of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), thereby largely suppressing the competitive reaction with oxygen that leads to the wasteful process of photorespiration (Bellasio et al., 2014; von Caemmerer & Furbank, 2003). For this function, C$_4$ photosynthesis is also referred to as a biochemical carbon-concentrating mechanism (CCM). Pyruvate resulting from the decarboxylation of C$_4$ acids diffuses back to the M cells, where it is regenerated into PEP by pyruvate orthophosphate dikinase (PPDK), using two ATP molecules. Some of the CO$_2$ released in BS cells diffuses out to M cells (called leakage), which incurs an additional ATP cost of C$_4$ photosynthesis (Bellasio & Griffiths, 2014a; Farquhar, 1983; Henderson et al., 1992).

C$_4$ photosynthesis is highly sensitive to limiting light intensities. Under low irradiance, the generation of ATP and NADPH slows down, limiting both the reductive pentose phosphate (RPP) cycle and the C$_4$ cycle. Since activities of RPP and the C$_4$ cycle are tightly balanced (Kromdijk et al., 2014) through light modulation (Bailey et al., 2007),
a slowdown of C₄ acid diffusion into the BS inevitably results in a decrease of CO₂ concentration in BS cells (C₄BS). Consequently, the ratio between CO₂ and O₂ concentration at the active sites of Rubisco decreases, favouring oxygenation over carboxylation, thereby lowering the biochemical efficiency (ATP per gross assimilation of CO₂) of C₄ photosynthesis (Furbank et al., 1990; Ubierna et al., 2013). In crop canopies, where up to 50% of the net CO₂ uptake is fixed by shaded leaves, (Baker et al., 1988; Long, 1993) light limitation plays an important role in decreasing canopy productivity (Kromdijk et al., 2010), and understanding the acclimation strategies of C₄ metabolism to light limitation is critical for increasing crop production to meet rising demands for food and fodder (Bellasio & Griffiths, 2014a; Evans et al., 1991).

Shading also limits C₄ photosynthesis at the cell level. M and BS cells both need light to power photosynthesis. The amount of light that can be harvested by the BS cell is limited by the size of the cell (i.e. by the BS optical cross section; Bellasio & Lundgren, 2016) and by light absorption in the M (with the concentric organization, BS cells are in effect shaded, meaning that light harvesting in the M reduces the light available to reach BS cells; Kramer & Evans, 2011), and depends on light quality. Green light is not strongly absorbed by chlorophyll, meaning that it can easily reach the BS, whereas blue light is all taken up by the upper chloroplasts and preferentially excites M cells (Evans et al., 2007). M cells use light to produce ATP and NADPH through linear electron flow (LEF), engaging both photosystems (PSs), whereas BS cells of NADP-ME plants are thought to produce mainly ATP through cyclic electron flow (CEF) around photosystem I (PSI). In this way, the relative absorption of excitation energy will influence the availability of ATP and NADPH. Bellasio and Griffiths (2014c) proposed that these environmentally driven shifts in ATP and NADPH production can be countered by adjusting ATP and NADPH consumption by carbon metabolism, with a surprising degree of flexibility, but only up to a defined limit, ultimately determining how the biochemical work is apportioned between the two cell types. However, the possibility that plants could respond to shift in energy availability by rearranging light reactions, in such a way that also the production of ATP and NADPH is regulated, received little attention.

We have recently shown that the photosynthetic apparatus of *Setaria viridis*, a model C₄ plant of NADP-ME subtype, is rearranged in response to low light in a cellspecific fashion, preferentially increasing the electron transport capacity of BS cells (Ermakova, Bellasio, et al., 2021). We hypothesized that this was a response to a shortage of ATP in the BS, which could depend on increased consumption resulting from dark reactions or on a lower rate of production caused by a shortage of light. Here we resolve these drivers and their causal relationship. First, we measure the anatomical properties of leaves from *S. viridis* plants grown under high light (HL plants) and low light (LL plants), and estimate how these would influence the light harvesting of the M and the BS. Then, we conduct comprehensive gas-exchange characterization under ambient and low-O₂ conditions, with the concurrent determination of PSII and PSI effective quantum yields, to estimate biochemical parameters and conductance to CO₂ diffusion at the M/BS interface (gBS). Finally, we develop a model integrating two electron transport chains with the light-harvesting properties of M and BS to study how the apportioning of light and carbon metabolism shifts in LL plants, and how these changes affect biochemical efficiency. We demonstrate that the decreased light interception in BS cells is effectively counteracted by an increase of CEF, uncovering another important link between the biochemical properties and structural characteristics of C₄ leaves.

**RESULTS**

**Leaf anatomy and chlorophyll**

In HL plants, BS chloroplasts were uniformly distributed inside the cells (Figure 1). In LL plants, BS chloroplasts had a clear centrifugal position, whereas M chloroplasts were specifically arranged along the cell walls, particularly in adaxial M cells. This was captured by the higher apparent absorbance of M cells of HL plants (Table 1). The increased number of chloroplasts in adaxial BS cells compared with

![Light microscopy images of the leaf cross sections used for anatomical measurements from *Setaria viridis* grown under high light (1000 μmol m⁻² sec⁻¹, HL) or low light (300 μmol m⁻² sec⁻¹, LL).](image-url)
Chlorophyll ($\mu$mol mol$^{-1}$) was decreased by 35% in LL plants: the BS relative to that absorbed in M was higher in HL plants compared with LL plants. The decrease in photosynthetic efficiency in LL plants, independently of O$_2$ level (Figures 2 and 3). Both HL and LL plants showed decreased Y(II) and increased Y(ND) at low C$_{\text{m}}$, probably representing an induction of non-photochemical quenching in the PSII antennae in response to the acidification of the thylakoid lumen. The acidification of the thylakoid lumen is induced by the downregulation of ATP synthase activity in response to the reduced consumption of ATP by the RPP cycle (Kanazawa & Kramer, 2002). Interestingly, Y(NA) at low C$_{\text{m}}$ was higher when measured at 2% O$_2$ (Figures 2h and 3h), pointing to a contribution of O$_2$ in oxidizing PSI via photorespiration and/or O$_2$ photorespiration (Sagun et al., 2021).

To aid the interpretation of light and CO$_2$ response curves, we fitted empirical and mechanistic models after Bellasio et al. (2016a). Fitted parameters in Table S1 show that, under ambient O$_2$, the main differences were dependent on a decrease of respiration under light ($R_{\text{LIGHT}}$), light compensation point (LCP), CO$_2$ compensation point ($\Gamma$) and BS conductance to CO$_2$ diffusion ($g_{\text{BS}}$) in LL plants. Curiously, the O$_2$ sensitivity of $R_{\text{LIGHT}}$, quantum yield, Y(CO$_2$)$_{\text{LL}}$, and carboxylation efficiency was opposite between HL and LL plants. The decrease in photosynthetic efficiency in LL plants under low concentrations of O$_2$ supports a more important role of O$_2$ in the photoprotection of LL plants (Sagun et al., 2021). A link between electron transport and $R_{\text{LIGHT}}$ is known to exist (Buckley & Adams, 2011), but is not characterized in C$_4$ plants. Of the fitted quantities, only $R_{\text{LIGHT}}$ and $g_{\text{BS}}$ were retained for model parameterization.

### Table 1 Anatomical and biochemical characteristics of leaves, mesophyll (M) and bundle sheath (BS) cells of *Setaria viridis* grown at high light (1000 μmol m$^{-2}$ sec$^{-1}$, HL) or low light (300 μmol m$^{-2}$ sec$^{-1}$, LL)

| Characteristic | HL | LL |
|---------------|----|----|
| Height at vein (μm) | 238 ± 12.2 | 205 ± 8.5* |
| Interveninal distance (μm) | 181.5 ± 4.8 | 165 ± 4.8* |
| Abaxial mesophyll height (μm) | 60.2 ± 3.3 | 46.0 ± 2.5* |
| Adaxial mesophyll height (μm) | 68.8 ± 3.2 | 69.7 ± 3.7 |
| Bundle sheath area (μm$^2$) | 9604 ± 1101 | 6719 ± 763* |
| Vein width (μm) | 98.1 ± 6.5 | 69.1 ± 5.11 |
| Chlorophyll (a + b) in the M (μmol m$^{-2}$) | 0.27 ± 0.01 | 0.38 ± 0.01* |
| Chlorophyll (a + b) in the BS (μmol m$^{-2}$) | 0.13 ± 0.01 | 0.25 ± 0.04* |
| Mesophyll surface area exposed to intercellular airspace, $S_M$ (m$^2$ m$^{-2}$) | 14.13 ± 0.4 | 9.12 ± 0.5* |
| Bundle sheath cells surface area per unit of leaf area, $S_{\text{BS}}$ (m$^2$ m$^{-2}$) | 2.21 ± 0.06 | 1.92 ± 0.04* |
| Leaf absorbance | 0.789 ± 0.02 | 0.855 ± 0.04* |
| Mesophyll apparent absorbance (from micrograph luminance) | 0.168 ± 0.01 | 0.117 ± 0.01* |
| Vascular bundle apparent absorbance (from micrograph luminance) | 0.288 ± 0.01 | 0.344 ± 0.02* |

Mean ± SE values are shown, $n = 20$ (except for: chlorophyll and leaf absorbance, $n = 3$; apparent absorbance, $n = 6$). Asterisks indicate statistically significant difference between two light regimes ($P < 0.05$).

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### Modelling of light distribution, light reactions and dark metabolism

Using measured anatomical characteristics, the BS/M chlorophyll ratio and leaf absorbance (Table 1), we modelled light penetration through the leaf profile according to Bellasio and Lundgren (2016). The absorbed light (A$\text{BS}$) in the BS relative to that absorbed in M was higher in HL plants: A$\text{BS}$(BS/M) was 0.414 for HL and 0.343 for LL (Table 1). Next, to gain a comprehensive understanding of the photosynthetic metabolism, we used A$\text{BS}$(BS/M), Y(III), $g_{\text{BS}}$ and $R_{\text{LIGHT}}$ (derived from the concurrent gas-exchange and chlorophyll fluorescence measurements; Table S1), to parameterize a purposely derived model of light reactions and dark metabolism. The fraction of electron flow through PSI following CEF in M and BS ($f_{\text{Cy3M}}$ and $f_{\text{Cy3BS}}$, respectively) was fitted to maximize assimilation, constrained to maintain PPDK and PEPCK activity in BS cells at zero to match immunoblotting (Figure 4).

Initially, we replicated in silico the leaf gas-exchange measurements under the ambient O$_2$ level (Figure 2). For both light and CO$_2$ response curves, the trends of CO$_2$ assimilation and Y(III) were captured well by the model. For assimilation, $R^2$ was 0.98 and 0.97 for the light curves or 0.95 and 0.81 for the A/C curves of HL and LL plants, respectively.
respectively. For Y(II), $R^2$ was 0.86 and 0.96 for the light curves or 0.91 and 0.76 for the $A/C_i$ curves of HL and LL plants, respectively. In the light response curves, the model captured a hyperbolic decrease of the measured Y(I) at high irradiance (Figure 2e) but not the initial dip at low irradiance. However, the modelled Y(I) was similar to the trend of $1 - Y(ND)$, perhaps only capturing Y(I) available through electron input from PSII but not accounting for the loss of Y(I) through the unavailability of acceptors, i.e. Y(NA). In CO$_2$ exported to the M for reduction a higher fraction of the total 3PGA produced in the BS response curves, the hyperbolic increase of Y(II) was captured, but Y(II) was slightly underestimated for C$_M$ values greater than 20 μmol mol$^{-1}$. Predicted Y(II) did not differ between HL and LL plants.

Next, we carried out a simulation of photosynthetic biochemistry using the specific parameterization of growth

Figure 2. Measured and modelled gas exchange and photosystems yield under ambient O$_2$ level. Symbols show response curves of CO$_2$ assimilation (a, b), quantum yield of photosystem II (c, d), quantum yield of photosystem I (e, f), and photosystem I donor side, Y(ND), and acceptor side, Y(NA), limitations (g, h) obtained for *Setaria viridis* grown under high light (HL, solid circles) or low light (LL, empty circles). Light curves measured under a reference CO$_2$ concentration of 420 μmol mol$^{-1}$ are shown in the left-hand panels, and CO$_2$ response curves obtained under constant irradiance of 1000 μmol m$^{-2}$ sec$^{-1}$ are shown in the right-hand panels. Mean ± SE, n = 3 biological replicates. C$_M$, CO$_2$ concentration in M cells. Corresponding curves obtained by switching the background gas to low O$_2$ are shown in Figure 3. Lines show modelled responses obtained through a combined biochemical model of light reactions and carbon metabolism of C$_4$ photosynthesis. Model parameters are listed in Table 2.

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light regimes (Table 2). LL plants had a 60% lower rates of the reactions of the assimilatory metabolism, as a result of the lower energy availability (Table 3, to aid comparison between light regimes we also expressed fluxes in multiples of gross assimilation). With the lower NADPH availability, LL plants exported a higher fraction of the total 3-phosphoglyceric acid (PGA) produced in the BS to the M (42% and 35%, respectively; Table 3). PGA is then reduced to trioses diffusing back to the BS. The rates of Rubisco carboxylation ($V_C$) and PEP carboxylation ($V_P$, which corresponds to the rate of malate export to BS cells and the backflux of pyruvate) were lower in LL plants. The decrease in $V_P$ caused a 25% decrease in CO2 concentration in the BS that was reflected by a higher ratio of Rubisco oxygenation relative to carboxylation ($V_O/V_C$, 4.9% in HL plants and 5.4% in LL plants; Table 3). However, there was a general

![Figure 3](image-url)

**Figure 3.** Measured gas exchange and photosystems yield at 2% O2. Light curves measured under a reference CO2 concentration of 420 μmol mol$^{-1}$ are shown in the left-hand panels; CO2 response curves obtained under constant irradiance of 1000 μmol m$^{-2}$ sec$^{-1}$ are shown in the right-hand panels. Symbols show response curves of CO2 assimilation (a, b), quantum yield of photosystem II (c, d), quantum yield of photosystem I (e, f), and photosystem I donor side, $Y(ND)$, and acceptor side, $Y(NA)$ limitations (g, h) obtained for *Setaria viridis* grown under high light (HL, solid circles) or low light (LL, empty circles). Mean ± SE, n = 3 biological replicates. $C_M$, CO2 concentration in M cells.
increase in PSII efficiency in LL plants that overweighed the effect on $V_o/V_c$. As a result, the average quantum yield increased by 38% on the basis of absorbed light (from 0.04 to 0.055 CO$_2$/quanta), and as much as 43% on the basis of incident light (from 0.031 to 0.044 CO$_2$/quanta), in LL plants compared with HL plants. LL plants gained more on the basis of incident light because of their higher leaf absorbance (captured in the measurements by $s'$, Table S1, and in the model inputs by $s_0$, Table 2).

**DISCUSSION**

*Setaria viridis* is a wild ancestor of *Setaria italica* (foxtail millet), a grain crop widely grown in China and India. It has gained favour for studying C$_4$ photosynthesis because it has a rapid life cycle, small stature, sequenced genome and an available genetic transformation method (Brutnell et al., 2010; Ermakova, Osborn, et al., 2021). As C$_4$ photosynthesis has evolved independently at least 70 times (Sage, 2017), diverse biochemical configurations exist in C$_4$ plants, and characterizing different strategies of shade acclimation is a prerequisite for identifying the best targets for improving crop performance. While the anatomical responses of C$_4$ plants to shading are relatively uniform or consistent (Pengelly et al., 2010; Ward & Woolhouse, 1986), the biochemical strategies appear to be diverse.

We recently showed that *S. viridis* grown at limiting irradiance deployed a suite of protein-level adjustments providing BS thylakoids with increased capacity for light harvesting and electron transport, pointing to an increased ATP demand in the BS (Ermakova, Bellasio, et al., 2021). Here, we were interested in resolving whether the observed rearrangements were triggered by increased ATP consumption from dark reactions or by a lower rate of production, perhaps due to the anatomical acclimation to growth under low light. Although acclimation to LL generally entails a downregulation of photosynthetic potential, here gas-exchange measurements showed that HL and LL plants reached strikingly similar rates of assimilation when measured at the same light or CO$_2$ levels. This intriguing invariance, which we repeatedly measured in plants from different growth batches, and that was previously observed independently in the same ecotype (Henry et al., 2019), may be a particular feature of *S. viridis*. Detailed analysis of the response curves through model fitting (Table S1) revealed some contrasting acclimation strategies: LL plants had lower carboxylation efficiency and lower Y($II$)$_{LL}$, but also lower respiration, than HL plants. We then characterized the leaf anatomy and found a reduction in BS size and in BS surface area per leaf area ($S_{BS}$) in LL plants. Our interpretation is that these anatomical changes contributed to the reduction of $g_{BS}$ (von Caemmerer et al., 2008), here estimated by fitting gas exchange and fluorescence data obtained under ambient and low concentrations of O$_2$. The reduction of $g_{BS}$ under LL conditions is consistent with previous results obtained through different proxies, including anatomy, isotopic discrimination, combined gas exchange and chlorophyll fluorescence (Pengelly et al., 2010; Ubierna et al., 2013). Interestingly, $g_{BS}$ was shown to decrease both in plants grown under LL conditions (Bellasio & Griffiths, 2014b) and in plants grown under HL conditions and subsequently transferred to LL, as occurs in crop canopies where older leaves are shaded by new growth (Bellasio & Griffiths, 2014a).

The decrease in $g_{BS}$ ameliorates the efficiency of C$_4$ photosynthesis. By hindering CO$_2$ leakage, it counters the decrease in CO$_2$ concentration in the BS and the increase of photorespiration occurring under LL (Kromdijk et al., 2014). However, the associated reduction in BS size (Table 1) has the undesirable consequence of decreasing the light harvesting in BS cells, limiting ATP generation and decreasing the operational plasticity under changing light conditions (Bellasio & Lundgren, 2016). LL plants...
| Symbol       | Description                                                                 | Unit                          | HL                      | LL                      | Source                                                                 |
|-------------|------------------------------------------------------------------------------|-------------------------------|-------------------------|-------------------------|----------------------------------------------------------------------|
| PPFD or $I_{in}$ | Photosynthetic photon flux density                                              | $\mu$mol m$^{-2}$ sec$^{-1}$ | 1000 or variable         | 1000 or variable         | Equal to measurements                                                |
| $C_M$       | CO$_2$ concentration in M cells                                               | $\mu$mol mol$^{-1}$          | Variable or 400          | Variable or 400          | –                                                                   |
| $O_M$       | O$_2$ concentration in M cells                                                | $\mu$mol mol$^{-1}$          | 210 000                 | 210 000                 | Equal to ambient                                                     |
| $g_{BS}$    | Bundle sheath conductance to CO$_2$ diffusion                                | mmol m$^{-2}$ sec$^{-1}$     | 0.0018                  | 0.0012                  | Gas-exchange curve fitting                                           |
| $\gamma^*$ | Half the reciprocal Rubisco specificity                                        | $\mu$mol m$^{-2}$ sec$^{-1}$ | 0.00031                 | 0.00031                 | Boyd et al. (2015)                                                   |
| $R_{\text{LIGHT}}$ | Respiration in light                                                       |                                | 1.93                    | 1.18                    | Gas-exchange curve fitting                                           |
| Chl(BS/M)   | Chlorophyll $(a + b)$ in the BS relative to M                                | (mmol m$^{-2}$ leaf$^{-1}$)  | 0.48                    | 0.66                    | Spectroscopic measurements (Table 1)                                |
| AB(BS/M)    | Fraction of light absorption in BS cells relative to M cells                 |                               | 0.414                   | 0.343                   | Light penetration and anatomy model (Bellasio & Lundgren, 2016)      |
| $Y(I)_{LL}$ | Yield of PSI extrapolated under zero PPFD and infinite $C_M$                |                               | 0.76                    | 0.73                    | 1.04 × $Y(I)_{LL}$                                                   |
| $s_0$       | Lumped energy conversion coefficient when $f_{\text{PEP}} = 0$ (Yin et al., 2009), similar to $a_q$ in other notations | $e^\text{−}$/quanta           | 0.440                   | 0.463                   | Fitted for $\frac{\text{d}A_{\text{CO}_2}}{\text{d}I_{\text{PPFD}}} = s'$ |
| $f_{\text{PEUDO NR M}}$ | Fraction of $J_1$ used by nitrate reduction in M cells                         |                               | 0.01                    | 0.01                    | Assumed low for simplicity                                           |
| $f_{\text{PEUDO NR BS}}$ | Fraction of $J_1$ used by nitrate reduction in BS cells                        |                               | 0.01                    | 0.01                    | Assumed low for simplicity                                           |
| $f_{\text{Q}}$ | Fraction of $J_1$ going through the Q-cycle                                 |                               | 1                       | 1                       | (Yin & Struik, 2012)                                                |
| $H$         | Stoichiometry of ATP synthase:protons required to synthesize ATP            | h$^+$/ATP                    | 4.67                    | 4.67                    | Hahn et al. (2018); Vollmar et al. (2009)                            |
| $Y(I)_{LL}$ | Yield of PSI extrapolated under zero PPFD                                     |                               | 1.0                     | 1.0                     | Yin and Struik (2012)                                               |
| $f_{\text{NDH M}}$ | Fraction of CEF through the NDH complex in the M                             |                               | 0.4                     | 0.4                     | See Parameterization                                                |
| $f_{\text{NDH BS}}$ | Fraction of CEF through NDH in BS cells                                       |                               | 0.7                     | 0.7                     | See Parameterization                                                |
| $f_{\text{CYC M}}$ | Fraction of $J_1$ following CEF in M cells                                    |                               | Fitted to max A         | Fitted to max A         |                                                                      |
| $f_{\text{CYC BS}}$ | Fraction of $J_1$ following CEF in BS cells                                     |                               | Fitted to max A         | Fitted to max A         |                                                                      |
| $\alpha_C$ | Slope of the non-rectangular hyperbola used to model $f(\text{CO}_2)$       | $\mu$mol m$^{-2}$ sec$^{-1}$ | 0.15                    | 0.15                    | Fitted to fluorescence                                              |
| $V_{DC}$    | y-intercept of the non-rectangular hyperbola used to model $f(\text{CO}_2)$ |                               | 1                       | 1                       | Assumed 1 for simplicity                                             |
| $\theta_C$ | Curvature of the non-rectangular hyperbola used to model $f(\text{CO}_2)$    |                               | 0.3                     | 0.3                     | Fitted to fluorescence                                              |
| $\alpha_V$ | Slope of the non-rectangular hyperbola used to model $f(\text{PPFD})$       |                               | 0.0004                  | 0.0004                  | Fitted to fluorescence                                              |
| $V_{DV}$    | y-intercept of the non-rectangular hyperbola used to model $f(\text{PPFD})$  |                               | 1                       | 1                       | Assumed 1 for simplicity                                             |
| $\theta_V$ | Curvature of the non-rectangular hyperbola used to model $f(\text{PPFD})$    |                               | 0.8                     | 0.8                     | Fitted to fluorescence                                              |
| $f_{\text{PEP}}$ | Fraction of PEP produced by PEPCK that is hydrolysed                           |                               | –                       | –                       | Irrelevant because $f_{\text{PEP}} = 0$                             |
| $f_{\text{PEPCK}}$ | Fraction of the activity of PECK relative to the maximum (that is the fraction of $J_{\text{ATP BS}}$ not used by the RPP and PCO cycle) |                               | 0                       | 0                       | Immunoblotting (Fig. 2)                                             |
| $f_{\text{RLIGHT}}$ | fraction of respiration in the light in BS cells relative to leaf level       |                               | 0.5                     | 0.5                     | von Caemmerer (2000)                                                |
| $f_{CS}$    | Fraction of carbohydrate synthesis in BS cells                               |                               | 0.5                     | 0.5                     | Bellasio (2017)                                                     |
| $f_{\text{MDH M}}$ | Fraction of M activity of MDH, relative to its maximum, defines the transition between NAD-ME and NADP-ME |                               | 1                       | 1                       | John et al. (2014)                                                 |
deployed a concerted suite of responses to counter a potential ATP starvation in the BS. First, whereas in HL plants the M chloroplasts were dispersed, in LL plants the M chloroplasts were arranged along the cell walls (Figure 1), similar to that observed in *Saccharum officinarum* grown under LL (Sales et al., 2018), which created characteristic optical corri-

The output shown is calculated at the growth PPFD (1000 for HL or 300 μmol m⁻² sec⁻¹ for LL), and expressed, except for $f_{\text{cyc}}$ and $\Phi$ (dimensionless) or $C$ (μmol mol⁻¹), in μmol m⁻² sec⁻¹, and in brackets as a fraction of gross assimilation under growth light (30.97 for HL and 13.24 μmol m⁻² sec⁻¹ for LL plants).

| Symbol | Description | HL | M | BS | LL |
|--------|-------------|----|---|----|----|
| $f_{\text{cyc}}$ | Fraction of electron flow through PSI ($J_i$) following CEF | 0.01 | 0.783 | 0.08 | 0.866 |
| $C$ | CO₂ concentration | 400 | 4100 | 400 | 3100 |
| $I_{\text{PPFD}}$ | Incident light (PPFD) | 707 (23.5) | 293 (9.71) | 223 (17.7) | 77 (6.08) |
| $I_r$ | Light absorbed by PSII | 310 (10.0) | 50 (1.63) | 100 (7.54) | 9.5 (0.718) |
| $I_t$ | Light absorbed by PSI | 238 (7.68) | 176 (5.69) | 79.1 (5.97) | 51.9 (3.92) |
| $J_i$ | Electron flow through PSII | 151 (4.88) | 25 (0.794) | 63.8 (4.82) | 6.07 (0.458) |
| $J_f$ | Electron flow through PSI | 153 (4.92) | 113 (3.65) | 69.2 (5.22) | 45.4 (3.43) |
| $J_{\text{NADPH}}$ | NADPH production rate (half the electron flow to NADPH) | 58.8 (1.90) | 7.09 (0.229) | 27.4 (2.07) | 0.948 (0.07) |
| $J_{\text{ATP}}$ | ATP production rate | 97.9 (3.16) | 80.2 (2.59) | 44.2 (3.34) | 32.5 (2.46) |
| $V_p$ | PEP carboxylation rate. In this study $f_{\text{NADH}} = 1$, so $V_p$ corresponds to the rate of malate flux to the BS, the rate of pyruvate flux to the M, the PPDK reaction rate in the M (PPDK reaction rate in the BS is fitted to be zero in this study) and the malic enzyme reaction rate | 36.7 (1.18) | 0 | 15.9 (1.20) | 0 |
| $L$ | Leakage rate of CO₂ out of the BS | −6.64 (−0.214) | 6.64 (0.214) | −3.21 (−0.242) | 3.21 (0.242) |
| $\Phi$ | Leakiness: CO₂ leakage rate out of the BS relative to $V_p$ | −0.18 | 0.18 | −0.20 | 0.20 |
| $V_C$ | Rubisco rate of carboxylation | 0 | 31.7 (1.02) | 0 | 13.6 (1.03) |
| $V_O$ | Rubisco rate of oxygenation | 0 | 1.54 (0.0496) | 0 | 0.735 (0.0555) |
| $PR$ | Rate of PGA reduction | 22.2 (0.716) | 43.0 (1.39) | 11.5 (0.868) | 16.4 (1.24) |
| $DHAP_{\text{p}}$ | Rate of DHAP entering the conversion phase of the RPP cycle | 0 | 55.5 (1.79) | 0 | 23.9 (1.81) |
| $RuP_{\text{phosph}}$ | Rate of RuP phosphorylation | 0 | 33.3 (1.07) | 0 | 14.3 (1.08) |
| $CS$ | Rate of carbohydrate synthesis | 4.85 (0.156) | 4.85 (0.156) | 2.01 (0.152) | 2.01 (0.152) |
| $R_{\text{Light}}$ | Rate of respiration in the light | 0.95 (0.031) | 0.95 (0.031) | 0.590 (0.047) | 0.590 (0.046) |
| $PGA_{\text{flux}}$ | Flux of PGA | 22.5 (0.726) | −22.5 (−0.726) | 11.7 (0.882) | −11.7 (−0.882) |
| $DHAP_{\text{flux}}$ | Flux of DHAP | −17.3 (−0.559) | 17.3 (0.559) | −9.48 (−0.716) | 9.48 (0.716) |

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transport processes in LL plants was effective in countering the limitations to ATP generation caused by a smaller BS, and what modifications to the metabolism of BS and M were required to maintain invariant levels of assimilation (Figure 2). We developed a model encompassing explicit anatomy and separate electron transport chains in the BS and M, parameterized specifically for HL and LL plants. We found that the increased chlorophyll concentration was not sufficient to counter the effect of smaller BS cells and, overall, LL plants absorbed 40% less light in BS cells relative to the total (from 23% under HL to 14% under LL; Table 3). However, the higher proportion of CEF (f_Cyc) in the BS of LL plants (Figure S3; Table 3) could partially compensate for the decrease in light absorption, so that the predicted ATP production rate, relative to the total, only decreased by about 5% (from 45% in the BS of HL plants to 42% in the BS of LL plants; Table 3). The predicted changes in f_Cyc – an emerging property of the fitted model mainly resulting from the reduction in the size of the BS – are in line with the increased content of NDH detected in LL BS cells (Ermakova, Bellasio, et al., 2021), again pointing to the key role of NDH in ATP production in BS cells. Hence, we demonstrated that: first, the upregulation of CEF (f_Cyc) is effective in mitigating decreased light interception in BS cells; and, second, that decreasing light interception in BS cells is a sufficient condition to require an increase of f_Cyc (Figure S3; Table 3). To our knowledge, this functional dependence was not demonstrated before, uncovering another important link between biochemical properties and the structural characteristics of C₄ leaves.

The NADPH production rate in BS cells relative to the total was predicted to decrease by 97% in LL plants (from 11% under HL to 0.3% under LL), driven both by the decrease in BS size and the increase in f_Cyc. This led to a 13% decrease in the rate of PGA reduction in BS cells relative to the total (from 66% under HL to 59% under LL), and a consequent relative increase of triose fluxes between M and BS cells (Table 3). By means of LEF, PSII activity in BS cells not only supplies NADPH production, but also replenishes the CEF electrons leaking to various electron sinks downstream of PSI (explicitly accounted for in the model as pseudocyclic electron flow, but not varied in this study). In this model output, LEF in the BS was predicted to be 21 μmol m⁻² sec⁻¹ (14% of the total leaf rate for HL plants) and 5.59 μmol m⁻² sec⁻¹ (9% of the total leaf rate for LL plants) (Table 3). The actual PSII activity in the BS thylakoid membranes measured by Ermakova, Bellasio, et al. (2021) matched the value predicted for LL plants (it was approx. 7% of the leaf rate), but in HL plants it was half of the LL value, much lower than the model prediction. This suggests that the predicted value of J₂ in the BS of HL plants cannot be supplied in vivo by electrons coming from water oxidation, but must be obtained via oxidation of NADPH, derived from malate imported from the M, through the NDH. This idea is supported by experimental observations. When supplied with malate, isolated BS strands of Z. mays retained PSI activity even in the presence of the PSI inhibitor. Further, the BS of Sorghum bicolor maintained CO₂ fixation under far-red illumination, which is unable to excite PSII (Ivanov et al., 2005; Osmond, 1974).

Theory shows that, under changing irradiance, flexibility in the apportioning of PGA reduction between BS and M cells can adjust the BS demand for NADPH, and the engagement of PEPCK and PPDK can attenuate the BS demand for ATP (Bellasio & Griffiths, 2014c; Furbank, 2011). Because PEPCK consumes ATP generated in the M and is tightly regulated by ATP availability, the expression of PEPCK in NADP-ME plants was predicted to increase light-harvesting plasticity, i.e. the capacity to efficiently harvest light of a broad range of intensities and spectral qualities (Bellasio & Griffiths, 2014c). Further, the activity of PPDK in the BS could compensate for a lack of PEPCK, making PEPCK engagement necessary when the activity of PPDK in the BS was reduced, and vice versa (Bellasio, 2017). Indirect evidence supports these predictions. It was shown that low-light-acclimated NADP-ME plants, like Z. mays and Saccharum officinarum, increased the total rate of PEPCK activity (Sales et al., 2018) or the activity of PEPCK relative to PEPC (Sharwood et al., 2014; Sonawane et al., 2018), and increased the pool of inactive PEPCK (data made available by courtesy of B.V. Sonawane, personal communication), presumably available to be activated by changing light conditions (Bailey et al., 2007).

Uniquely, S. viridis did not express PEPCK under HL or LL conditions (Figure 4). Nor did we find detectable levels of PPDK in the BS (Figure 4), in line with previous reports (John et al., 2014; Schlüter & Weber, 2020). Although Z. mays grown under LL overexpressed LHCCI subunits and PaeB in BS cells (Drozak & Romanowska, 2006), it did not show increased oxygen activity or changes in the supramolecular organization of PSII (Rogowski et al., 2019; Romanowska et al., 2006). In contrast, S. viridis had a striking plasticity in acclimating light reactions, probably sufficient to avoid the necessity to alter PEPCK and PPDK expression (Figure 4). Importantly, and in contrast to the nine species (four NADP-ME species, including Z. mays and Saccharum officinarum, two NAD-ME species and two PEPCK species) studied by Sonawane et al. (2018), the acclimation of light reactions in S. viridis did not compromise quantum yield (Table S1). Therefore, the outstanding capacity of S. viridis to rearrange light reactions under LL is a highly efficient acclimation strategy, setting S. viridis aside from other NADP-ME plants and representing a so-far overlooked innovation in C₄ evolutionary history.

**CONCLUSION**

Using a purposely developed model, parametrized with original anatomical, biochemical and gas-exchange data,
we analytically and mechanistically solved the causality link between anatomy and biochemistry. The optical cross section determined the absorption of light in the BS and the M, the rate of electron transport, the engagement of CEF and LEF, the rate of ATP and NADPH generation, and ultimately the apportioning of carbon metabolism between M and BS cells. To counter ATP starvation, *S. viridis* adjusted the electron transport processes, boosting cycling electron flow in the BS. Although quantifying the impact in field conditions will require additional experiments, the striking capacity of *S. viridis* to acclimate light reactions is perhaps the most efficient shade-tolerance strategy, potentially leading to novel possibilities for crop improvement.

**EXPERIMENTAL PROCEDURES**

**Plants**

*Setaria viridis* plants (A10 ecotype) were grown in 2-L pots filled with commercial soil mix (Debco, [https://www.lovethegarden.com.au-en/debco](https://www.lovethegarden.com.au-en/debco)) supplemented with 1 g L⁻¹ of slow-release fertilizer (Osmocote; Scotts, [https://www.scotts.com](https://www.scotts.com)). Plants were grown in controlled chambers with 28°C day, 24°C night, 60% humidity and 16 h of illumination at an intensity of 1000 or 300 μmol m⁻² sec⁻¹, provided by halogen incandescent lamps (42 W, 2800 K, warm white, clear glass; CLA, [https://www.clairighting.com.au](https://www.clairighting.com.au)) and Pentron Hg 4-fluorescent tubes (54 W, 4100 K, cool white; Osram Sylvania, [https://www.osram.us](https://www.osram.us)), and reached by hanging a shade cloth above some of the plants. Growth light spectra are shown in Figure S2. All measurements were performed on the youngest fully expanded leaves sampled before flowering, between 15 and 25 days after germination.

**Leaf anatomy**

Resin-embedded cross sections were prepared and imaged according to Pengelly et al. (2010). Quantification of anatomical parameters was performed using [ImageJ](https://imagej.nih.gov/) on an equal number of secondary and tertiary veins, as described by Bellasio and Lundgren (2016). Calculations of the M surface area exposed to the intercellular airspace (S_M) and the BS surface area per unit of leaf area (S_S/B) were made as described by Pengelly et al. (2010) using the curvature correction factor of 1.43 from Evans et al. (1994). Apparent absorbance was calculated as the log₁₀ of the ratio between the luminance of the background divided by the luminance of the tissue, both processed with [ImageJ](https://imagej.nih.gov/) as described by Bellasio and Lundgren (2016) and averaged over two regions for n = 6 replicates. Leaf absorbance was the complement to one of leaf reflectance and transmittance, both measured with a Li-Cor 1800-12 integrating sphere (LI-COR, [https://www.lci-cor.com](https://www.lci-cor.com)) coupled to a Li-1900 light sensor (Li-Cor), following the manufacturer’s instructions for calibration and calculations.

**Chlorophyll**

Total chlorophyll was extracted from frozen leaf discs ground using TissueLyser II (Qiagen, [https://www.qiagen.com](https://www.qiagen.com)) in 80% acetone, buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-KOH (pH 7.8). Chlorophyll a and b content was measured at 750, 663.3 and 646.6 nm, and calculated according to Porra et al. (1989). The fraction of total leaf chlorophyll in BS cells was determined from the chlorophyll a/b ratios in BS and M cells, as described by Ermakova, Bellasio, et al. (2021).

**Western blotting and immunolocalization**

Protein isolation from leaves, gel electrophoresis and Western blotting were performed as described by Ermakova et al. (2019). PEPCK antibodies (Agrisera, [https://www.agrisera.com](https://www.agrisera.com)) were used according to the manufacturer’s protocol.

Immunodetection of PPDK on lightly fixed leaf cross sections was performed according to Ermakova, Arrivault, et al. (2021). Sections were treated with 1:100 PPDK primary antibody (Karki et al., 2020), 1:200 Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Life Technologies, now ThermoFisher Scientific, [https://www.thermo Fisher.com](https://www.thermo Fisher.com)) and 0.05% calcofluor white to stain cell walls. The fluorescence signal was captured with a Leica DM5500 microscope (Leica Microsystems, [https://www.leica-microsystems.com](https://www.leica-microsystems.com)) equipped with a Leica DFC700XT camera using LEICA APPLICATION SUITE 4.12. Fluorescence was detected at 505–565 nm for PPDK (excitation 490–510 nm, YFP Filter Cube; Leica) and at 450–490 nm for cell walls (excitation 330–370 nm, A4 Filter Cube; Leica).

**Simultaneous gas exchange, chlorophyll fluorescence and P700 measurements**

Gas exchange, fluorescence and P700⁺ absorbance changes were measured simultaneously with the set-up of Bellasio and Farquhar (2019) on n = 3 biological replicates. Briefly, a portable gas-exchange system (LI6400XT; Li-Cor) was modified to operate at low CO₂ concentrations (see [licor.com](https://www.licor.com)) and fitted with a 6400-06 PAM2000 adapter (courtesy of Susanne von Caemmerer), holding a fibre probe in the upper leaf cuvette distant enough to minimize shading. Leaves were aligned without overlapping their edges to fill the cuvette. Light was provided by a bespoke red-blue light source, adjusted to provide approximately 90% red and 10% blue light, positioned to illuminate the leaf uniformly. Light intensity was measured through an in-chamber gallium arsenide photodiode, calibrated using a Li-250 light sensor (Li-Cor). Neoprene gaskets were used on both sides of the cuvette. A mixture with 2% O₂ was prepared by mixing ambient air and N₂ using a bespoke gas mixing unit (kindly assembled by Suan Chin Wong). This mix of ambient air was CO₂-scrubbed with soda lime and humidified to a dew point of 15°C upstream of the inlet to maintain water vapour pressure deficit around 1 kPa. CO₂ was added from a large cylinder (BOC, [https://www.boc.com.au](https://www.boc.com.au)), using the CO₂ injection unit of the LI6400XT. Mass flow leaks (Boesgaard et al., 2013) were monitored with a gas flow meter, as detailed by Bellasio et al. (2016b).

Both PSII and PSI yields were measured with a Dual PAM-F (Walz, [https://www.walz.com](https://www.walz.com), courtesy of Dean Price). PSII activity was assessed with the pulse amplitude modulated fluorescence method using 620 nm measuring light of 9 μmol m⁻² sec⁻¹ (Schreiber et al., 1986). The redox state of P700, the reaction centre of PSI, was assessed by detecting the absorbance of the cation at 830 nm with a dual wavelength unit (830/875 nm, following the method of Klughammer and Schreiber (1994)). Saturating pulses of red light (635 nm) at 20 000 μmol m⁻² sec⁻¹ were used. First, Pₐ, the maximal level of P700⁺, was recorded upon the application of a saturating pulse on top of the far-red light, and Pₛ, the minimal P700⁺ signal, was recorded after that saturating pulse. Photosynthetic parameters (P, the steady-state P700⁺ signal; Pₛ, the maximal P700⁺ signal under light; Fₛ, the steady-state fluorescence signal; Fₐ, the maximal fluorescence signal under light) were monitored by the application of the saturating pulse at
different irradiances, CO₂ and O₂ concentrations. The effective quantum yield of PSI, Φ(III), was calculated according to Genty et al. (1989). The effective quantum yield of PSI, Φ(III), and the non-photochemical yields of PSI caused by donor, Φ(D), or acceptor, Φ(A), side limitation were calculated as described by Klughammer and Schreiber (2008).

Four photosynthetic response curves (A/PPFD and A/C, curves, under ambient and low concentrations of O₂) were measured at 25°C in day-long experiments. A/C curves were measured first, under a PPFD of 1000 μmol m⁻² sec⁻¹ and imposing reference CO₂ concentrations of 20, 40, 60, 80, 100, 200, 300, 400, 500, 600, 800, 1000 μmol mol⁻¹ and a minimum of 120 sec between steps; A/PPFD curves were measured under reference CO₂ concentrations of 420 μmol mol⁻¹, imposing PPFDs of 1500, 1000, 200, 500, 150, 100, 75, 50, 30 μmol m⁻² sec⁻¹ and a minimum of 180 sec between steps. The order between ambient and low O₂ was inverted each day. The flow rate was 400 μmol sec⁻¹; CO₂ diffusion through the gaskets was compensated by lengthening the tubing of the LI6400XT reference gas (Bellasio & Farquhar, 2019). Gas-response curves were analysed using the protocol of Bellasio et al. (2016a).

CO₂ concentration at the M carboxylation sites was calculated as C₅ = C₄ - A/DM where mesophyll conductance (g₄m) was 1.5 and 1.2 mol m⁻² sec⁻¹ for HL and LL, respectively, derived by adjusting the data of Ubierna et al. (2017) for S₅ (Table 1).

C₄ photosynthesis model

Biochemical models may include formulations describing a variety of processes (e.g. electron transport), but at any given time only the formulation describing the process that is limiting photosynthesis in that circumstance will be representative (outputs are called ‘actual’ rates), whereas others are not (outputs are called ‘potential’ rates; Farquhar et al., 1980). The mismatch between formulations can sometimes be useful, for instance, to estimate stomatal conductance in C₃ (Farquhar & Wong, 1984) and C₄ plants (Bellasio et al., 2017), but typically it generates ambiguity in curve fitting (e.g. fluorescence; reviewed in Bellasio et al., 2016b) and difficulties in locating the cut-off points (Gu et al., 2010). To avoid discontinuities, we merged enzyme limitations into a light-limited model by introducing a function describing the quenching of Y(III) depending on C₄M, analogous to the classical PPFD-dependence of the electron transport rate. This function depends on the familiar biochemical quantities V₄MAX and Kₐ, but we have not derived an explicit expression at this stage.

The model is organized to simulate two compartments representing M and BS cells (Figure 5): each harvests light to drive a distinct electron transport chain (Note S1; Figure S1). In both electron transport chains, the ratio between the rates of ATP and NADPH production is varied via adjustment of CEF (through the parameter fCEF), which may flow through PGRS/PGLR1 or the NAD (P)H dehydrogenase-like complex (NDH, adjusted through the parameter fNDH). When fCEF is adjusted, the proportion of light absorbed by the photosystems varies to maintain invariance in the sum of the light absorbed by the two (equations described in supporting information of Yin et al., 2004). This is of key importance when modelling energetics and CEF engagement under limiting light (Bellasio, 2019) because, otherwise, supplemental CEF would be driven by an increase in light absorbed (but s would be invariant, main text of Yin et al., 2004). The reducing power requirements for nitrogen reduction and for the water–water cycle are explicitly accounted for as a fraction of pseudo-cyclical electron flow, after Yin and Struik (2012), but we did not use the functionality in this study. A key innovation is that the rate of oxygen evolution in the BS is calculated from the actual rate of electron transport in both compartments, instead of being assumed to scale to assimilation through the parameter α, as in all the models derived from Berry and Farquhar (1978).

Both ATP and NADPH drive the reactions of carbon metabolism, described following Bellasio and Griffiths (2014c), McQualter et al. (2016) and Bellasio (2017). Although the NADPH available in the BS is consumed by the RCP and the photorespiratory cycles, the ATP available in the M and any ATP surplus in the BS are consumed by the C₄ cycle, until all the ATP and NADPH available have been consumed. In this way, the partitioning between cycles and the proportioning of metabolic work between M and BS cells emerges directly from the rate of ATP and NADPH produced by light reactions, instead of being assumed through the parameter x, as in all the approaches following Berry and Farquhar (1978). In contrast to Bellasio (2017), where all intermediate photosynthetic types were modelled, here C₄ photosynthesis is fully expressed, such that the glycine decarboxylase complex (GDC) and Rubisco are both fully compartmentalized to BS cells (Bellasio, 2017). However, C₄ subtypes can be changed seamlessly, forming a continuous biochemical space that can be explored by varying two parameters: fNDH, representing the engagement of MDH in M cells and governing the trajectory between the NADP-ME subtype and the NAD-ME subtype, and fPEPCK, representing the capacity of PEPCK to consume the ATP available in BS cells and representing the level of PEPCK expression relative to other carboxylases (we will explore these functionalities in dedicated studies). In the decarboxylation of malate, the co-products NADH and NADPH are assumed to be equivalent or interconvertible. This may be underpinned by an actual biochemical interconversion, mediated by the intercellular operation of the malate shuttle (Furbank, 2011), or simply by the capacity of malic enzyme (ME) to dock both cofactors, as suggested by early studies (Kanai & Edwards, 1973).

The M and BS cells are connected by an exchange of metabolites (aspartate, ASP; alanine, ALA; dihydroxyacetone phosphate, DHAP; malate, MAL; phosphoenolpyruvate, PEP; 3-phosphoglyceric acid, PGA; pyruvate, PYR; and CO₂) that diffuse obeying mass-balance constraints. Diffusion is non-limited, except that of CO₂, restrained by a finite conductance (g₃BS) that allows higher CO₂ concentrations in BS cells (C₃BS) based on classical underpinnings (Berry & Farquhar, 1978). Glutamate and αKG do not diffuse directly between M and BS cells, but exchange amino- groups with ALA and PYR (Mallmann et al., 2014; Pick et al., 2011; Schlüter et al., 2019). If reactions can proceed in the opposite direction, they are assumed to proceed only in the direction with positive balance. Respiration is a shared process between M and BS cells. To avoid a futile cycle consisting of concurrent glycolysis and carbohydrate synthesis, respiration is assumed to be supplied by new assimilates (PGA), in line with Bellasio and Griffiths (2014c). The ATP and NADH produced during respiration are neglected, assumed to be consumed by basal metabolism. Any ATP and NADH residual imbalances produced during respiration in the light are absent, supported by the analysis of Buckley and Adams (2011), who proposed that they would be dissipated by alternative oxidases. The final product of photosynthesis is a generic triose carbohydrate, the destiny of which is not followed further by the model.

Details of the functions describing the reaction rates and CO₂ and O₂ concentrations in M and BS compartments are provided in Note S2, the balance of reducing power consumption and production in the M and BS are provided in Note S3 and metabolite flows are provided in Note S4. Model sensitivity analysis is shown in Table S2.

Parameterization

Parameters were either derived from measurements (in which case the data were randomized and averaged for HL and LL.
Figure 5. Schematic of the processes included in the model. Metabolites are in black. Fluxes are depicted by black arrows and pink symbols that directly link to the equations reported in Notes S1-S4. Key processes are briefly described in bright green. The leaf is divided into mesophyll (M) and bundle sheath (BS) compartments. Incident PPFD ($I_{inc}$) may reach M ($I_{inc,M}$) or BS ($I_{inc,BS}$) cells, depending on anatomical characteristics and the size of the light-harvesting machinery. A fraction $I$ is absorbed by PSI or PSII ($I_x$ or $I_y$, respectively). Light reactions (for more details see Figure S1) result in the production of NADPH and ATP, which are consumed by carbon metabolism encompassing C4 and C3 activity. The C4 cycle reactions appear in the middle of the M and BS compartments. CO2 is initially hydrated to bicarbonate (the point of entry is highlighted in yellow) and fixed by PEP carboxylase (PEPC) at the rate of 2 ATP to form oxaloacetate (OAA). This may be reduced to malate (MAL) or to aspartate (ASP) through transamination (αKG) and is deaminated to OAA (the exchange of amino groups with α-ketoglutarate (αKG) is implied) and may be decarboxylated by PEP carboxykinase (PEPCK) or by malic enzyme (ME). The regeneration of PEP is shared between M and BS cells, depending on energy availability. The C3 metabolism appears at the bottom, partitioned between M and BS compartments. Rubisco carboxylation and oxygenation reactions ($V_o$ and $V_c$) consume RuBP and produce PGA and PGLA and are fully compartmentalized to BS cells. PGLA is recycled through the photorespiration cycle, eventually regenerating PGA. This is consumed by respiration ($R_{respir}$), assumed to be entirely supplied by newly assimilated PGA, and is reduced ($PR$) to triose phosphate (DHAP), which is a substrate of carbohydrate synthesis (CS). The final product of photosynthesis is a generic triose carbohydrate (highlighted in yellow). The majority of DHAP enters the sugar conversion phase of the reductive pentose phosphate (RPP) cycle, exclusive to the BS. Metabolites for which fluxes are calculated are listed in the middle and are assumed to be positive when occurring in the normal direction, indicated by the arrows. The EXCEL workbook provided renders outputs according to this scheme.

plants), taken from dedicated studies or assumed. Light penetration in the leaf was simulated through a separate optical model, freely downloadable from the supporting information of Bellasio and Lundgren (2016). The number of layers of adaxial M cells was 289 for HL plants and 340 for LL plants; the number of layers of the vein was 458 for HL plants and 345 for LL plants; the number of layers of abaxial M cells was 253 for HL plants and 225 for LL plants; the fraction of interveinal distances occupied by the vein was 0.56 for HL plants and 0.53 for LL plants, all derived from anatomical measurements (Table 1). The
absorbance of the adaxial M cells was set to equal that of M cells following the observation that BS extensions are absent in *S. viridis*; the absorbance of the vein relative to M cells (*khv/kM*) was 1.65 for HL plants and 2.73 for LL plants, calculated by dividing the measured BS/M ratio of chlorophyll content per square leaf, by the measured ratios of BS/M areas, 0.29 for HL and 0.24 for LL; reflectance was 0.067 for HL plants and 0.081 for LL plants (Table 1).

The combined model of light and dark reactions has 29 input quantities: three define the environmental characteristics, PPFD, C and θ; 17 define light reactions, Y(I) and Y(II) are the output of light reactions; six define dark reactions, fNDH, fPPDK, fLIGHT, fCS, fMDH, and three more, gBS, AB(0C/M), RLIGHT, are not grouped. AB(0C/M) was taken from the light penetration model described above, and RLIGHT and gBS were derived from gas-exchange and fluorescence measurements with the procedure described by Bellasio et al. (2016a) (Table S1). Y(I) and Y(II) were derived by slightly increasing the maximum values of Y(I) in the sunny, so that Eqn S11 would return Y(II) under ambient CO2 concentration and zero PPFD, α, VCC, fC, αV, VC, Vh, and fV were derived by fitting the fluorescence data shown in Figure 2. s0 is the theoretical maximum value of s when fPSI is zero, adjusted for: s′ = s0/[1 – 1.32 s0] modelled to fit measured values of s′ (Table S1). The Q cycle was assumed to be obligate, making fQ = 1; Y(I) was assumed to be 1, after Yin and Struik (2012). The behaviour of fNDH, fPPFD, and f假NDH in response to CO2 and PPFD is not well known, and therefore we assumed a low value for simplicity. The stoichiometry of the ATP synthase behaviour of f was assumed for the complex have not yet been made, and are difficult to imagine. Here fNDH was set to 0.4 in M cells and 0.7 in BS cells following the observation that NDH- and PGR5-mediated electron flows are both operating in C4 plants (Takabayashi et al., 2005), while keeping in mind that the relative ratio of NDH to PSI was higher in BS cells than in M cells (Ernakova, Bellasio, et al., 2021). fC, fC, and fC were fitted to maximize A at each light intensity, subject to the constraint that the activity of PPDK in BS cells is zero; fitted values are shown in Figure S3. fL and fC were set to 0.5 after von Caemmerer (2000) and Bellasio (2017). fPPDK was set to zero to match immunoblotting (Figure 4). fPSI is irrelevant under the previous assumption. fNDH sets the transition between NAD-ME and NADP-ME subtypes and was assumed to be 1 (Gutierrez et al., 1974; John et al., 2014).

**Statistical analysis**

The relationship between the mean values for HL and LL plants was tested using a two-tailed, heteroscedastic Student’s t-test, at *P* < 0.05 (MICROSOFT EXCELS 2016, Microsoft, https://www.microsoft.com). Error propagation in the model was not analysed.

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**AUTHOR CONTRIBUTIONS**

ME and CB conceived the project. CB developed the model and ran the simulation. CB and ME performed the experiments, analysed the data and wrote the article.  

**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest associated with this work.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Schematic of the light reactions model.  
**Figure S2.** Spectra of growth light.  
**Figure S3.** The fitted fraction of CEF.  
**Table S1.** C4 photosynthesis parameters.  
**Table S2.** Sensitivity analysis.  
**Note S1.** A model for light reactions: ATP and NADPH production rate.  
**Note S2.** Dark reactions and metabolite diffusion.  
**Note S3.** Balancing NADPH demand in BS cells.  
**Note S4.** Metabolite Fluxes between M and BS cells, and amino-group balancing.

**OPEN DATA BADGES**

This article has earned Open Data and Open Materials badges. Data are available in Supporting Information. Models are available on GitHub at: https://github.com/chandrabellasio/C4-Electron-Transport-and-biochemistry.

**DATA AVAILABILITY STATEMENT**

Data are made available in Supporting Information, and the model coded in R and Microsoft Excel is freely available on GitHub at the link https://github.com/chandrabellasio/C4-Electron-Transport-and-biochemistry.

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