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The response of mesophyll conductance to short-term variation in CO₂ in the C₄ plants *Setaria viridis* and *Zea mays*

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Abstract

Mesophyll conductance (gₘ) limits rates of C₃ photosynthesis but little is known about its role in C₄ photosynthesis. If gₘ were to limit C₄ photosynthesis, it would likely be at low CO₂ concentrations (pCO₂). However, data on C₄-gₘ across ranges of pCO₂ are scarce. We describe the response of C₄-gₘ to short-term variation in pCO₂, at three temperatures in *Setaria viridis*, and at 25 °C in *Zea mays*. Additionally, we quantified the effect of finite gₘ on leakiness (ϕ) and the potential limitations to photosynthesis imposed by stomata, mesophyll, and carbonic anhydrase (CA) across pCO₂. In both species, gₘ increased with decreasing pCO₂. Including a finite gₘ resulted in either no change or increased ϕ compared with values calculated with infinite gₘ depending on whether the observed ¹³C discrimination was high (*Setaria*) or low (*Zea*). Post-transitional regulation of the maximal PEP carboxylation rate and PEP regeneration limitation could influence estimates of gₘ and ϕ. At pCO₂ below ambient, the photosynthetic rate was limited by CO₂ availability. In this case, the limitation imposed by the mesophyll was similar or slightly lower than stomata limitation. At very low pCO₂, CA further constrained photosynthesis. High gₘ could increase CO₂ assimilation at low pCO₂ and improve photosynthetic efficiency under situations when CO₂ is limited, such as drought.

Keywords: A-Ci curves, carbonic anhydrase, CO₂, C₄ photosynthesis, diffusional limitations, in-vitro V₉₅ₐ₅, leakiness, mesophyll conductance, *Setaria viridis*, *Zea mays*.

Introduction

In C₄ plants photorespiration is reduced by concentrating CO₂ around Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) (Edwards and Walker, 1983; Hatch, 1987; Sage, 2004). In Kranz-type C₄ plants this is achieved with a compartmentalized two-carboxylation process: (1) in the cytosol of mesophyll cells, bicarbonate (HCO₃⁻) and phosphoenolpyruvate are fixed into four-carbon acids by phosphoenolpyruvate carboxylase (PEPC) (Hatch et al., 1967); and (2) in chloroplasts of the bundle-sheath cells the concentrated CO₂ released from the decarboxylation of these acids is fixed by Rubisco. Mesophyll conductance (gₘ) describes the movement of CO₂ from stomata across the intercellular spaces to the sites of first carboxylation, which are the chloroplast stroma or mesophyll cytosol in C₃ and C₄ species, respectively (Evans and von Caemmerer, 1996). There is extensive research describing gₘ in C₃ species; however, C₄-gₘ is poorly understood because it is difficult to estimate. Traditionally gₘ was assumed to be larger in C₄ compared to C₃ species, but most recent studies suggest that values for C₄-gₘ correspond to higher-end C₃-gₘ reports, and that C₄-gₘ reacts similarly to C₃-gₘ with...
regards to variation in factors such as leaf age and temperature (Barbour et al., 2016; Osborn et al., 2017; Ubierna et al., 2017). If $C_{a}g_{m}$ is lower than previously thought, that could affect derivations of other key parameters such as leakiness ($\phi$, the proportion of C fixed by PEP that subsequently leaks out the bundle-sheath cells). Leakiness cannot be directly measured and is commonly estimated from observations and models of $^{13}$C discrimination ($\Delta^{13}$C) (Farquhar, 1983; Farquhar and Cernusak, 2012). Historically, $g_{m}$ is generally assumed to be infinite when solving for $\phi$ from $\Delta^{13}$C; however, this simplification and estimates of $\phi$ would be compromised if $g_{m}$ is finite and low.

Mesophyll conductance has long been recognized as a significant limitation for $C_{3}$ photosynthesis (Evans, 1983; Evans et al., 1986; Evans and Terashima, 1988), limiting photosynthesis as much as stomatal conductance (Warren, 2008). It is unclear if $g_{m}$ limits $C_{4}$ photosynthesis as ambient $pCO_{2}$. If $g_{m}$ were to limit $C_{4}$ photosynthesis, it would likely only be at very low $pCO_{2}$. However, not much is known about the variation of $C_{4}$ with $pCO_{2}$. In the $C_{4}$ grass *Setaria viridis*, $g_{m}$ derived with the $^{18}$O discrimination ($\Delta^{18}$O) method increased as $pCO_{2}$ decreased, although the variation was not significant (Osborn et al., 2017). Some reports have shown that in $C_{3}$ species $g_{m}$ increases with short-term exposure to decreasing $pCO_{2}$ (Bongi and Loreto, 1989; Loreto et al., 1992; Flexas et al., 2007, 2008; Hassiotou et al., 2009; Bunce, 2010; Douthe et al., 2011; Tazoe et al., 2011). However, others have suggested that $C_{4}$ is insensitive to changes in $pCO_{2}$ (Loreto et al., 1992; Tazoe et al., 2009). It has been hypothesized that the observed $C_{4}$ response to $pCO_{2}$ might result from a significant chloroplast resistance (Tholen and Zhu, 2011; Tholen et al., 2012) or artifacts in the calculations (Gu and Sun, 2014).

In $C_{4}$ plants, $g_{m}$ has been estimated with the $\Delta^{13}$O method (Gillon and Yakir, 2000a, 2000b; Barbour et al., 2016; Osborn et al., 2017; Ubierna et al., 2017) and the *in vitro* maximal PEP carboxylation rate ($V_{pmax}$) method (Ubierna et al., 2017). The latter method solves for the $pCO_{2}$ in the mesophyll cells ($C_{a}$) needed to simultaneously match modeled and measured rates of CO$_{2}$ assimilation and $\Delta^{13}$C when the models are parameterized with *in vitro* $V_{pmax}$ as determined in a crude leaf extract. Values derived for $g_{m}$ with the $\Delta^{13}$O and *in vitro* $V_{pmax}$ methods were similar in two $C_{4}$ species measured over a range of temperatures (Ubierna et al., 2017). The *in vitro* $V_{pmax}$ method also allows the implementation of two modeling alternatives: carbonic anhydrase (CA)-saturated and CA-limited. They differ in the calculation of PEP carboxylation rate as a function of CO$_{2}$ or HCO$_{3}$ for the CA-saturated and -limited scenarios, respectively. Ubierna et al. (2017) found no difference between CA-limited and CA-saturated estimates of $g_{m}$ at ambient $pCO_{2}$, but CA limitation is expected at low $pCO_{2}$.

In this study, we calculated $g_{m}$ using the *in vitro* $V_{pmax}$ method across a range of $pCO_{2}$ in two $C_{4}$ grasses, one economically important (*Zea mays*) and the other the adopted model system for studying $C_{4}$ photosynthesis (*S. viridis*). Measurements were performed at three temperatures (10, 25, and 40 °C) in *Setaria* and at 25 °C in *Zea*. Our objectives were to: (1) describe the response of $C_{a}g_{m}$ to short-term variation in $pCO_{2}$; (2) evaluate the impact of disequilibrium between CO$_{2}$ and HCO$_{3}$ at a range of $pCO_{2}$ and temperatures; (3) investigate if $g_{m}$ represents a limitation to $C_{4}$ photosynthesis across $pCO_{2}$; and (4) assess the impact of finite $g_{m}$ on $\phi$ calculations.

### Materials and methods

**Plant material**

Seeds of *Z. mays* (var. Truckers Favorite, Victory Seed Company, Oregon, USA) were grown in a greenhouse supplemented with artificial lighting at the School of Biological Sciences at Washington State University, Pullman, WA (USA) during August to October 2011. Seeds of *S. viridis* (A-010) were grown in a controlled environment growth chamber (Enconair Ecological GC-16) in 2013. Plants used for measurements were 4 and 6 weeks old for *Zea* and *Setaria*, respectively. *Zea* was fertilized with 17-3-6 NPK and weekly additions of 4 g l$^{-1}$ solution of 10% Fe-DPTA (Sprint 330, Becker Underwood, IA, USA). *Setaria* was treated weekly with Peters 20-20-20 (J. R. Peters, Inc., Allentown, PA, USA). For all plants, the photon flux density was $\approx$5000 μmol m$^{-2}$ s$^{-1}$, the day length was 14 h, and the temperature was 25–28/20–25 °C for day/night.

**Coupled gas exchange and isoflux measurements**

The system used for measurements has been described in detail in Ubierna et al. (2013, 2017). Briefly, a LI-6400XT open gas exchange system assembled with a 6400-22L conifer chamber fitted with a LI-6400–18 RGB light source (Li-Cor, Lincoln, NE, USA) was coupled with a tunable-diode laser absorption spectroscopy (TDLAS, TGA 200A, Campbell Scientific, Inc. Logan, UT, USA). The entire gas exchange system was placed in a growing cabinet (Pericival Scientific, Perry, IA), where the temperature was varied to match leaf temperature ($T_{l}$) settings. The TDLAS data were calibrated with the concentration series method (Tazoe et al., 2011; Ubierna et al., 2013) using two calibration gases, one measured at different [CO$_{2}$] that spanned the gas exchange reference and sample lines. Each measurement cycle included five to seven TDLAS sequences of zero air, calibration gases, reference, and sample lines measured for 40 s each. Data from the last three sequences were averaged and used for calculations.

Young fully-expanded leaves of *Setaria* and *Zea* were acclimated for ~1 h with chamber conditions of $C_{a}$ (ambient CO$_{2}$ supply to the chamber) = 35 Pa, 21% O$_{2}$, and photosynthetically active radiation (PAR) =2000 μmol m$^{-2}$ s$^{-1}$. Then, $C_{a}$ was varied in steps, and gas and $^{13}$C isotopic exchange were measured simultaneously. In *Setaria* ($n=4$) $C_{a}$ was set at 5, 7, 10, 12, 14, 19, 28, 38, 56, and 93 Pa, and measurements were performed at $T_{l}$=10, 25 and 40 °C. In *Zea* ($n=3$), $C_{a}$ was set at 9, 14, 19, 35, 56, 84, and 112 Pa, and $T_{l}$=25 °C. In both species the measurements were performed in the sequence ambient – low – ambient – high $pCO_{2}$.

**Enzyme-limited $C_{4}$ photosynthesis model for CA-limited or CA-saturated conditions**

The enzyme-limited $C_{4}$ photosynthesis rate is (von Caemmerer, 2000):

$$A = \frac{-b - \sqrt{b^2 - 4ac}}{2a},$$  
Eqn 1

where:

$$a = 1 - \frac{\alpha K_{c}}{\tau_{u} K_{o}},$$  
Eqn 2

$$b = \frac{\alpha K_{c}}{\tau_{u}} - \frac{\alpha C_{a}}{\tau_{u}},$$  
Eqn 3

$$c = \frac{\alpha C_{a}}{\tau_{u}} - 4 \tau_{u} C_{a}.$$

Eqn 4
where $\alpha (=0)$ is the fraction of PSII activity in the bundle-sheath cells (von Caemmerer, 2000); $\omega_{bs}$ is the ratio of $O_2$ and CO$_2$ diffusivities and solubilities, 0.047 at 25 °C but variable with temperature (Yin et al., 2016); $g_{bs}$ is the bundle-sheath conductance, 0.0164 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (Ubierna et al., 2013) or variable; $O_{bs}$ is the O$_2$ partial pressure in the mesophyll (19.5 kPa, which corresponds to 21%); $R_d$ is the non-photorespiratory CO$_2$ released in the dark, assumed to equal measured rates of dark respiration after 30 min of dark adaptation, which at 25 °C were 1.89 and 1.06 µmol m$^{-2}$ s$^{-1}$ in Zea and Setaria, respectively, but were also measured at each temperature; $R_m$ is the mesophyll mitochondrial respiration rate, $R_m=0.5R_d$ (von Caemmerer, 2000); $\gamma^*$ is half of the reciprocal of Rubisco specificity, and equals 0.5/S$_{CO}$ (von Caemmerer, 2000), where S$_{CO}$ is the Rubisco CO$_2$/O$_2$ specificity. $K_r$, $K_c$, and $K_O$ are the Michaelis–Menten constants of Rubisco for CO$_2$ and O$_2$, respectively. S$_{CO}$, $K_r$, $K_c$, and $K_O$ were determined in vitro at 25 °C in Zea (S$_{CO}$=2147 Pa Pa$^{-1}$, $K_r$=96 Pa, $K_c$=49 683 Pa, R.A. Boyd, Washington State University, pers. comm.) and Setaria (S$_{CO}$=1310 Pa Pa$^{-1}$, $K_c$=121 Pa, $K_O$=29 200 Pa; Boyd et al., 2015). Their values at different temperatures were obtained using the temperature functions of Boyd et al. (2015). For $V_{cmax}$ (maximal Rubisco carboxylation rate) we used in vivo values calculated as described in Ubierna et al. (2017) or as specified otherwise. The calculation of $C_m$ (pCO$_2$ in the mesophyll cells) will be discussed subsequently.

CA-saturated and CA-limited models differ as follows.

(1) The calculation of PEP carboxylation rate ($V_p$):

$$ V_p = \begin{cases} \text{CA saturated} & \frac{C_m V_{\text{max}}}{C_m + K_p} \\ \text{CA limited} & \frac{[\text{HCO}_3^-] V_{\text{max}}}{K_{CA} + [\text{HCO}_3^-]} \end{cases} $$

where the maximal PEP carboxylation rate ($V_{\text{max}}$) was measured in vitro at 25 °C in Zea (184 µmol m$^{-2}$ s$^{-1}$, R. A. Boyd, pers. comm.) and in Setaria (450 µmol m$^{-2}$ s$^{-1}$, Boyd et al. 2015) and varied with temperature as described in Boyd et al. (2015). For all species, the Michaelis–Menten constant of PEPC for CO$_2$ ($K_p$) was modeled with the temperature response and value at 25 °C (60.5 µM HCO$_3^-$) from Boyd et al. (2015). The $K_{CA}$ was calculated as previously discussed (Jenkins et al., 1989; Hatch and Burnell, 1990; Boyd et al., 2015) for details see Ubierna et al. (2017).

If the rate of PEP regeneration is limiting, then $V_p$ is (von Caemmerer, 2000):

$$ V_p = \min\{V_p \text{ calculated with Eqn 5}, 5V_p\} $$

where $V_{pr}$ is the PEP regeneration rate (Peisker, 1986; Peisker and Henderson, 1992). We arbitrarily set $V_{pr}$ to 64 and 59 µmol m$^{-2}$ s$^{-1}$ in Setaria and Zea, respectively, which corresponded to twice the maximum measured net assimilation rate, $A$.

(2) The calculation of the ratio $V_p/V_h$, where $V_h$ is hydration rate:

$$ \begin{cases} V_p = \frac{\text{CA saturated}}{0} \\ \text{CA limited} & \frac{V_h}{C_m K_{CA}} \end{cases} $$

where $K_{CA}$ is the rate constant of CA for CO$_2$, that at 25 °C was 65.5 and 124 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ in Zea and Setaria, respectively (R.A. Boyd pers. comm., Boyd et al., 2015), varying with temperature as described in Boyd et al. (2015).

**Measurements and models of discrimination**

The observed photosynthetic discrimination against $^{13}$C ($\Delta_{13}^{\text{obs}}$) is calculated as (Evans et al., 1986):

$$ \Delta_{13}^{\text{obs}} = \frac{C_{m} - C_{\text{out}}}{1 + \delta_{\text{out}} - \delta_{m}} $$

where $C$ and $\delta$ are the $^{12}$CO$_2$ mol fraction and the $\delta^{13}$C of the CO$_2$, respectively, in dry air in and out the chamber.

The theoretical model for $\Delta^{13}$C is (Farquhar and Cernusak, 2012):

$$ \Delta_{13}^{\text{theo}} = \frac{1}{1 - t} \left[ a_{m} C_{i} - C_{m} \right] + a_{i} C_{i} - C_{m} = \frac{1 + t}{1 - t} \left[ a_{m} C_{i} - C_{m} \right] + a_{i} C_{i} - C_{m} $$

Values and calculations of the variables included in this equation have been discussed before (i.e. Ubierna et al., 2017) and can also be found in Supplementary Methods S1 at JXB online.

**Calculation of mesophyll conductance ($g_m$)**

Following Fick’s law of diffusion:

$$ g_m = \frac{A}{C_i - C_m}, $$

where the $C_m$ is calculated for two case scenarios, CA-saturated and CA-limited, resulting in CA-sat $g_m$ and CA-lim $g_m$ values. In both cases, $C_m$ is derived with the in vitro $V_{\text{max}}$ method as the $C_m$ that needs to be combined with in vitro $V_{\text{max}}$ to match measurements and predictions of $A$ and $\Delta^{13}$C (Eqns 1, 9); details on these calculations have been provided in Ubierna et al. (2017). The CA-sat and CA-lim options are introduced through the calculation of $V_p$ and $V_h/V_b$ (Eqns 5–7).

**Limitations to photosynthesis**

To calculate the limitation on CO$_2$ assimilation by either finite stomatal conductance ($L_s$), by mesophyll conductance ($L_m$), or by carbonic anhydrase ($L_{CA}$), we adapted to C$_4$ photosynthesis an approach previously used for C$_3$ photosynthesis. This compares $A$ when all conductances are finite with the $A$ estimated assuming that the conductance related with the limitation of interest is infinite (Farquhar and Sharkey, 1982; Warren et al., 2003). In all cases $A$ was calculated with Eqn 1 and assuming:

(a) $A_{s}$ (expected $A$ with all limitations, = measured photosynthetic rate): finite $g_i$ and $g_m$, CA-lim model.

(b) $A_{i}$ (expected $A$ if there were no stomatal limitations): infinite $g_i$ ($C_i=C_m$), finite $g_m$, CA-lim model.

(c) $A_{m}$ (expected $A$ if there were no mesophyll limitations): infinite $g_m$ ($C_m=C_i$), $g_i$ as measured, CA-lim model.

(d) $A_{CA}$ (expected $A$ if there were no CA limitations): $g_i$ as measured, $g_m$ finite, CA-sat model.
Then $L_v$, $L_m$, and $L_{CA}$ were calculated as:

\[ L_v = 100 \times \frac{A - A_{all}}{A_v} \]  
\[ L_m = 100 \times \frac{A - A_{all}}{A_m} \]  
\[ L_{CA} = 100 \times \frac{A_{CA} - A_{all}}{A_{CA}}. \]

\[ \text{Eqn 11} \]
\[ \text{Eqn 12} \]
\[ \text{Eqn 13} \]

Calculation of leakiness ($\phi$)

The $C_4$ photosynthesis model (von Caemmerer, 2000) calculates $\phi$ as:

\[ \phi = \frac{g_{ms}(C_{bs} - C_m)}{V_p}, \]

\[ \text{Eqn 14} \]

where $C_{bs}$, the $pCO_2$ in the bundle-sheath cells, is (von Caemmerer, 2000):

\[ C_{bs} = \frac{\gamma' O_i + K_C \left(1 + \frac{O_i}{K_C} \right) \left( \frac{A + R_d}{V_{max}} \right) - \frac{A - R_m}{g_{ms}}}{1 + \frac{A + R_d}{V_{max}}}, \]

\[ \text{Eqn 15} \]

where $O_i$ is the O$_2$ partial pressure in the bundle-sheath cells. From $\Delta^{13}$C (Eqn 9), $\phi$ is solved as:

\[ \phi = \frac{C_{bs} - C_m}{C_m} \times \frac{\Delta_{13}^{13} C_{bs} - \bar{\phi}(C_{bs} - C_m)}{[1 + \beta_i(C_{bs} - C_m) + a_m(C_{bs} - C_m)]} \]

\[ \text{Eqn 16} \]

where $\beta_i$ (combined effects of Rubisco fractionation, and fractionations associated with respiration and photorespiration) and $\beta_s$ (combined fractionation during PEP carboxylation, hydration, and respiration) are calculated as (Farquhar, 1983; Cousins et al., 2006):

\[ \beta_i = \beta_i \left(1 - \frac{0.5fV_p}{V_v} \right), \]

\[ \text{Eqn 17} \]

\[ \beta_s = \beta_s \left(1 - \frac{V_p}{V_v} \right) + \left(\epsilon_i + h\right) \frac{V_v}{V_p} - \frac{eR_m}{V_p}. \]

\[ \text{Eqn 18} \]

A description of other variables included in Eqns 16–19 can be found in Supplementary Methods S1.

To evaluate the effect of $g_{ms}$ on calculations of $\phi$ we implemented four model scenarios, which differed in values for $g_{ms}$, calculation of $V_p$, or constrained imposed. Model 1 used in vitro $V_{pmax}$ and $g_{ms}$ finite and equal to the values for CA-lim $g_{ms}$ presented in the Results; Model 2 used in vivo $V_{pmax}$ and $g_{ms}$ infinite; Model 3 was the same as Model 1 but the solution was only constrained by $A$ and not $\Delta^{13}$C; and Model 4 was the same as Model 1 but with $V_p$ calculated with Eqn 6, which introduces a PEP regeneration limitation. The in vitro $V_{pmax}$ method calculates $g_{ms}$ by solving the system of two equations formed by the models of $A$ and $\Delta^{13}$C. Therefore, once a solution is found, $\phi$ values calculated with either Eqn 14 or 16 are identical. This is the case for Models 1, 2, and 4; however, in Model 3, which is constrained only by $A$, $\phi$ was obtained only with Eqn 14. All four modeling scenarios described above used the CA-limited calculations (Eqns 5–7).

At ambient $pCO_2$, $\phi$ was also calculated with a simplified equation derived from $\Delta^{13}$C assuming that $C_{bs}$ is much larger than $C_m$ and that hydration and assimilation fluxes are large ($V_f/V_v=0$, and $V_p=0$, where $V_o$ is oxygenation rate):

\[ \phi = \frac{\Delta_{13}^{13} C_{bs} - \bar{\phi}(C_{bs} - C_m)}{1 + \beta_i(C_{bs} - C_m) + a_m(C_{bs} - C_m)} + \frac{a_m + \bar{\beta}}{C_m}, \]

\[ \text{Eqn 19} \]

where $\bar{\beta}_i$ and $\bar{\beta}_s$ are (von Caemmerer et al., 2014):

\[ \bar{\beta}_i = \frac{eR_m}{A + 0.5R_d}, \]

\[ \text{Eqn 20} \]

\[ \bar{\beta}_s = \frac{e0.5R_d}{A + 0.5R_d}. \]

\[ \text{Eqn 21} \]

Statistical analyses

Statistical analyses were performed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA). Differences between CA-lim $g_{ms}$ and CA-sat $g_{ms}$ were investigated using t-tests ($H_o$: CA-lim $g_{ms}$/CA-sat $g_{ms}=1$). The effect of CO$_2$ supply on CA-lim $g_{ms}$ was analysed using repeated measurements ANOVA. Data were log-transformed to meet normality criteria. In Setaria we used PROC MIXED with: plant as the repeated measurement; $pCO_2$, temperature, and their interaction as fixed effects; a covariance structure of compound symmetry; and we applied Kenward–Roger’s approximation to correct the denominator degrees of freedom (Arnau et al., 2009). In Zea, we used PROC ANOVA with the statement REPEAT.

Results

A--C$_4$ curves and observed $^{13}$C photosynthetic discrimination

Under all leaf measurement temperatures ($T_L$), the rate of net photosynthesis ($A$) in Setaria increased with $C_i$ as the $pCO_2$ supplied increased from ~5 Pa to ambient air values (~35 Pa) and then leveled off (Fig. 1A). At all $pCO_2$, increasing $T_i$ resulted in larger $A$ (Fig. 1B). In Zea, $A$ also increased with increasing $C_i$ and reached a maximum at ambient air $pCO_2$ decreasing at higher $pCO_2$ (Fig. 1B).

At ambient $pCO_2$ and 25 °C, $\Delta_{13}^{13}$ was larger in Setaria (4.5 ± 0.1‰) than in Zea (3.1 ± 0.2‰) (Fig. 1C, D). In Zea, the $\Delta_{13}^{13}$ was low at ambient air $pCO_2$ and increased at lower or higher $C_i$ (Fig. 1D). However, in Setaria, $\Delta_{13}^{13}$ remained constant with $C_i$ when $T_i$=25 °C, but decreased as $C_i$ increased both at 40 and 10 °C (Fig. 1C).

Mesophyll conductance calculated assuming CA-saturated or CA-limited conditions

For both species and at all temperatures, the ratio CA-lim $g_{ms}$/CA-sat $g_{ms}$ = 1 when $pCO_2$ was above ambient (Fig. 2). As $pCO_2$ decreased, CA-lim $g_{ms}$ became larger than CA-sat $g_{ms}$; the differences increased with temperature and were larger in Zea than in Setaria. In Setaria, CA-lim $g_{ms}$ and CA-sat $g_{ms}$ were significantly different ($P<0.05$) at all $pCO_2$ at 40 °C, at all $pCO_2$ except at ambient and the measurement just above
ambient at 25 °C, and at the largest pCO₂ at 10 °C (Fig. 2A). In *Zea*, CA-lim \(g_m\) and CA-sat \(g_m\) were significantly different \((P<0.05)\) at all pCO₂/ambient air (Fig. 2B).

In *Setaria*, the under-estimation of \(g_m\) by ignoring the CA limitation was very small (maximum of 5%, CA-lim \(g_m\)/CA-sat \(g_m<1.1\); Fig. 2A). However, in *Zea*, the CA-lim \(g_m\)
calculated at the lowest $p_{CO_2}$ was 20 ± 8% larger than CA-sat $g_m$ at 25 °C. Because CA limitation was relevant at low $p_{CO_2}$, for subsequent analyses we use the CA-lim $g_m$ values for all species, temperatures, and $p_{CO_2}$.

**CO₂ response of mesophyll conductance**

The CA-lim $g_m$ significantly increased as $p_{CO_2}$ decreased in *Setaria* at all temperatures ($P<0.0001$) and in *Zea* at 25 °C ($P<0.0004$) (Fig. 3). At ambient $p_{CO_2}$ and 25 °C, CA-lim $g_m$ values (mean±SE) were 2.00 ± 0.10 μmol m⁻² s⁻¹ Pa⁻¹ in *Setaria*, and 2.43 ± 0.13 μmol m⁻² s⁻¹ Pa⁻¹ in *Zea*. At the lowest $p_{CO_2}$ measured (~5–9 Pa) and 25 °C, the CA-lim $g_m$ increased to 6.30 ± 0.32 and 16.20 ± 5.74 μmol m⁻² s⁻¹ Pa⁻¹ in *Setaria* and *Zea*, respectively. Values for $C_m$ across $C_i$ can be found in Supplementary Fig. S1.

To compare the magnitude of the change in CA-lim $g_m$ across species and temperatures, CA-lim $g_m$ was normalized by dividing each value at a given temperature and $p_{CO_2}$ by CA-lim $g_m$ at ambient $p_{CO_2}$ at that temperature (Fig. 3 D–F). At 25 °C, the increase in CA-lim $g_m$ with decreasing $p_{CO_2}$ was steeper in *Zea* than in *Setaria* (Fig. 3E). In *Setaria*, the $g_m$ $p_{CO_2}$ response was greatest at 40 °C and there was little difference between the 25 and 10 °C curves.

**Limitations to photosynthesis**

At elevated $p_{CO_2}$ assimilation rate was not limited by diffusion or substrate availability, as indicated by $L_s$, $L_m$, and $L_{CA} = 0\%$ for both species and all temperatures (Fig. 4). However, below ambient $p_{CO_2}$, the diffusional limitation to $A$ increased exponentially with decreasing $p_{CO_2}$. The data in Fig. 4 show the different limitations as a function of the amount of substrate available: $C_a$, $C_i$, and $C_m$ for $L_s$, $L_m$, and $L_{CA}$, respectively. In *Setaria*, diffusional limitations were lower at 10 °C than at any other temperature. Comparing *Zea* and *Setaria* at 25 °C, they had similar $L_s$ but $L_m$ was larger in *Setaria* than in *Zea*. For example, when $C_a=9$ Pa, $L_s=23\%$ and $19\%$ in *Setaria* and *Zea*, respectively. The corresponding $C_i$ at this $C_a$ was 5 Pa for both species, whereas $L_m$ was almost double in *Setaria* (23%) compared to *Zea* (12%) (Fig. 4C, D). In both species, $L_{CA}$ was small in comparison with $L_s$ and $L_m$, and rapidly decreased below 5% as $p_{CO_2}$ increased.

$$T_L = 40 °C \quad T_L = 25 °C \quad T_L = 10 °C$$

![Fig. 3.](image) The response of carbonic anhydrase-limited mesophyll conductance (CA-lim $g_m$) to changes in $p_{CO_2}$ inside the leaf ($C_i$) in (A, C) *Setaria viridis* (circles) and (B) *Zea mays* (white squares). *Setaria* was measured at three leaf temperatures, as indicated at the top of the figure. *Zea* was measured at $T_L=40 °C$. For comparison, the available literature reports for $\Delta^{18}O$-$g_m$ for different species and temperatures are included, as indicated in the keys: Ubierna et al. (2017) *Setaria* measured at $T_L=40 °C$, $T_L=25 °C$, and $T_L=10 °C$; Osborn et al. (2017) *Setaria* measured at $T_L=25 °C$; Barbour et al. (2016) *Setaria* measured with block temperature of 30 °C; Ubierna et al. (2017) *Zea* measured at $T_L=25 °C$; Barbour et al. (2016) *Zea* measured with block temperature of 30 °C. For all species and temperatures CA-lim $g_m$ significantly varied with $p_{CO_2}$. (D–F) The CO₂ response of normalized $g_m$ calculated by dividing individual values by the $g_m$ at ambient $p_{CO_2}$ at each temperature. Values are means ±SE; $n=4$ in *Setaria*, $n=3$ in *Zea*. The arrows indicate the values at ambient $p_{CO_2}$: black, *Setaria*; grey, *Zea*. 
Leakiness ($\phi$)

Values of $\phi$ across $p$CO$_2$ for Setaria and Zea at 25 °C calculated under different modeling assumptions are shown in Fig. 5. When $g_m$ was finite and variable with $p$CO$_2$ (Model 1), $\phi$ increased from low to high $p$CO$_2$, with a range of 0.16–0.59 in Zea and 0.45–0.76 in Setaria. Assuming that $g_m$ was infinite and $V_p$max variable with $p$CO$_2$ (Model 2) removed the $p$CO$_2$ response of $\phi$ and generally decreased $\phi$ at all $p$CO$_2$ in Setaria, but only at large $p$CO$_2$ in Zea. Model 3 resulted in nearly identical $\phi$ to Model 2 using the same finite $g_m$ as Model 1 but with the solution constrained by only the photosynthesis model. However, this scenario failed to predict $\Delta^{18}$O$_{bs}$ (see Supplementary Fig. S2). Imposing a PEP regeneration rate ($V_{pr}$) limitation of 64 and 59 μmol m$^{-2}$ s$^{-1}$ in Setaria and Zea, respectively (Model 4), decreased $\phi$ compared to the results with Model 1 in Setaria but resulted in no change in Zea. Interestingly, at $p$CO$_2$ ≤ambient air, values for $\phi$ were similar across models in Zea, but they differed in Setaria. The values of $V_{p}$max, $V_{c}$max, $V_{pr}$, $V_{c}$, $C_{bs}$, and $g_{bs}$ used in these four models are reported in Supplementary Fig. S2.

For comparison we also present $\phi$ at ambient $p$CO$_2$ calculated with the simplified Eqn 19 and assuming either $g_m$ finite or infinite. For both species, $\phi$ calculated with Eqn 19 was not different to values obtained with the complete Eqn 16 when $g_m$ was finite (compare black lines and black symbols in Fig. 5) and when $g_m$ was infinite (compare grey dashed line and clear symbols).

Discussion

Calculation of mesophyll conductance and model parameterization

Mesophyll conductance ($g_m$) was derived with the in vitro $V_{p}$max method (Ubierna et al., 2017). Estimations of $g_m$ with this method were similar to $\Delta^{18}$O$_{bs}$ across temperatures (Ubierna et al., 2017) and across $p$CO$_2$ (Kolbe and Cousins, 2018). Potential errors in $g_m$ originating from inaccurate model parameterization of the in vitro $V_{p}$max method were tested with a sensitivity analysis using Setaria data at three
temperatures and across pCO₂ (see Supplementary Fig. S3). Halving in vitro \( V_{p}\max \) increased \( g_{m} \) by <20% at large pCO₂ and almost doubled it at low pCO₂ and high temperature. Alternatively, doubling in vitro \( V_{p}\max \) decreased \( g_{m} \) by <15% at all pCO₂ and temperatures (Supplementary Fig. S3J-L). This demonstrates that uncertainties in in vitro \( V_{p}\max \) affect absolute values of \( g_{m} \) but not the trend of increasing \( g_{m} \) with decreasing pCO₂. The sensitivity analysis also demonstrated that variations up to ±50% in \( K_{p}, K_{C}, \) or \( K_{CA} \) resulted in negligible (when pCO₂ zambient) or small (at low pCO₂) errors in \( g_{m} \) calculations at any temperature (Supplementary Fig. S3A-I) and did not affect the observed trend of \( g_{m} \) with pCO₂.

In C₃ plants, it has been suggested that large \( g_{m} \) values reported for low pCO₂ might be an artifact of uncertainties in parameters such as \( R_{d} \), \( \Gamma^{*} \), and \( b_{i} \) (Gu and Sun, 2014). The simulations with different values for \( R_{d} \) (see Supplementary Fig. S4A, B) or \( b_{i} \) (Supplementary Fig. S4C, D) resulted in variations in \( g_{m} \) of <6% and did not affect the trend of increasing \( g_{m} \) with decreasing pCO₂. Ubierna et al. (2017) demonstrated that \( g_{m} \) is largely independent of values of \( g_{s} \) or \( V_{c}\max \) and this is also illustrated in Supplementary Fig. S2.

**CA-limited versus CA-saturated models to estimate \( g_{m} \)**

The substrate for the initial carboxylation by PEPC is HCO₃⁻ and not CO₂. However, \( V_{p} \) is often calculated in terms of CO₂, because the hydration of CO₂ (\( V_{h} \)) generally happens very fast when catalysed by CA (Stryer, 1988). We refer to this case as the CA-saturated model. In contrast, the CA-limited model calculates \( V_{p} \) as a function of HCO₃⁻. The value of HCO₃⁻ is calculated with \( C_{a}, V_{h}, V_{p}\max \), and a series of rate constants (see Ubierna et al., 2017, for details). Producing the same \( V_{p} \) with the CA-limited and the CA-saturated calculations requires larger \( C_{m} \) for the former than the latter, and the difference could potentially be large if \( V_{p} \) is low. Subsequently, neglecting the hydration step, as in the CA-saturated calculations, can result in under-estimation of \( C_{m} \) and \( g_{m} \). The terminology CA-saturated or -limited refers to the modeling of \( V_{p} \) and how this affects the calculated \( C_{m} \) value, but it does not imply different roles of CA in the photosynthetic process. Ubierna et al. (2017) found no difference between CA-sat \( g_{m} \) and CA-lim \( g_{m} \) at ambient pCO₂; however, the aim here is to compare these calculations for a range of pCO₂.

In both species and at all temperatures, the difference between CA-sat \( g_{m} \) and CA-lim \( g_{m} \) was negligible for pCO₂ >ambient (Fig. 2). However, as pCO₂ decreased, CA-lim \( g_{m} \) became larger than CA-sat \( g_{m} \), especially at high temperatures and in Zea. In this species ignoring the hydration step resulted in under-estimating \( g_{m} \) by as much as 20%, whereas in Setaria the under-estimation was <5%.

The larger differences at high temperatures can be explained by the temperature response of \( K_{CA} \), which increases from 10 to 30 °C but plateaus above that (Boyd et al., 2015). Species differences can be explained by different \( K_{CA} \) values and CO₂ availability to CA. Firstly, \( K_{CA} \) in Setaria (124 μmol m⁻² s⁻¹ Pa⁻¹) was double the value for Zea (65.5 μmol m⁻² s⁻¹ Pa⁻¹). Below ambient pCO₂, Setaria and Zea had similar A, \( g_{s} \), and \( C_{i} \). Sustaining similar A in these two species requires larger \( C_{a} \) in Zea than in Setaria because of the lower in vitro \( V_{p}\max \) value in the former (148 μmol m⁻² s⁻¹) versus the latter (450 μmol m⁻² s⁻¹). Therefore, in Zea the lower \( K_{CA} \) and in vitro \( V_{p}\max \) was counterbalanced by increased CO₂ availability to CA through higher \( g_{m} \). Osborn et al. (2017) also suggested large \( g_{m} \) as a mechanism to increase CO₂ assimilation rate at low pCO₂.
At low pCO₂ or in species with low Kₐ, ignoring the hydration step results in under-estimation of gₘ. However, the error is insignificant at pCO₂ above ambient or in species with large Kₐ, such as Setaria. The hydration step should be included for accurate determination of gₘ at low pCO₂ in species with low Kₐ and/or high A, such as C₄ grasses (Cousins et al., 2008), especially at high temperatures.

Values for CA-lim gₘ and variation with pCO₂

Across pCO₂ and temperatures, CA-lim gₘ ranged from 0.6 ± 0.1 to 9.3 ± 1.5 μmol m⁻² s⁻¹ Pa⁻¹ in Setaria, and 0.6 ± 0.1 to 16.2 ± 5.7 μmol m⁻² s⁻¹ Pa⁻¹ in Zea (Fig. 3). In Zea, photosynthetic rate declined above ambient pCO₂, indicating deactivation at low Cᵰ that did not fully recover when pCO₂ supply was returned to ambient levels (Fig. 1B). This could have introduced some bias in the CA-lim gₘ values calculated at high pCO₂. Nevertheless, the CA-lim gₘ values were used at pCO₂ ≥ ambient, because above ambient, photosynthesis was not restricted by diffusional limitations (Fig. 4).

To validate CA-lim gₘ values, they were compared with literature reports for the same species obtained with the alternative Δ¹⁸O method (Barbour et al., 2016; Osborn et al., 2017; Ubierna et al., 2017; Fig. 3). In Zea, there was a good agreement between Δ¹⁸O-gₘ (Barbour et al., 2016; Ubierna et al., 2017) and CA-lim gₘ (Fig. 3B). A recent study in Zea by Kolbe and Cousins (2018) also found agreement between Δ¹⁸O-gₘ and in vitro Vₚₘₐₓ gₘ across a range of pCO₂, although both estimations of gₘ deviated at very low pCO₂. In Setaria, Δ¹⁸O-gₘ (Barbour et al., 2016; Osborn et al., 2017; Ubierna et al., 2017) was larger than our CA-lim gₘ results (Fig. 3A–C). This discrepancy could have originated if in vitro Vₚₘₐₓ gₘ was over-estimated, and more studies exploring gₘ variation and assessing the impacts of the method are needed.

In Zea at 25 °C and in Setaria at three temperatures, the CA-lim gₘ increased with short-term exposure to decreasing pCO₂. Increasing gₘ with decreasing pCO₂ has also been observed in C₃ species (Bongi and Loreto, 1989; Loreto et al., 1992; Flexas et al., 2007, 2008; Hassiotou et al., 2009; Bunce, 2010; Douthe et al., 2011; Tazoe et al., 2011), although there are also a few studies that have concluded there is no change (Loreto et al., 1992; Tazoe et al., 2009). There are only two studies that have presented C₄ gₘ across pCO₂. In Osborn et al. (2017), Δ¹⁸O-gₘ values for Setaria increased with decreasing pCO₂ but the trend was not significant. In Zea, Kolbe and Cousins (2018) found a significant increase in Δ¹⁸O-gₘ with decreasing pCO₂.

The initial slope of an A-Cᵰ curve can be modified with either Cₘ (gₘ) or Vₚₘₐₓ (see Supplementary Fig. S5). Therefore, there may be a value for Vₚₘₐₓ that would cancel out the trend in CA-lim gₘ. However, this is not the case if Vₚₘₐₓ is independent of pCO₂, and cancelling the observed trend in CA-lim gₘ would require Vₚₘₐₓ to decrease with increasing pCO₂ (Supplementary Fig. S6). There is evidence showing that CO₂ levels affect the phosphorylation state of PEPC and PEPCK, and therefore variation of in vivo Vₚₘₐₓ across pCO₂ could be expected (Bailey et al., 2007). However, the CO₂ response of photosynthetic rate was found to be no different between wild-type and transgenic plants with low PEPC phosphorylation (Furumoto et al., 2007). Much of the post-translational modifications that presumably lower Vₚₘₐₓ would probably occur when CO₂ is saturating and some other factor limits C₄ photosynthesis. At ambient pCO₂ and below it is generally thought, although not known, that PEPC is operating at Vₚₘₐₓ. The fact that Δ¹⁸O-gₘ data have demonstrated a similar trend of increasing gₘ with decreasing pCO₂ (Kolbe and Cousins, 2018) points to a constant Vₚₘₐₓ value. Nevertheless, if fast in vivo regulation of Vₚₘₐₓ occurs it could alter values and trends in gₘ. In reality, there might be a combination of both fluctuations in gₘ and Vₚₘₐₓ in response to short-term variation in pCO₂. Future work should investigate in vivo regulation of Vₚₘₐₓ and its impact on gₘ calculations.

Limitation to photosynthesis at low pCO₂

C₄ photosynthesis saturates at ambient pCO₂ and A was not limited by diffusion, as indicated by Lₐ, Lₘ, and L_A = 0% for both species and all temperatures (Fig. 4). However, below ambient air pCO₂, diffusional limitations constrained CO₂ assimilation and increased exponentially with decreasing pCO₂. As shown in Fig. 1 and Supplementary Fig. S5, in both species the CO₂ responsive part of the A-Cᵰ curve corresponded to Cᵰ below ~10 Pa. This raises the question of whether C₄ plants operate below this threshold. In laboratory experiments, high irradiance and N fertilization shifted the operational Cᵰ down to the CO₂ responsive part of the A-Cᵰ curve (Ghannoum et al., 1997; Ghannoum and Conroy, 1998). Additionally, moderate water stress decreased Cᵰ in several C₄ species, although under severe drought declines in A precluded Cᵰ from getting very low (Ghannoum, 2009, and references herein). Under ambient air pCO₂, Cᵰ<11 Pa were reported for Zea grown in FACE-type experiments (Leakey et al., 2004; Markelz et al., 2011), and Sorghum bicolor grown in an open field reached Cᵰ/C₄=0.2 after two consecutive water-stress cycles (Steduto et al., 1997). Therefore, under certain growth conditions, CO₂ availability may limit C₄ photosynthesis.

Interestingly, Setaria and Zea displayed different behavior at low pCO₂. At low pCO₂, Zea was more efficient because it achieved high A despite lower Vₚₘₐₓ and Kₐ by decreasing diffusional limitations and sustaining greater Cₘ with high gₘ. The high gₘ at low pCO₂ could increase or maintain photosynthesis at low Cᵰ and could improve photosynthetic rates under situations that result in low CO₂ availability, such as drought.

In both species, the conversion of CO₂ into bicarbonate as catalysed by CA was fast enough that the hydration rate only limited A at low pCO₂ (L_a=6–16% for Cₘ<4 Pa, Fig. 4). Such low Cₘ is unlikely to occur, even under drought conditions. At these very low pCO₂, the hydration rate (Vₖ) was comparable to rates in CA-depleted transgenic plants (Supplementary Fig. S7). For example, in Setaria at 25 °C, Vₖ decreased from 581 μmol m⁻² s⁻¹ at ambient pCO₂ to 100 μmol m⁻² s⁻¹ at the lowest pCO₂ measured. Using values from Osborn et al. (2017) at 25 °C and ambient pCO₂ to calculate Vₖ as Cₘ×Kₐ resulted in 1215 and 142 μmol m⁻² s⁻¹.
for the wild type and CA-depleted transgenic, respectively. Osborn et al. (2017) concluded that in Setaria at low pCO₂, gₘ posed a greater limitation than CA activity. Our study confirms that gₘ is a major determinant of photosynthetic capacity at low pCO₂ and CA further constrains assimilation rates only at very low pCO₂. However, the CA limitation at low pCO₂ will be exacerbated at higher temperatures as the hydration rate is less able to keep up with the increase in PEPC activity (Boyd et al., 2015).

Leakiness (ϕ)

Leakiness is often estimated from comparing models and measurements of Δ¹³C assuming gₘ is infinite (Pengelly et al., 2010; Ubierna et al., 2011, 2013) or large (Kromdijk et al., 2010). Values of ϕ vary by as much as 0.04–0.9 (for a compilation of values and review of methods see Kromdijk et al., 2014), although for most species under most conditions ϕ=0.2–0.3 (Cousins et al., 2006; Kromdijk et al., 2010; Pengelly et al., 2010; Ubierna et al., 2013; Bellasio and Griffiths, 2014).

In our study, considering gₘ to be finite had a different effect on the calculation of ϕ for Setaria and Zea. At ambient air pCO₂ and 25 °C, both Setaria and Zea had similar gₘ (2.00 and 2.43 μmol m⁻² s⁻¹ Pa⁻¹, respectively). However, while ϕ in Zea was the same whether gₘ was finite or infinite, in Setaria, accounting for a finite gₘ doubled ϕ (Fig. 5, compare Models 1 and 2). This high ϕ in Setaria was driven by constraints imposed by the Δ¹³C model rather than the photosynthesis model. This is illustrated by the comparison of Models 2 and 3 (Fig. 5). Both models predicted the same A and ϕ, but Model 2 used gₘ finite (and in vitro Vₚmax) and Model 3 assumed gₘ infinite (and in vivo Vₚmax). However, Model 3 failed to predict Δ₁³C (see Supplementary Fig. S2). Forcing the solution to satisfy both models of A and Δ₁³C resulted in increases in ϕ in Setaria, but not in Zea.

This can be explained through the relationship between Δ¹³C and Cₚ/Cₐ, which is illustrated in Fig. 6 for different values of ϕ. Increasing Cₚ/Cₐ results in either increased or decreased Δ¹³C depending on whether ϕ is low (≤0.3) or high (Henderson et al., 1992; von Caemmerer et al., 2014). When Δ₁³C = 4.4–2.6 C/Cₐ = 2.9% in our data set at 25 °C and ambient pCO₂, increasing Cₚ/Cₐ results in decreased ϕ; meanwhile the opposite is true when Δ₁³C < 4.4–2.6 C/Cₐ. The value aₘ + (aₘ – aₚ)(C/Cₐ) represents the intercept of the line Δ¹³C versus Cₚ/Cₐ when Cₛ and boundary layer conductance are large and ternary effects are ignored. At ambient air pCO₂ and 25 °C, Δ₁³C = 3.1% in Zea. Therefore, varying Cₚ/Cₐ resulted in minimal changes in ϕ (compare black triangle and circle in Fig. 6). However, in Setaria, Δ₁³C = 4.5% and therefore low Cₚ/Cₐ translated into large ϕ (compare grey triangle and circle in Fig. 6). The photosynthesis model demonstrated that this increase in ϕ was achieved by increased Vₚ and gₘ (see Supplementary Fig. S2).

It is questionable that Setaria operates with ϕ=0.7, and it is seemingly unreasonable that it does. Because Δ¹³C is mostly determined by Cₚ/Cₐ and ϕ, low Cₚ/Cₐ forces the increase in ϕ. But are there any other parameters in the discrimination equation that could be manipulated in order to predict large Δ¹³C with low Cₚ/Cₐ without large ϕ? Calculations of ϕ with the complete (Eqn 16) and simplified (Eqn 19) models suggest that, at least at ambient pCO₂, this was not the case. The simplified calculation of ϕ produced values similar to the complete model, suggesting that at ambient air pCO₂ or above, modifying parameters such as Cₜₐₐ, bₚ, or bₜ within their current definition did not result in large changes in Δ¹³C.

In addition to the possible post-translational regulation of Vₚmax, PEP regeneration (Vₚ) may also influence Vₚ (Eqn 6) and estimates of ϕ. In our calculations, Vₚ=64 μmol m⁻² s⁻¹ decreased ϕ in Setaria by 0.3 and resulted in slightly larger gₘ values at high pCO₂ but no change at low pCO₂ (compare Models 1 and 4 in Fig. 5 and Supplementary Fig. S2). In fact, at low pCO₂ it is expected that Vₚ would not limit Vₚ and would have no effect on estimates of gₘ or ϕ under these conditions. Changes in ϕ in response to pCO₂ or other conditions are possible if Vₚ is allowed to vary, although at present Vₚ variation across species, temperatures, or pCO₂ is unknown. The Vₚ values that would be needed to remove the observed trend in gₘ with pCO₂ are shown in Supplementary Fig. S8. Introducing a value for Vₚ implies decoupling Vₚ from Cₙ (gₘ). In other words, the required Vₚ value to support the measured A could be achieved by choosing the adequate Vₚ rather than by varying Cₙ. This would also further complicate estimations of ϕ from Δ¹³C as Vₚ is not often measured and is not incorporated into the Δ¹³C models.

Our calculations assume that theoretical models of photosynthesis and discrimination represent the actual photosynthetic

![Fig. 6. Δ¹³C (Eqn 9) as a function of Cₚ/Cₐ for different ϕ values (indicated by the numbers at the end of each line). For calculations we used values of 37, 36, 20, and 1364 Pa for Cₚ, Cₐ, Cₚ, and Cₚ, respectively; t=0.0058, bₚ=-4.49%, and bₚ=29.87%. These values correspond to the mean values measured or calculated in Setaria at 25 °C and ambient pCO₂. Black symbols represent data for Zea and grey symbols for Setaria. For both species, ϕ was calculated assuming either gₘ, infinite (triangles) or gₘ=2.00 and 2.43 μmol m⁻² s⁻¹ Pa⁻¹ in Setaria and Zea, respectively (circles).]
process; any inaccuracy in the models will introduce error in the calculated $g_m$. We have evaluated one common modelling simplification, the effect of CA limitation, and also the impact of uncertainty on input parameters. Additionally, we have used two contrasting species to illustrate the sensitivity of $\varphi$ to $g_m$. Although a complete analysis of $\varphi$ is beyond the scope of this work, this should be undertaken in future studies together with investigations on PEP regeneration limitations. Other future focal points for research include: investigating $\text{in vivo}$ and $\text{in vitro}$ $V_{\text{max}}$ values and variation across species and environmental conditions; and compiling leaf structure, CA, aquaporins, or other data that could reveal potential mechanisms behind observed $g_m$. patterns.

**Supplementary data**

Supplementary data are available at JXB online.

Methods S1. Model of $^{13}$C discrimination in C₄ species.

Table S1. Gas exchange values for $C_i$ and $A$, and calculated values for $C_m$ and CA-limit $g_m$ in *Setaria viridis* and *Zea mays* at 25 °C and variable CO₂ supply.

Fig. S1. $C_m$ across $C_i$ in *Setaria viridis* at three temperatures, and in *Zea mays* at 25 °C.

Fig. S2. Description of the models used to evaluate the effect of $g_m$ in calculations of $\varphi$.

Fig. S3. Sensitivity of calculations of CA-limit $g_m$ in *Setaria viridis* to uncertainty in input parameters.

Fig. S4. Impact of $R_g$ and $b_5$ in the calculation of CA-limit $g_m$ in *Setaria viridis* at 25 °C.

Fig. S5. Measured versus modeled response of $A$ to $C_i$ at 25 °C in *Setaria viridis* and *Zea mays* for different values of $V_{\text{max}}$ and $g_m$.

Fig. S6. Values for $\text{in vivo}$ $V_{\text{max}}$ across $C_i$ in *Setaria viridis* calculated when CA-limit $g_m$ is constant with $p\text{CO}_2$.

Fig. S7. $V_{\text{h}}$ across $C_i$ in *Setaria viridis* at three temperatures.

Fig. S8. Values for $V_p$ across $C_i$ in *Setaria viridis* calculated when CA-limit $g_m$ is constant with $p\text{CO}_2$.

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