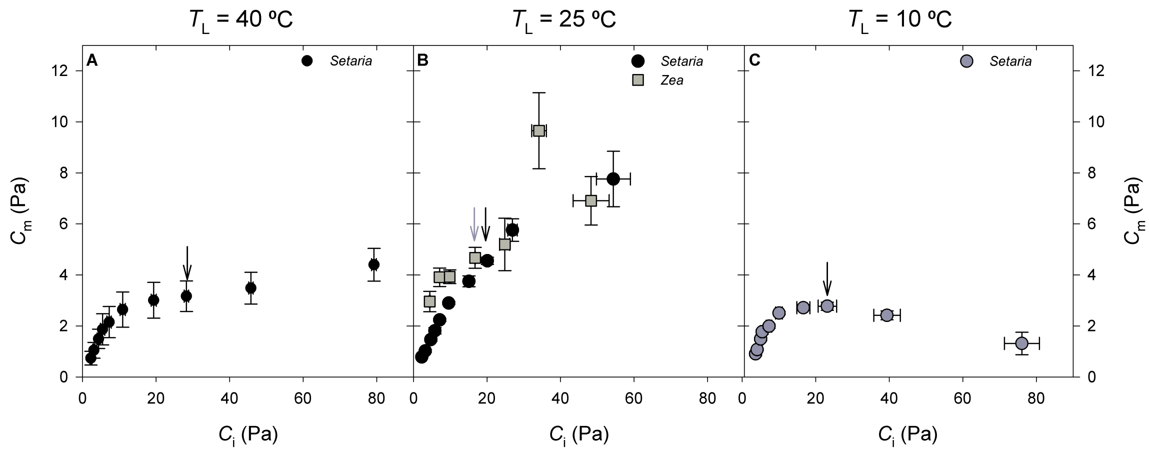
**Supplementary Figure S1.** Values for $pCO_2$ in the mesophyll ($C_m$, Pa) calculated with the carbonic anhydrase limited calculations across the $pCO_2$ inside the leaf intercellular spaces ($C_i$, Pa). Data are for *Setaria viridis* (black circles) at three temperatures ($T_L$ = 40 ºC, panel A; $T_L$ = 25 ºC, panel B; $T_L$ = 10 ºC, panel C) and for *Zea mays* (grey squares) at $T_L$ = 25 ºC. Values are means ± 1SE, *n* = 4 in *Setaria* and *n* = 3 in *Zea*. Arrows indicate values at ambient $pCO_2$.



**Supplementary Figure S2.** Description of the models used to evaluate the effect of mesophyll conductance ($g_m$) in calculations of leakiness (ϕ). Model 1: uses *in -vitro* $V_{pmax}$ and $g_m$ finite and equal to the values presented in Fig. 3 of the main manuscript; Model 2: uses *in-vivo* $V_{pmax}$ and $g_m$ infinite; Model 3: is the same than Model 1 but the solution was only constrained by $A$ and not $\Delta^{13}C$; Model 4: is the same than Model 1 but with $V_p = \min\left(V_{pr}, \frac{[HCO_3^-]V_{pmax}}{[HCO_3^-]+K_p}\right)$, where $V_{pr}$ = 64 and 59 μmol $m^{-2}s^{-1}$ in *Setaria* and *Zea*, respectively. Several parameters that describe $C_4$ photosynthesis are plotted across the $pCO_2$ inside the leaf intercellular spaces ($C_i$, Pa), namely: maximal PEP carboxylation rate ($V_{pmax}$, μmol $m^{-2}s^{-1}$), mesophyll conductance ($g_m$, μmol $m^{-2}s^{-1}Pa^{-1}$), maximal Rubisco carboxylation rate ($V_{cmax}$, μmol $m^{-2}s^{-1}$), bundle-sheath conductance ($g_{bs}$, μmol $m^{-2}s^{-1}Pa^{-1}$), $pCO_2$ in the bundle-sheath cells ($C_{bs}$, Pa), PEP carboxylation rate ($V_p$, μmol $m^{-2}s^{-1}$) and Rubisco carboxylation rate ($V_c$, μmol $m^{-2}s^{-1}$). All models applied the CA-limited calculations to *Setaria virids* and *Zea mays* data measured at 25 ºC.

The models of $A$ (photosynthetic rate, μmol $m^{-2}s^{-1}$) and $\Delta^{13}C$ ($^{13}C$ discrimination, ‰) include four variables − $C_m$, $V_{pmax}$, $g_{bs}$ and $V_{cmax}$− that are grouped in the pairs ($C_m$, $V_{pmax}$) and ($g_{bs}$, $V_{cmax}$), with the exception of the crossed term $g_{bs}C_m$ that is very small. This explains why one pair can be calculated as a function of the other pair rather than as a function of the individual values for each variable (detailed explanation can be found in Ubierna *et al.*, 2017). To illustrate that individual values chosen for $V_{cmax}$ and $g_{bs}$ do not affect the calculation of $g_m$ or ϕ, the four models were applied in two set of conditions: considering $V_{cmax}$ either variable (**A**) or constant (**B**) with $pCO_2$. In **A** we used *in-vivo* $V_{cmax}$, and in **B** we used $V_{cmax}$ = 34 and 31 μmol $m^{-2}s^{-1}$ for *Setaria* and *Zea*, respectively. Therefore, Figure S2 includes the following sub-figures:

Fig. S2.A.1. Models 1 to 4 in *Setaria virids* when $V_{cmax}$ is variable with $pCO_2$
Fig. S2.A.2. Models 1 to 4 in *Zea mays* when $V_{cmax}$ is variable with $pCO_2$
Fig. S2.B.1. Models 1 to 4 in *Setaria virids* when $V_{cmax}$ is constant with $pCO_2$
Fig. S2.B.2. Models 1 to 4 in *Zea mays* when $V_{cmax}$ is constant with $pCO_2$

**Figure S2.A.1 Models 1 to 4 in *Setaria virids* when $V_{cmax}$ is variable with $pCO_2$**

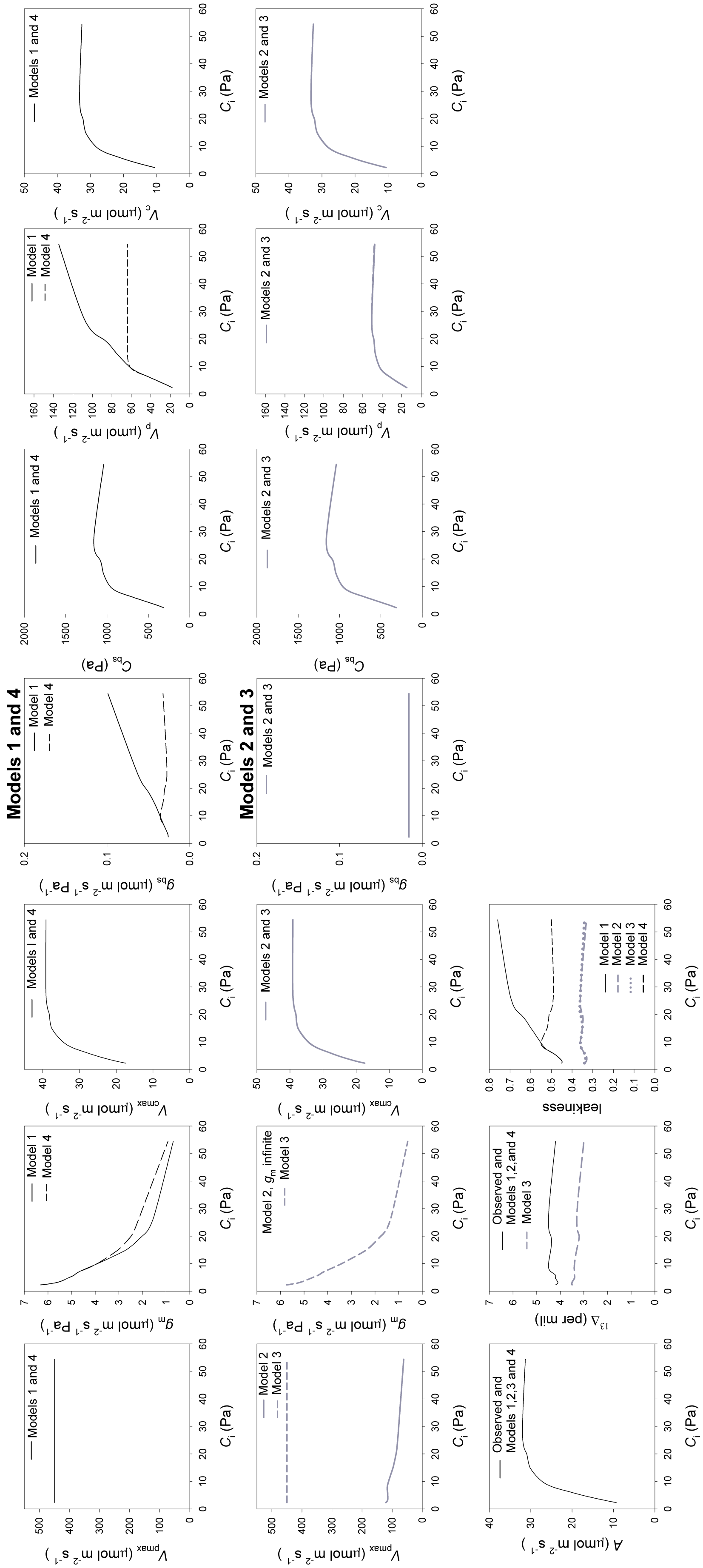


**Figure S2.A.2 Models 1 to 4 in *Zea mays* when $V_{cmax}$ is variable with $pCO_2$**

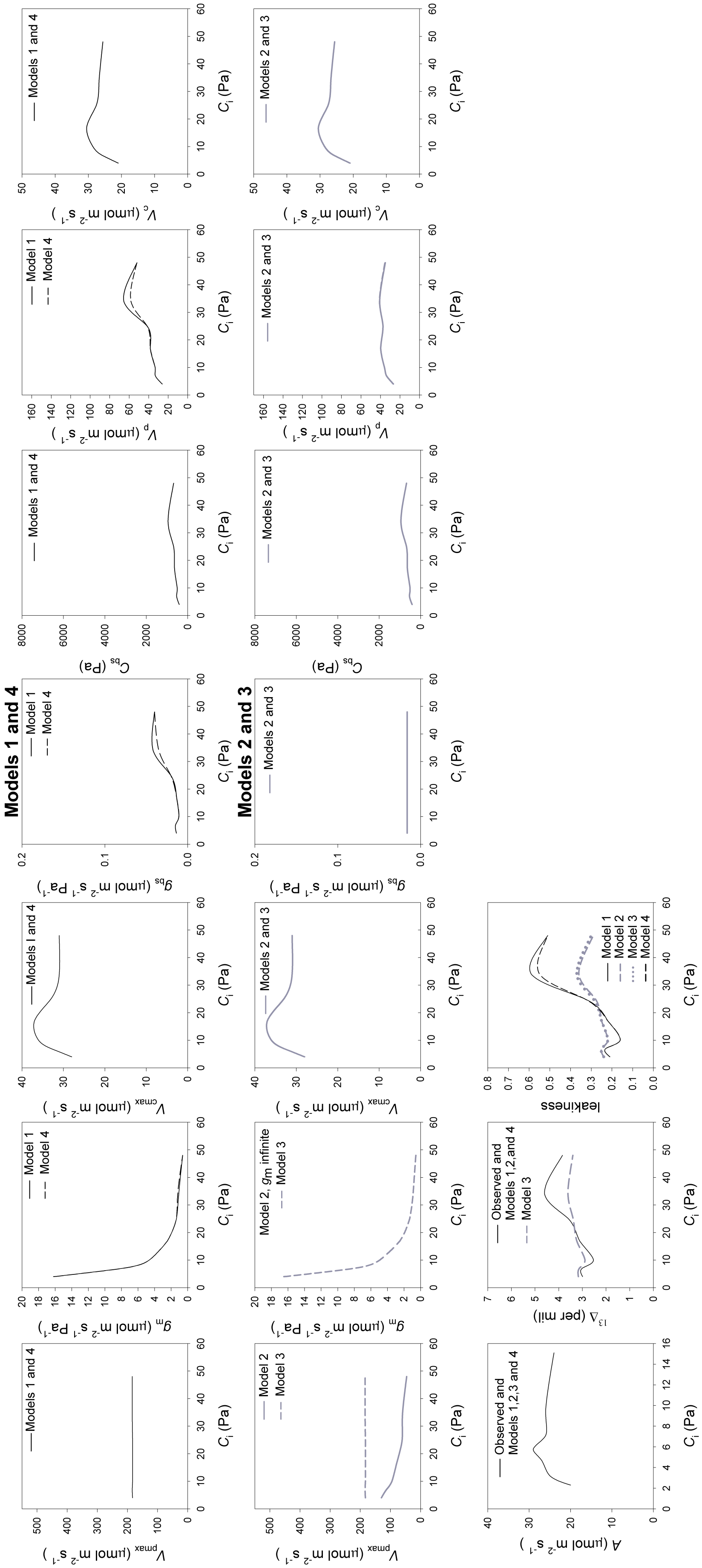


**Figure S2.B.1 Models 1 to 4 in *Setaria virids* when $V_{cmax}$ is constant with $pCO_2$**

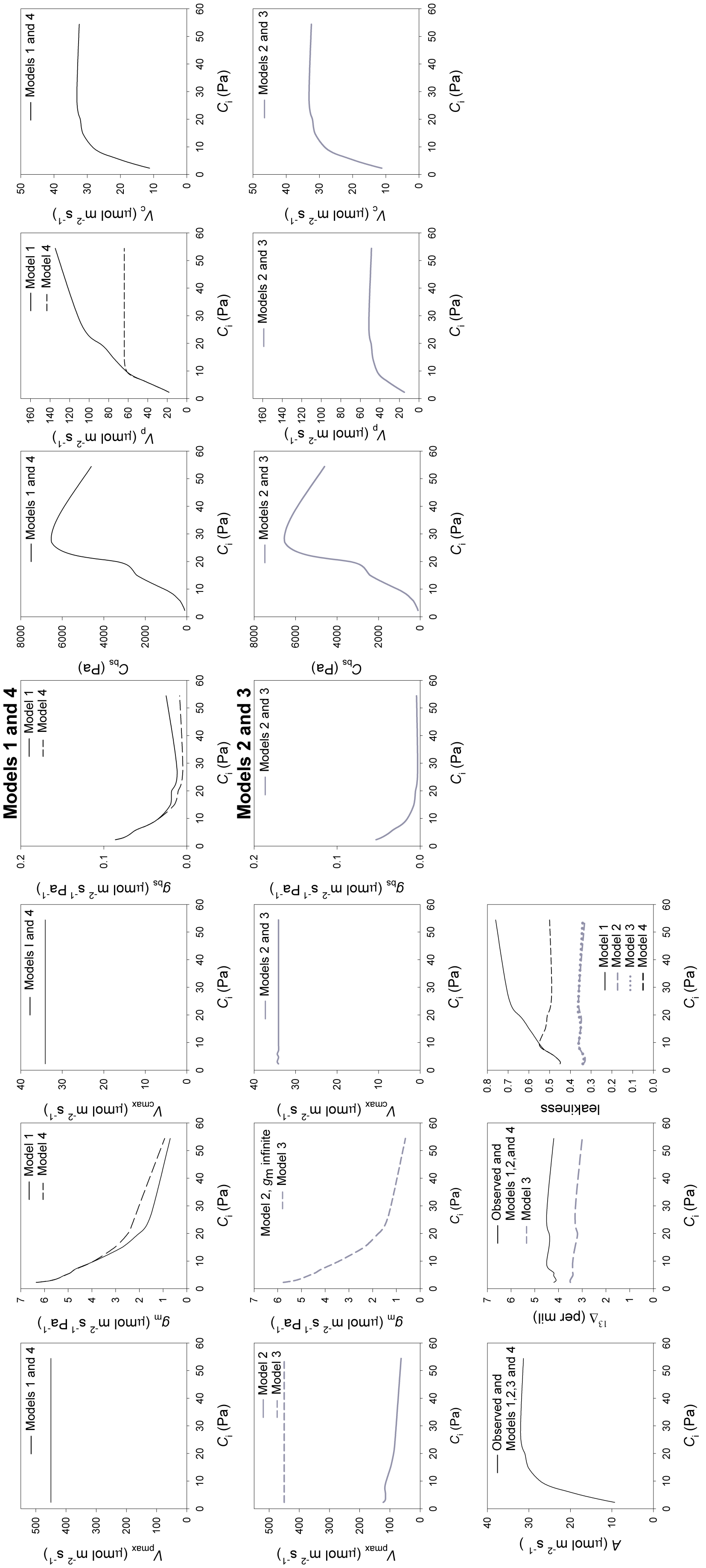
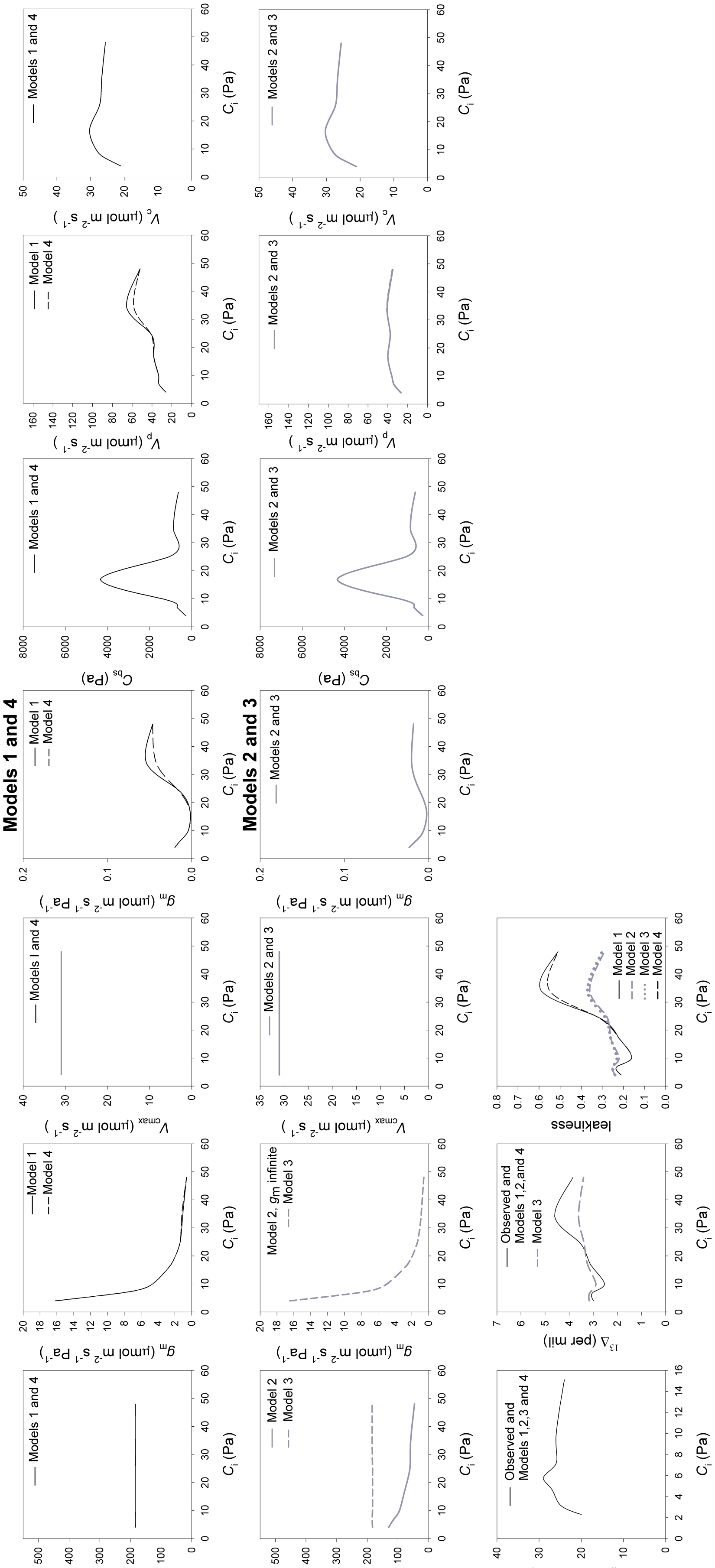
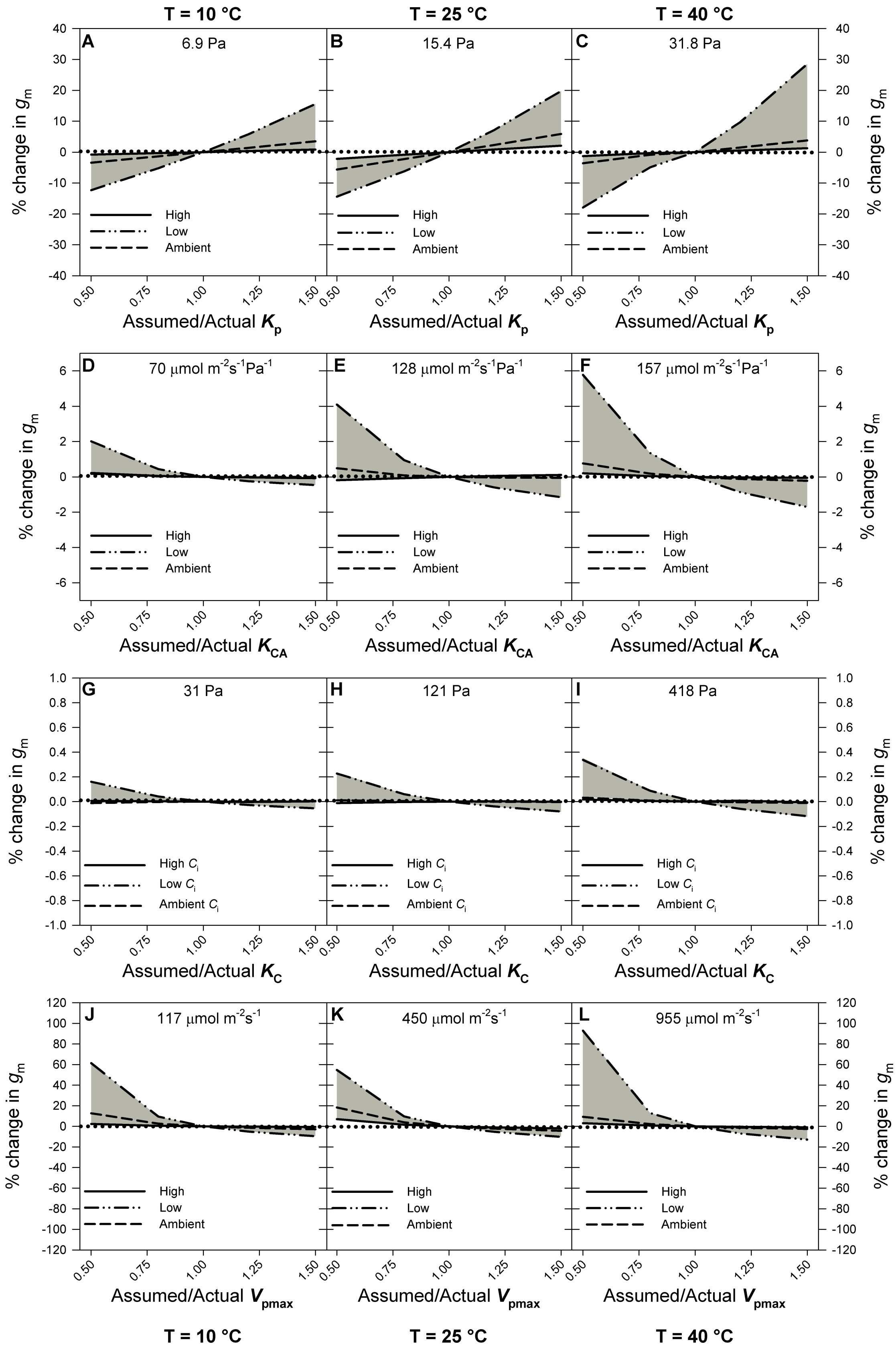


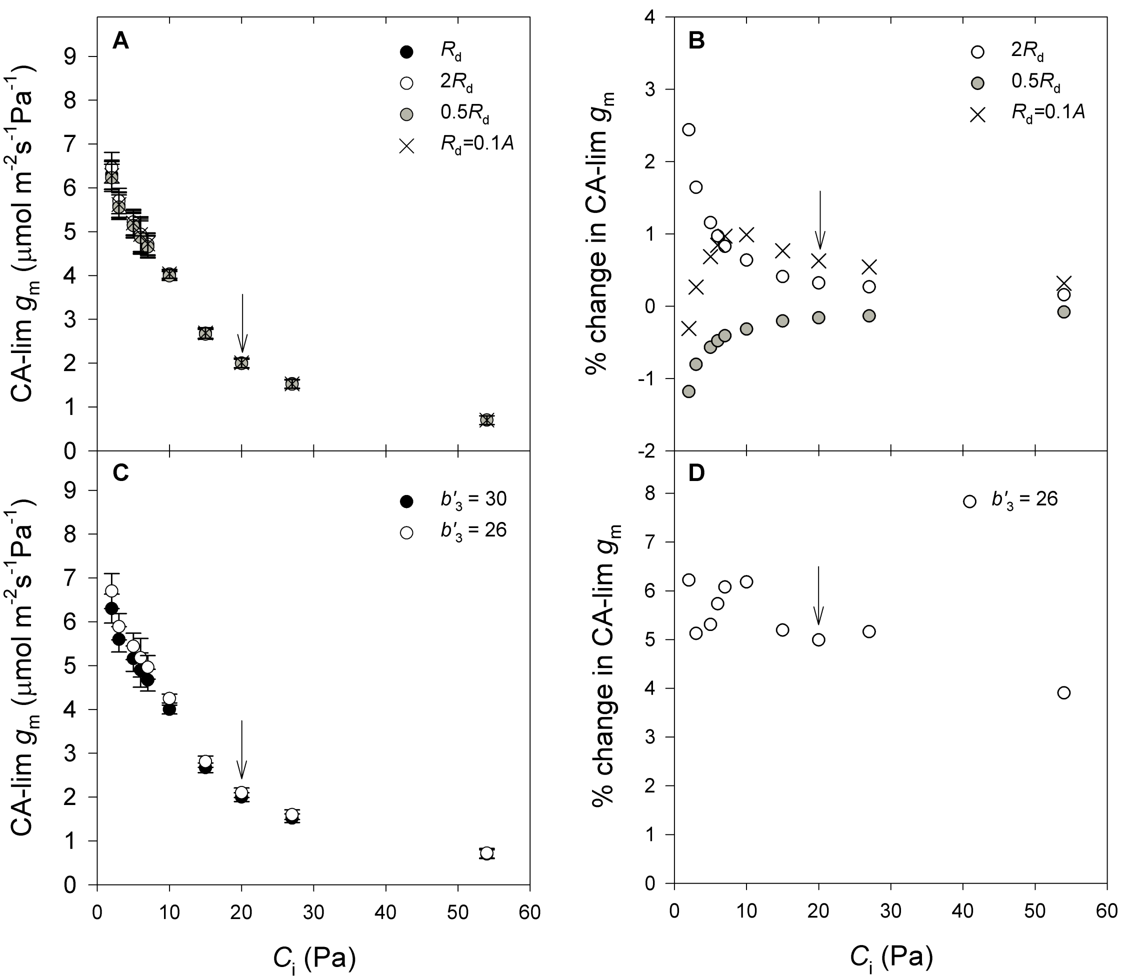
**Figure S2.B.2 Models 1 to 4 in *Zea mays* when $V_{cmax}$ is constant with $pCO_2$**



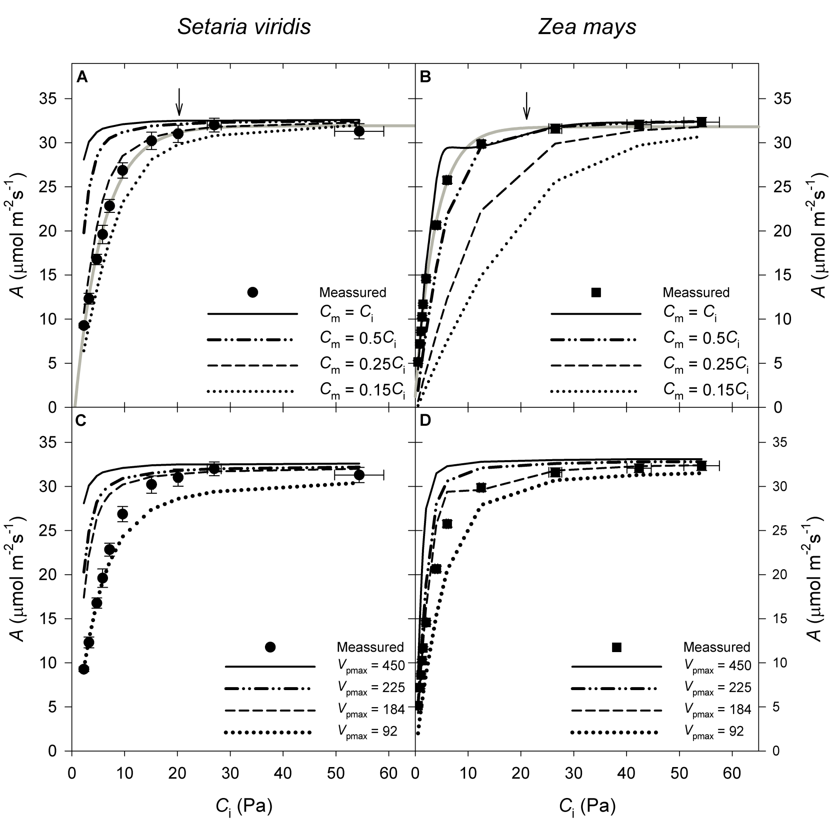
**Supplementary Figure S3.** Sensitivity of calculations of carbonic anhydrase limited mesophyll conductance (CA-lim $g_m$) in *Setaria viridis* to uncertainty in values for Michaelis-Menten constant of PEPC ($K_P$, **A - C**), rate constant of CA for $CO_2$ ($K_{CA}$, **D - F**), Michaelis-Menten constant of Rubisco ($K_C$, **G- I**), and maximal PEP carboxylation rate ($V_{pmax}$, **J - L**). Calculations were performed at three temperatures (10, 25 and 40 ºC) and three $pCO_2$ ($C_a$ = 93, 38 and 5-10 Pa for high - solid line -, ambient - dashed line -, and low - dashed and dotted line -, respectively). The numbers on top of each panel represent the actual parameter value at each temperature. Each parameter was varied ± 20 and ± 50% of its actual value and the % change in $g_m$ was calculated by comparing CA-lim $g_m$ results calculated with the assumed versus the actual parameter value.


T = 10 °C
T = 25 °C
T = 40 °C
A 6.9 Pa
B 15.4 Pa
C 31.8 Pa
% change in $g_m$
High
Low
Ambient
Assumed/Actual $K_p$
D 70 μmol m$^{-2}$s$^{-1}$Pa$^{-1}$
E 128 μmol m$^{-2}$s$^{-1}$Pa$^{-1}$
F 157 μmol m$^{-2}$s$^{-1}$Pa$^{-1}$
Assumed/Actual $K_{CA}$
G 31 Pa
H 121 Pa
I 418 Pa
High $C_i$
Low $C_i$
Ambient $C_i$
Assumed/Actual $K_C$
J 117 μmol m$^{-2}$s$^{-1}$
K 450 μmol m$^{-2}$s$^{-1}$
L 955 μmol m$^{-2}$s$^{-1}$
Assumed/Actual $V_{pmax}$

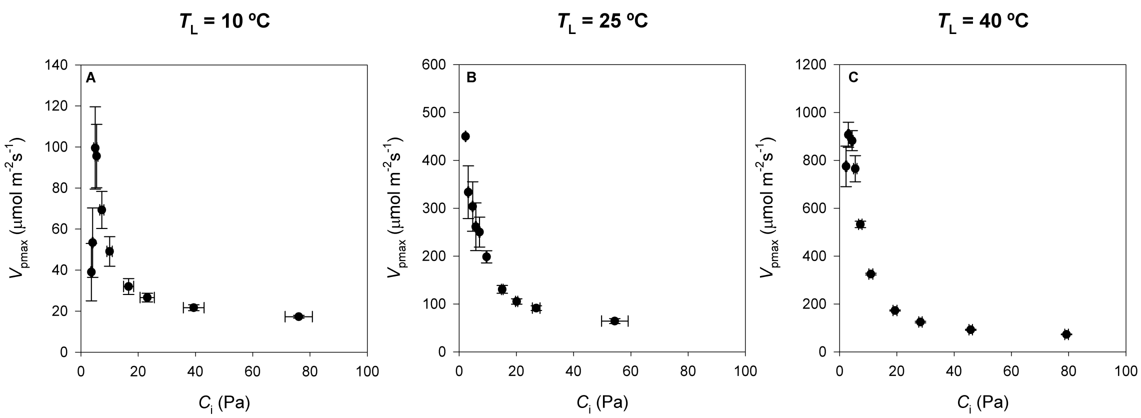
**Supplementary Figure S4.** Impact of $R_d$ (non-photorespiratory $CO_2$ released in the dark) and $b'_3$ (Rubisco fractionation) in the calculation of carbonic anhydrase limited mesophyll conductance (CA-lim $g_m$) in *Setaria viridis* at 25 ºC. **A**) and **C**) means ± 1SE, $n$ =4. **B**) and **D**) % change in CA-lim $g_m$. The reference CA-lim $g_m$ values for calculating the percentages were CA-lim $g_m$ calculated with measured $R_d$ in **B** and CA-lim $g_m$ calculated with $b'_3$ = 30‰ in **D**. Arrows indicate values at ambient $pCO_2$.



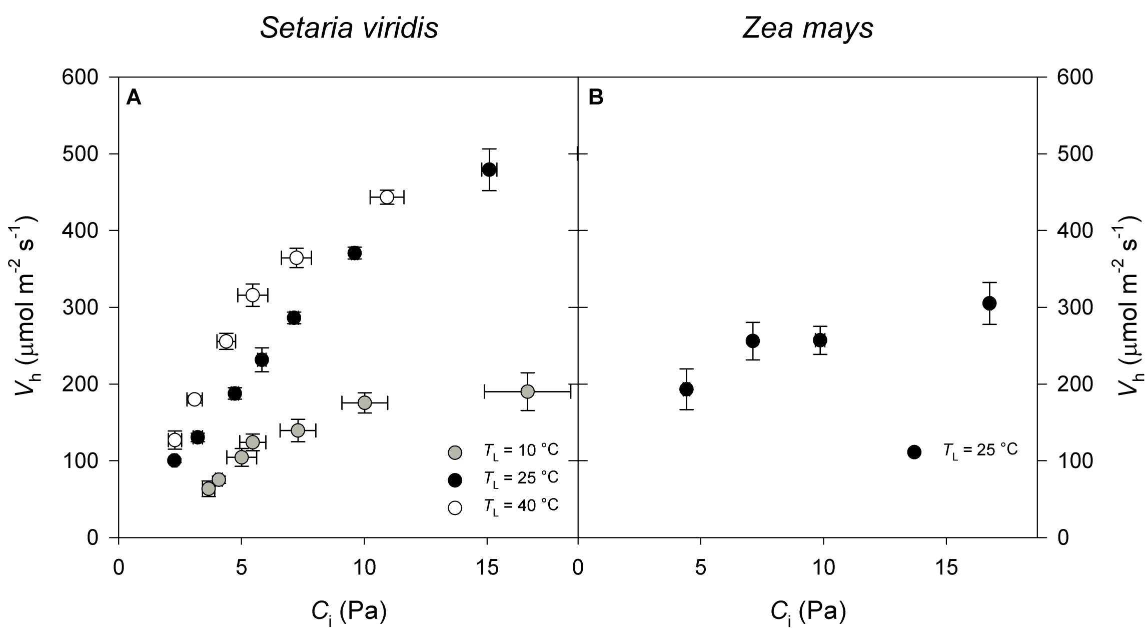
**Supplementary Figure S5.** Measured (symbols) versus modeled (black lines) response of photosynthetic rate ($A$) to variation in the leaf intercellular $CO_2$ partial pressure ($C_i$) at 25 ºC in *Setaria viridis* (**A**, **C**) and *Zea mays* (**B**, **D**). In *Zea*, the data are for a different set of 4 plants than the set used in the main document which includes more data points at low $pCO_2$. In all panels modeled $A$ was calculated with Eqn 1, assuming CA-limiting and $V_{cmax} \approx A_{max} + R_d$. In panels **A** and **B** modeled $A$ was calculated assuming $V_{pmax}$ = 450 (*Setaria*) or 184 µmol m$^{-2}$ s$^{-1}$ (*Zea*) and $C_m$ equal to $C_i$ (solid line), 0.5$C_i$ (dashed-dotted), 0.25$C_i$ (dashed) or 0.15$C_i$ (dotted). In panels **C** and **D** modeled $A$ was calculated assuming $C_m = C_i$ and $V_{pmax}$ equal to 450 µmol m$^{-2}$ s$^{-1}$ (solid line), 225 µmol m$^{-2}$ s$^{-1}$ (dashed-dotted), 184 µmol m$^{-2}$ s$^{-1}$ (dashed) or 92 µmol m$^{-2}$ s$^{-1}$ (dotted). Values are means ± 1SE, $n$ = 4. The arrows indicate the values at ambient $pCO_2$. The grey lines in panels **A** and **B** are the function fitted to the means $A = a(1 - e^{-bC_i}) + c$. Function fitting was done with SigmaPlot 11.0 (Systat Software Inc., San Jose, California, USA). *Setaria*: $a$ = 35.64 ± 1.18, $b$ = 0.19 ± 0.01, $c$ = -3.72 ± 1.26, $r^2$ = 1.00, $F(2,7)$ = 1059, $P$ < 0.0001. *Zea*: $a$ = 30.59 ± 0.52, $b$ = 0.26 ± 0.01, $c$ = 1.22 ± 0.51, $r^2$ = 1.00, $F(2,9)$ = 2280, $P$ < 0.0001.



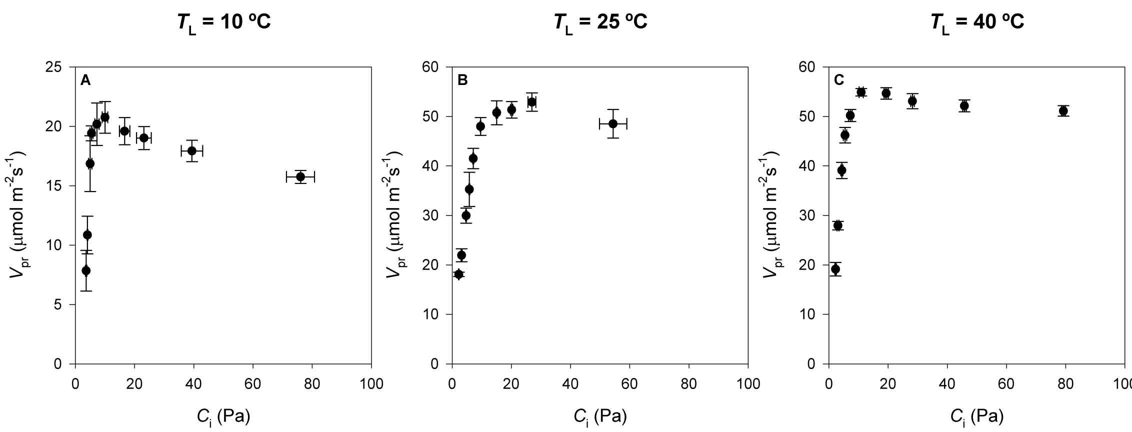
**Supplementary Figure S6.** Variation in maximum PEP carboxylation rate ($V_{pmax}$, μmol $m^{-2}s^{-1}$) across $pCO_2$ that is required to match observations of $A$ and $\Delta^{13}C$ in *Setaria viridis* with their theoretical predictions assuming that mesophyll conductance (CA-lim $g_m$) is constant with $pCO_2$ and equal to 2.7 ± 0.3, 6.3 ± 0.3 and 9.5 ± 1.4 μmol $m^{-2}$ $s^{-1}$ $Pa^{-1}$ at 10, 25, and 40 ºC, respectively. These values correspond to the maximum CA-lim $g_m$ values at each temperature derived for *Setaria viridis* with the *in-vitro* $V_{pmax}$ method. Values are means ± 1SE, $n$ = 4.



**Supplementary Figure S7.** Hydration rate ($V_h = C_m * K_{CA}$) in function of $pCO_2$ inside the leaf intercellular spaces ($C_i$) in *Setaria viridis* (**A**) and *Zea mays* (**B**). Values for three leaf temperatures ($T_L$) are presented in *Setaria*: 10 ºC (grey circles), 25 ºC (black circles) and 40 ºC (white circles), and for $T_L$ = 25 ºC in *Zea*. Values are means ± 1 SE, $n$ = 4 (*Setaria*) or 3 (*Zea*). The values for the rate constant of carbonic anhydrase (CA) for $CO_2$ at 25ºC are: $K_{CA}$ = 65.5 µmol $m^{-2}$ $s^{-1}$ $Pa^{-1}$ (*Zea*) and $K_{CA}$ = 124 µmol $m^{-2}$ $s^{-1}$ $Pa^{-1}$ (*Setaria*) (R.A. Boyd unpublished, Boyd *et al.*, 2015) and varying with temperature as described in Boyd *et al.* (2015).



**Supplementary Figure S8.** PEP regeneration rate ($V_{pr}$) as a function of $pCO_2$ inside the leaf intercellular spaces ($C_i$) needed to match observations of $A$ and $\Delta^{13}C$ in *Setaria viridis* with their theoretical predictions assuming that mesophyll conductance (CA-lim $g_m$) is constant with $pCO_2$ and equal to 2.7 ± 0.3, 6.3 ± 0.3 and 9.5 ± 1.4 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ at 10, 25, and 40 ºC, respectively. These values correspond to the maximum CA-lim $g_m$ values derived in *Setaria viridis* at each temperature with the *in-vitro* $V_{pmax}$ method. Values are means ± 1SE, $n$ = 4.




## References


**von Caemmerer S**. 2000. *Biochemical models of leaf photosynthesis*. Collingwood, Australia: CSIRO publishing.

**Boyd RA, Gandin A, Cousins AB**. 2015. Temperature responses of $C_4$ photosynthesis: biochemical analysis of Rubisco, Phospho*enol*pyruvate carboxylase, and Carbonic anhydrase in *Setaria viridis*. *Plant Physiology* **169**, 1850–1861.

**Ubierna N, Gandin A, Boyd RA, Cousins AB**. 2017. Temperature response of mesophyll conductance in three $C_4$ species calculated with two methods: $^{18}O$ discrimination and *in-vitro* $V_{pmax}$. *New Phytologist* **214**, 66-80.