Optimal coordination and reorganization of photosynthetic properties in C₄ grasses

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Running title: Optimal coordination in C₄ photosynthesis
Conflict of interest: None declared.

Paper type: Original Article
Word count: 5368
Number of figures: 7
Number of tables: 1

Supplementary material:
Supplementary Material I: supplementary dataset
Number of supplementary figures: 4
Number of supplementary tables: 11

Funding:
HZ and this research is supported by the NOAA Climate and Global Change Postdoctoral Fellowship Program, administered by UCAR's Cooperative Programs for the Advancement of Earth System Science (CPAESS) under award #NA18NWS4620043B and is also supported by the Dissertation Completion Fellowship provided by the Graduate Division of School of Arts and Sciences, University of Pennsylvania. BH is supported by NSF-IOS award 1856587.
Abstract

C₃ and C₄ are major functional types in terrestrial biosphere models, with photosynthesis traits as important input parameters. The evolution of C₄ required reorganizations of Calvin-Benson-cycle and coordination of C₄-cycle enzymes, resulting in divergences of physiological traits between C₃ and C₄. In addition, photosynthesis further optimized after the evolution of C₄ causing diversification within C₄ lineages due to different evolutionary histories. We combined optimality modeling, physiological measurements and phylogenetic analysis to examine how various aspects of C₄ photosynthetic machinery were reorganized and coordinated within C₄ lineages and as compared to closely-related C₃ in grasses.

Optimality models and measurements indicated a higher maximal electron transport to maximal Rubisco carboxylation ratio (Jₓₘₓ/Vₓₘₐₓ) in C₄ than C₃, consistent with the optimal prediction to maximize photosynthesis. The coordination between Calvin-Benson and C₄ cycles (Vₓₘₐₓ/Vₓₘₐₓ), however, is in line with the optimal modeling results under 200 ppm, as opposed to current CO₂. Such inconsistencies can be explained by a slowly declining assimilation rate beyond optimal Vₓₘₐₓ/Vₓₘₐₓ. Although rapid coordination occurred early in C₄ evolution, C₄ is still under optimizing processes and photosynthetic measures have continued to increase across time. Lastly, better understandings of Jₓₘₓ/Vₓₘₐₓ, Vₓₘₐₓ/Vₓₘₐₓ and fluorescence-based-electron-transport proffer enhanced approaches to parameterize terrestrial biosphere models.

Key words C₄ photosynthesis, electron transport, grass, optimality, Jₓₘₓ/Vₓₘₐₓ, Vₓₘₐₓ/Vₓₘₐₓ, evolution

Introduction

C₃ and C₄ are major photosynthesis pathways and important functional types identified in the
terrestrial biosphere models, e.g., Earth System Models (ESMs) and Land Surface Models (LSMs) (Booth et al., 2012; Schaefer et al., 2012; Croft et al., 2017; Rogers et al., 2017).

Photosynthetic traits and parameters of C₃ and C₄ photosynthesis, e.g., maximal Rubisco carboxylation rate ($V_{c_{max}}$) and maximal electron transport ($J_{max}$), are crucial inputs in these models. Such parameters, as well as the $J_{max}/V_{c_{max}}$, are well-documented for C₃ photosynthesis, however, the C₄ photosynthesis are still underrepresented currently due to lack of empirical reports and estimation (Bellasio et al., 2016). The evolution of C₄ occurred over an extended period that encompassed many different climate change scenarios; therefore C₄ could respond to the future climate change differently than C₃ (Ward et al., 1999; Leakey et al., 2009; Wittmer et al., 2010; Morgan et al., 2011; Reich et al., 2018). Additionally, it has recently been proposed that by taking a lineage-based, or evolutionary, approach to ESM and LSM parameterization that a more realistic approach to functional diversity can be captured by these large-scale models (Griffith et al. 2020). Thus, it is crucial to identify the trait divergences of C₄ photosynthesis from C₃, especially with regard to the photosynthetic parameters.

C₄ photosynthesis evolved as a response to inefficiencies of C₃ photosynthesis that are exacerbated under certain environmental conditions: low CO₂, drought, high temperature and high light (Ehleringer & Monson, 1993; Ehleringer et al., 1997; Edwards and Smith, 2010; Zhou et al., 2018). Rubisco, the CO₂ carboxylating enzyme of the Calvin-Benson (CB) cycle also assimilates O₂ as the first reaction of photorespiration and consequently reduces CB cycle efficiency up to 30% in C₃ species (Ehleringer et al., 1991; Bauwe et al., 2010; Raines 2011). The C₄ pathway concentrates CO₂ around Rubisco and dramatically reduces photorespiration by segregating atmospheric CO₂ uptake by Phosphoenolpyruvate carboxylase (PEPc) and CB-cycle into two compartments within the leaf. The operation of C₄
cycle does have additional ATP costs for which C₃ plants do not remunerate (Hatch, 1987).

In sum, the assembly of C₄ photosynthesis broke the balance between CO₂ carboxylation and electron transport of the CB cycle, which existed in C₃ ancestors: C₄ photosynthesis elevates the efficiency of CO₂ carboxylation at the expense of using more energy from electron transport (Sage, 2001, 2004, 2016; Christin & Osborne, 2014; Lundgren et al., 2019). Thus, a further reorganization of resource allocation between Rubisco carboxylation and electron transport and the coordination between CB and C₄ cycle should occur along with, or as a consequence of, C₄ formation. Furthermore, selection optimized the function of the C₄ through intermediate steps through adjusting physiological traits (Christin & Osborne, 2014; Sage, 2016) and different C₄ lineages evolved at different time points and endured different evolutionary histories. We therefore expect diversification in photosynthetic traits and parameters among C₄ lineages. If C₄ photosynthesis is continuously under extended optimization after its formation (Edwards, 2019; Heyduk et al., 2019), such diversification would also represent evolutionary trends between photosynthetic parameters and evolutionary time/ages. In the current study, we examine how various aspects of C₄ photosynthetic machinery— nitrogen allocation between Rubisco carboxylation and electron transport, and CB and C₄ cycle— were reorganized and coordinated within C₄ lineage, as well as compared to closely-related C₃ species, and whether the coordination of C₄ machineries are on a walk toward or have already reached the optimal state.

The relative ratio among $V_{\text{cmax}}$, $J_{\text{max}}$ and maximal PEP carboxylation rate ($V_{\text{pmax}}$) could represent the coordination within CB cycle and between CB and C₄ cycles. The resource allocation between the Rubisco carboxylation and electron transport is represented by $J_{\text{max}}/V_{\text{cmax}}$. Although $J_{\text{max}}/V_{\text{cmax}}$ has been empirically measured in numerous C₃ species (Wullschleger, 1993) and examined with optimal modeling results of $J_{\text{max}}/V_{\text{cmax}}$ for C₃
(Walker et al., 2014; Kromdijk & Long, 2016; Quebbeman & Ramirez, 2016), there have
been far fewer measurements or predictions in C₄. The coordination among CB cycle and the
C₄ cycle could be represented by the relationships among $V_{cmax}$, $J_{max}$ and $V_{pmax}$. As a
relationship between $J_{max}$ and $V_{cmax}$ has been established above, $V_{pmax}/V_{cmax}$ could be taken
into consideration to depict a complete picture of the functional coordination (Yin et al.,
2016).

In C₃ plants $J_{max}/V_{cmax}$ displays acclimation with changing environmental factors
(temperature, CO₂, light, and water availability; Onoda et al., 2005; Rodriguez-Calcerrada et
al., 2008; Kromdijk & Long, 2016; Yin et al., 2018), and it is expected that $J_{max}/V_{cmax}$ and
$V_{pmax}/V_{cmax}$ might also vary in C₄. Sage and McKown (2006) proposed that C₄ may show less
plasticity and acclimation in phenotypical traits in response to global climate change, due to
their complex anatomical and biochemical features, e.g., the structural and physiological
integration of the mesophyll-bundle sheath complex. Combining theoretical predictions and
empirical examination of how $J_{max}/V_{cmax}$ and $V_{pmax}/V_{cmax}$ vary with environment could
elucidate the acclimation capability C₄ and further show if acclimation occurs in an optimal
manner. Such an understanding of C₄ responses to changing climate would reduce the
uncertainty of global carbon flux prediction (Beerling & Quick, 1995; Quebbeman &
Ramirez, 2016; Croft et al., 2017; Rogers et al., 2017).

In addition to the coordination between CB and C₄ cycle detailed above, we expect a
coordination between cyclic electron transport and total electron transport. Cyclic-electron
transport, which produces ATP only, has been proposed to be enhanced C₄ plants as
compared to C₃ plants to fulfill the extra ATP requirements of C₄ cycle (Takabayashi et al.,
2005; Nakamura et al., 2013; Munekage, 2016; Munekage & Taniguchi, 2016; Yin & Struik,
Chlorophyll fluorescence has been widely used for C3 species to estimate the $J_{\text{max}}$ (Yin et al., 2009; Bellasio et al., 2016); however, chlorophyll fluorescences, representing the electron transport associated with linear electron transport, differs between C3 and C4 species. Lack of details in the proportion of linear electron transport in total electron transport in C4 species, therefore, hindered the use of fluorescence method to estimate electron transport in C4 species. We expected such a ratio will be lower in C4 than in C3. Establishing this ratio will provide for an additional tool to estimate $J_{\text{max}}$ using fluorescence measurements, as well as $V_{\text{cmax}}$ and $V_{\text{pmax}}$ for C4 species.

In the current study, we first used physiological models coupling photosynthesis, hydraulics and nitrogen stoichiometry to predict the optimal $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$. Then, we performed in vivo experiments to estimate these parameters empirically on grass lineages including C3 and C4 species selected from the PACMAD clade (Grass Phylogeny Working Group II, 2012; Spriggs et al., 2014) and compared these to published in vitro measurements. By sampling multiple independent origins of C4 species within a phylogenetic context (Edwards et al., 2007; Cavender-Bares et al., 2009), we were able to use phylogenetic comparative methods to examine the divergence of traits between C3 and C4 species and, to detect whether there are continuous evolutionary trends. In sum, we used optimality modeling, physiological measurements and evolutionary comparative methods to examine evolutionary trends, the approach to optimality, and to gain a better formal understanding of $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ in C4 photosynthesis to reduce uncertainty in modeling the carbon/nitrogen cycle, vegetation dynamics and productivity. Specifically, we aimed to test and examine the following hypotheses and questions. (1) Reorganization in resource allocation between Rubisco carboxylation and electron transport and coordination between CB cycle and C4 cycle in C4 resulted in higher $J_{\text{max}}/V_{\text{cmax}}$ and lower flr-ETR/$J_{\text{max}}$ than C3. (2)
C₄ values for $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ are optimized for current environment conditions. (3) Although evolving millions of years ago, selection has continued to optimize C₄ after the evolution of the CCM, and this will yield a positive evolutionary trend between photosynthesis parameters and evolutionary age. (4) To examine the acclimation capability of C₄ species using predictions and measurements of how $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ vary with environmental change.

**Materials and Methods**

**Plant material**

We cultivated 35 closely related species, including nine C₃ and 26 C₄. The species belong to nine independent origins of closely-related C₃ and C₄ lineages. Detailed cultivation information is the same as Zhou et al. (2020). We, then, extracted the dated phylogenetic tree for the species from the dated phylogenetic trees (Spriggs et al., 2014) (Fig. S1).

**Physiological Modeling**

**Optimal $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$** Based on the C₃ and C₄ models constructed in Zhou et al. (2018), which incorporates the soil-plant-air water continuum into traditional C₃ and C₄ photosynthesis models (Farquhar et al., 1980; von Caemmerer, 2000), we added stochiometric correlations between photosynthesis parameters and nitrogen. Using such a framework, we can model the optimal $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ simultaneously considering the following nitrogen stoichiometry:

The total nitrogen is the sum of different components (Evans, 1989):

$$N_{\text{org}} = N_{\text{p}} + N_{\text{E}} + N_{\text{R}} + N_{\text{S}} + N_{\text{O}},$$

(1)
in which \( N_P \) represents the nitrogen in pigment proteins, \( N_E \) represents the nitrogen for the electron transport system, \( N_R \) represents the nitrogen of Rubisco, \( N_S \) represents nitrogen in soluble proteins except for Rubisco, and \( N_O \) represents additional organic leaf nitrogen not invested in photosynthetic functions.

In order to model the optimal \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \), we need to consider the nitrogen stoichiometry among \( J_{\text{max}} \), \( V_{\text{cmax}} \) and \( V_{\text{pmax}} \). We used empirical relationships found in previous studies (Evans & Poorter, 2001; Niinemets & Tenhunen, 1997; Quebbeman & Ramirez, 2016):

\[
N_P + N_E = 0.079 J_{\text{max}} + 0.0331 \chi, \tag{2}
\]

\[
N_S = \nu J_{\text{max}}, \tag{3}
\]

\[
N_R = V_{\text{cmax}} /(6.25 \times V_{\text{cr}} \times \xi), \tag{4}
\]

\[
N_{\text{PEP}} = V_{\text{pmax}} /(6.72 \times V_{\text{pr}} \times \xi), \tag{5}
\]

\( \chi \) is the concentration of chlorophyll per unit area (\( \mu \text{mol Chl m}^{-2} \)), 0.079 is in mmol N s (\( \mu \text{mol)\}^{-1} \)), and 0.0331 is in mmol N (\( \mu \text{mol Chl)\}^{-1} \)), \( \nu \approx 0.3 \) (mmol N s (\( \mu \text{mol)\}^{-1} \)). \( V_{\text{cr}} \) is the specific activity of Rubisco (the maximum rate of RuBP carboxylation per unit Rubisco; \( \approx 20.5 \mu \text{mol CO}_2 (g \text{ Rubisco})^{-1} \text{s}^{-1} \)) and 6.25 is grams RuBisCO per gram nitrogen in RuBisCO.

\( V_{\text{pr}} \) is the specific activity of PEPC, that is, the maximum rate of RuBP carboxylation per unit PEPC (\( \approx 181.7 \mu \text{mol CO}_2 (g \text{ PEPC})^{-1} \text{s}^{-1} \)), 6.72 is grams PEPC per gram nitrogen in PEPC (calculated from the amino acids composition of Fujita et al., 1984), and \( \xi \) is the mass in grams of one millimole of nitrogen equal to 0.014 g N (mmol N)\(^{-1} \).

Further, we simplify the equation (2) by assuming there is a coordination of resource allocation between chlorophyll and electron transport for saturated light intensity, which determines the \( J_{\text{max}} \). We make this assumption for the light saturated condition and use the empirical equation of Croft et al. (2017) to equation (2)
\[ \chi = \frac{1000 J_{\text{max}}}{2.49} / \eta, \]  

(6)

where \( \eta \) is the average molar mass for chlorophyll (900 g/mol). Thus,

\[ N_{\text{org}} - N_0 = 0.079 J_{\text{max}} + 0.0331 \chi + v J_{\text{max}} + \frac{V_{\text{cmax}}}{6.25 \times V_{\text{cr}} \times \xi} + V_{\text{pmax}} / (6.72 \times V_{\text{pr}} \times \xi), \]  

(7)

When the light intensity varies, the following function is used to adjust the electron transport rate (Ögren & Evans, 1993):

\[ J = \frac{l_2 + J_{\text{max}} - \sqrt{(l_2 + J_{\text{max}})^2 - 4 l_2 J_{\text{max}}}}{2 \theta}. \]  

(8)

Also, all the photosynthetic parameters are temperature-sensitive (Zhou et al., 2018).

In the optimal modeling processes, we set \( N_{\text{org}} - N_0 \) as constant of 129 mmol N m\(^{-2}\) (which yield a \( V_{\text{cmax}} = 39 \) μmol m\(^{-2}\) s\(^{-1}\), \( J_{\text{max}} = 195 \) μmol m\(^{-2}\) s\(^{-1}\) and \( V_{\text{pmax}} = 78 \) μmol m\(^{-2}\) s\(^{-1}\), if assuming \( J_{\text{max}}/V_{\text{cmax}} = 5 \) and \( V_{\text{pmax}}/V_{\text{cmax}} = 2 \)). Using these models, we modeled the assimilation rates with different \( J_{\text{max}}/V_{\text{cmax}} \) from 1 to 8 of 0.01 interval and different \( V_{\text{pmax}}/V_{\text{cmax}} \) from 0.5 to 5 of 0.01 to find the globally optimal assimilation rate with respect to both \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \). The corresponding \( J_{\text{max}}/V_{\text{cmax}} \) or \( J_{\text{max}}/V_{\text{cmax}} \) under the highest assimilation rates represent the optimal ratios. Then, we also model the locally optimal \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \) when constraining the corresponding \( V_{\text{pmax}}/V_{\text{cmax}} \) and \( J_{\text{max}}/V_{\text{cmax}} \) with the average measured values respectively.

Using the model described above, we were able to model the optimal \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \) under different environmental gradients: CO\(_2\) of 200, 300, 400, 500 and 600 ppm; VPD and \( \psi_S \) of (0 MPa, 0.15) (0.625, -0.5), (1.25, -1), (1.875, -1.5), and (2.5, -2); light intensity of 2000, 1600, 1200, 800 and 400 μmolm\(^{-2}\)s\(^{-1}\); temperature of 15, 20, 25, 30 and 35 °C. Since there is potential uncertainty for stochiometric relationships, we performed sensitivity analysis for the total nitrogen (from 100% to 50% with 10% interval of the regular nitrogen) for optimal \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \) results and we also performed sensitivity
analysis for stoichiometry of PEPC by varying the $1/(6.72\times V_{pr} \times \xi)$ term in Eq. 5 from 50% to 800%. For the C₃ pathway, all the modeling process are similar with the C₄ and a same value of $N_{org} - N_0$ is used, except that a simplified version of equation (7) is used as below (Quebbeman & Ramirez, 2016):

$$N_{org} - N_0 = 0.079J_{max} + 0.0331 \chi + v J_{max} + \frac{V_{cmax}}{6.25 \times V_{cr} \times \xi}, \quad (9)$$

Effects of decreasing $V_{cmax}$ Using the model, we also simulated the effect of decreasing $V_{cmax}$ on the assimilation rate of both C₃ and C₄ pathway. In this modeling process, we hold $J_{max}$ and other photosynthetic parameters constant as the initial modeling condition as above, but varying the $V_{cmax}$ to 100%, 90%, 80%, 70%, 60% and 50% of the original values of C₃.

Gas exchange and fluorescence measurements

We measured $A/C_i$ curves using a LI-6400XT (LI-COR Inc., Lincoln, NE, USA) for all the species by setting the CO₂ concentrations as 400, 200, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 400, 500, 600, 700, 800, 1000, 1200, 1400 ppm under light intensity of 2000 µmolm⁻²s⁻¹. Data were recorded when the intercellular CO₂ concentration equilibrated 2-5 minutes. Fluorescence was measured along with $A/C_i$ curves using a 2 cm² fluorescence chamber head. After the change of CO₂ concentration, the quantum yield was measured by multiphase flash when $A$ reached a steady state (Bellasio et al., 2014). The leaf temperatures were controlled at 25°C, VPD varied at 1-1.7kPa and the flow rate of 500 µmol s⁻¹ for all the measurements. The cuvette was covered by Fun-Tak to lessen leakiness. We used the estimation method in Zhou et al. (2019) for $A/C_i$ curves to estimate in vivo $V_{cmax}$, $J_{max}$, and $V_{pmax}$ with one slight methodological change. Since it is thought that the region of $V_{cmax}$ limit $A/C_i$ is very narrow in C₄ species, we assigned the Cᵢ regions limited by carbonic
anhydrase and \( V_{\text{cmax}} \) with a very low criteria of 5 Pa or below. We let the data points with \( C_i \) ranging from 5 to 60 Pa \( \text{CO}_2 \) to be freely determined by which of the four potential limitation states to minimize the estimation error. Using this method, we avoided the potential bias of including optimal perspectives to the estimation method which could occur when directly assigning the cross points co-limited by \( V_{\text{cmax}}, V_{\text{pmax}} \) and \( J_{\text{max}} \). Fluorescence results were used to calculate the flr-ETR (Genty et al., 1989).

Furthermore, in vivo \( V_{\text{cmax}} \) estimated from \( A/C_i \) could be a source of uncertainty in \( \text{C}_4 \) species. In order to provide additional support to the measured \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \) value, we collected in vitro measured values for \( V_{\text{cmax}} \) and \( V_{\text{pmax}} \) from previous research, which includes eleven studies with 87 averaged results reported under current and varying environmental conditions (Supplementary Material I). Since it is impossible to obtain in vitro \( J_{\text{max}} \) and the estimation of \( J_{\text{max}} \) from \( A/C_i \) curves is considered reliable, we also obtain the corresponding \( A/C_i \) curves from these studies if they were reported, to obtain the \( J_{\text{max}} \). The combination of in vivo and in vitro measurements should yield a good representation of current \( J_{\text{max}}/V_{\text{cmax}} \) and \( V_{\text{pmax}}/V_{\text{cmax}} \) states in the \( \text{C}_4 \) plants.

**Chlorophyll measurements and leaf nitrogen**

Chlorophyll were measured using the spectrophotometer method (Porra et al., 1989). We cut the fresh leaves of species into pieces of 0.5 mm long (total leaf area was measured) and submerged the fragments into DMF. After all the Chlorophyll was extracted and the leave turned white, the supernatant was used to measure the absorption under 663.8 nm and 646 nm. Total Chlorophyll concentrations were calculated using the equation of Porra et al. (1989). We measure leaf nitrogen contents for each sample using the CHNOS analyzer (ECS4010, Costech Analytical Technologies, Inc., Valencia, CA).
Phylogenetic analysis

We fitted each of the photosynthetic parameters ($V_{\text{cmax}}$, $J_{\text{max}}$, $J_{\text{max}}/V_{\text{cmax}}$, Total Chl, flr-ETR, flr-ETR, $V_{\text{pmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$) to six different evolutionary models falling into Brownian Motion model and Ornstein-Uhlenbeck Model using the R package of “mvMORPH” (Table S1). The small-sample-size corrected version of Akaike information criterion (AICc, the lower AICc, the better fit) and Akaike weights (AICw, the higher AICw, the better fit) were used as criteria to figure out the best-fitted model. We used likelihood-ratio test (LRT) method to test whether one model variant performs significantly better than others and to determine whether there are significant differences between C$_3$ and C$_4$ species. We also extract the evolutionary ages for each C$_4$ species from the dated phylogeny (Spriggs et al., 2014). We regressed the above photosynthetic traits with evolutionary ages to detect potential evolutionary trends.

Results

In vivo and in vitro $J_{\text{max}}/V_{\text{cmax}}$ follow the global optima in C$_4$, but $V_{\text{pmax}}/V_{\text{cmax}}$ does not.

In vivo and in vitro $J_{\text{max}}/V_{\text{cmax}}$ are consistent with the optimal predictions under current CO$_2$ conditions, but $V_{\text{pmax}}/V_{\text{cmax}}$ fell in to the optimal range under CO$_2$ of 200 ppm. The global optima modelling results indicated maximal photosynthesis at the $J_{\text{max}}/V_{\text{cmax}}$ of 5.6, which is relatively constant across different CO$_2$ concentrations, while the optimal range for $V_{\text{pmax}}/V_{\text{cmax}}$ for C$_4$ species is 1.4-2.2 at CO$_2$ of 200 ppm, but decreases to 1-1.6 when CO$_2$ reaches 400 and 600 ppm (Fig. 1b, d, f). The averaged in vitro and in vivo $J_{\text{max}}/V_{\text{cmax}}$ are consistent with the global optimal predictions under CO$_2$ of 400 ppm (Fig. 1, Fig. 2a, Supplementary Material I), as well as the locally optimal predictions controlling $V_{\text{pmax}}/V_{\text{cmax}}$ at the in vivo and in vitro level (Fig. 2). However, the averages of in vitro and in vivo
$V_{\text{pmax}}/V_{\text{cmax}}$ are beyond the optimal predictions of global optima at CO$_2$ of 400 ppm, but the measurement results are consistent with the optimal condition at CO$_2$ of 200 ppm (Fig. 1, 3, 4, S2, Supplementary Material I). The 3D images and the contour plots also illustrate that when $J_{\text{max}}/V_{\text{cmax}}$ is at the optimal range, beyond the optimal range of $V_{\text{pmax}}/V_{\text{cmax}}$, the assimilation surface is quite flat: photosynthesis declines, but quite slowly; however, when $V_{\text{pmax}}/V_{\text{cmax}}$ drops outside of the optimal ranges, there are sharp decreases of photosynthesis (Fig. 1). In vitro measurements indicated large variations of $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ at the species level, and in vivo results, within a small scale of variation, fell completely into the range of in vitro results (Fig. 2a and 3a).

**C$_4$ species have higher $J_{\text{max}}/V_{\text{cmax}}$ and lower flr-ETR/$J_{\text{max}}$ than C$_3$ species.**

Phylogenetic analysis shows the $J_{\text{max}}/V_{\text{cmax}}$ follows the Ornstein-Uhlenbeck model with a higher stable state for C$_4$ species and a lower stable state for C$_3$ (Table 1; Fig. 1a). Such an empirical relationship is consistent with the optimal modeling predictions for C$_3$ and C$_4$. We looked further into how such a higher $J_{\text{max}}/V_{\text{cmax}}$ in C$_4$ species is reached by comparing individual empirical parameters. C$_4$ species has significantly higher stable states of $J_{\text{max}}$, but significantly lower stable states of $V_{\text{cmax}}$ and nitrogen content than their closely-related C$_3$ (Table 1). In order to examine the potential effects of decreasing $V_{\text{cmax}}$ on assimilation rate, we held the $J_{\text{max}}$ as constant and changed the $V_{\text{cmax}}$ from 100% to 50% of the original C$_3$ parameter values in the C$_3$ and C$_4$ models. A decrease in $V_{\text{cmax}}$ will significantly decrease the assimilation rates of C$_3$ species from 10°C to 35°C under different CO$_2$ concentrations, while decreasing $V_{\text{cmax}}$ has little effects on the assimilation rates of C$_4$ species (Fig. 5).

The flr-ETR/$J_{\text{max}}$ was significantly higher in C$_3$ species than that in their closely related C$_4$ species, with the stable states of 1.08 and 0.64 for C$_3$ and C$_4$ respectively (Table 1).
Phylogenetic analysis indicated C$_3$ and C$_4$ species had the similar stable states of ETR, but C$_4$ has a higher total electron transport rate, $J_{\text{max}}$ (Table 1).

The positive evolutionary trends of photosynthetic parameters

Plotting the photosynthetic parameters with evolutionary ages, extracted from a dated phylogeny for the multiple lineages, allows us to look for further evolutionary trends in C$_4$ and their closely related C$_3$ species. Regressions of evolutionary age versus photosynthetic traits provide signals for long-term directional trends in photosynthetic machinery following the establishment of C$_4$ photosynthesis (Fig. 6). $A_{\text{max}}$, $J_{\text{max}}$, total chlorophyll, $V_{\text{cmax}}$ and $V_{\text{pmax}}$ showed significant positive correlations with evolutionary age in C$_4$ species, but not C$_3$ species, while nitrogen, $J_{\text{max}}/V_{\text{cmax}}$, $V_{\text{pmax}}/V_{\text{cmax}}$ and flr-ETR/$J_{\text{max}}$ did not show significant correlation with the evolutionary age.

Optimal variation of $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ with environmental conditions.

To understand how the $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ varied theoretically in response to environmental changes (Fig. 7a, b, Fig. S3a, b), we calculated their optimal value for varying CO$_2$ concentrations, water limitations, temperatures, and light intensities. The optimal $J_{\text{max}}/V_{\text{cmax}}$ is predicted to increase linearly in C$_3$ and, but increase a little bit and maintain relatively constant in C$_4$ species with increasing CO$_2$ concentration (Fig. 7a). The optimal $J_{\text{max}}/V_{\text{cmax}}$ decreases similarly in both C$_3$ and C$_4$ species along with increasing water limitation and increases similarly with decreasing light intensity and increasing temperatures (Fig. 7).

The changes of $J_{\text{max}}/V_{\text{cmax}}$ with water limitation, light intensity, and temperature are non-linear, with the rate-of-change increasing greatly after a threshold (water limitation of $\psi_S$=-1, VPD=1.25, the light intensity of 800 $\mu$mol m$^{-2}$ s$^{-1}$ and temperature of 30 °C). The optimal $V_{\text{pmax}}/V_{\text{cmax}}$ decreases along with the increase of the CO$_2$ concentration, especially when CO$_2$
increases from 200 ppm to 300 ppm, but the change is little when CO$_2$ is above 400 ppm (Fig. 7c, S3c). However, $V_{pmax}/V_{cmax}$ is relatively constant with the varying of water limitation conditions and light intensity (Fig. 7c,d, S3c,d). $V_{pmax}/V_{cmax}$ decreases with the rise in temperature from 15 to 35 °C (Fig. 7d, S3d).

**Sensitivity analysis for optimal $J_{max}/V_{cmax}$ and $V_{pmax}/V_{cmax}$**

Since there is a great variation of total nitrogen content in plants and there is uncertainty in the stoichiometry of PEPC, we performed sensitivity analysis and found the optimal modeling of $J_{max}/V_{cmax}$ and $V_{pmax}/V_{cmax}$ are robust. For C$_3$ photosynthesis, optimal $J_{max}/V_{cmax}$ increases slightly with decreasing total nitrogen (7.7% increase of $J_{max}/V_{cmax}$ with 50% reduction in total nitrogen), while for C$_4$, optimal $J_{max}/V_{cmax}$ decreases with total nitrogen (16.2% increase of $J_{max}/V_{cmax}$ with 50% reduction in total nitrogen) (Fig. S4a). The optimal $V_{pmax}/V_{cmax}$ increases somewhat with decreasing total nitrogen (8.7% increase of $V_{pmax}/V_{cmax}$ with 50% reduction in total nitrogen) (Fig. S4a). The optimal $J_{max}/V_{cmax}$ is relatively constant with the change of stoichiometry of PEPC from 50% to 200%, but increases as the stoichiometry increases from 200% to 800% (Fig. S3b). The optimal $V_{pmax}/V_{cmax}$ is relatively robust with the change of stoichiometry of PEPC.

**Discussion**

Our modeling efforts provide an explanation for the observed variation in $J_{max}/V_{cmax}$ and $V_{pmax}/V_{cmax}$, and why $V_{pmax}/V_{cmax}$ appears to be optimized for the lower bounds of atmospheric CO$_2$ of the Pleistocene. Our reported value of $V_{pmax}/V_{cmax}$ are comparable with previous studies (Kubien *et al.*, 2003; Pengelly *et al.*, 2010; Yin *et al.*, 2011, 2016; Pignon and Long, 2020), and two recent papers also indicated that the coordination between CB and C$_4$ cycles might represent a legacy of ancient low CO$_2$ conditions (Sundermann *et al.*, 2018; Pignon and...
Long, 2020). All extant C₄ species have gone through a low CO₂ bottleneck over the last 5379 million years (Edwards et al., 2010). This bottleneck may have resulted in a strong selection
380 to increase $V_{\text{pmax}}/V_{\text{cmax}}$ to maintain a high assimilation rate under the low CO₂ of glacial
381 maxima (~200 ppm). As CO₂ has risen, first with the beginning of the Holocene interglacial,
382 and then again with the continual burning of fossil fuels, $V_{\text{pmax}}/V_{\text{cmax}}$ remained constant and
383 consequently exceeded the optimal $V_{\text{pmax}}/V_{\text{cmax}}$ at higher CO₂. The effects of a too-high
384 $V_{\text{pmax}}/V_{\text{cmax}}$ on assimilation rate are, however, minimal, thus the selection against increasing
385 $V_{\text{pmax}}/V_{\text{cmax}}$ was likely weak. The explanation directly rests on the topology of assimilation
386 surface: when $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ are lower than the optimal states, assimilation rate
387 declines greatly; but when $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ exceed the optimal states, the decrease
388 of assimilation rate is minimal. Similarly, if a high $J_{\text{max}}/V_{\text{cmax}}$ was selected for a given species
389 under, for example, growth at relatively low light conditions, the high $J_{\text{max}}/V_{\text{cmax}}$ could well
390 be maintained for a long time even when moving to a high light environment. Such small
391 changes in assimilation rate may not change fitness enough to drive evolution toward optimal
392 states in natural plants; artificial selection and manipulation to change the $J_{\text{max}}/V_{\text{cmax}}$ and
393 $V_{\text{pmax}}/V_{\text{cmax}}$ toward the optimal states, however, might show potential in regard to increasing
394 total assimilation rate and productivity (Walker et al., 2018; Pignon and Long, 2020).
395
397 It has been hypothesized that after the evolution of the full C₄ CCM that selection across
398 various habitats would select for further physiologically optimal adjustments (Williams et al.,
399 2013; Stata et al., 2019); we find unequal support for such adjustments through time as
400 several physiological measures were positively related to evolutionary age, but there were no
401 trends with photosynthetic coordination. From this, we conclude that initially there was very
402 strong selection for coordination between the CB cycle, light reactions and the C₄ CCM, and
403 that changes in secondary or tertiary traits led to an increase in, for example, maximum CO₂
assimilation rate through time. Zhou et al. (2020) illustrated that hydraulic traits could be just such an example of the secondary traits: the formation of C$_4$ pathway allowed for lower stomatal conductance which triggered the decline of leaf hydraulic conductance and capacitance over evolutionary time to further maximize the assimilation rate. Additionally, anatomical organization within the leaf, like the ratio of mesophyll cells to bundle-sheath cells, the 3D arrangement of cells and shifts in intercellular airspace could also be selected upon through time towards a more optimal C$_4$ photosynthetic machine (Wang et al., 2017; Alonso-Cantabrana et al., 2018; Edwards, 2019).

Higher $J_{\text{max}}/V_{\text{cmax}}$ in C$_4$ than that in C$_3$ indicated a change in resource allocation, namely nitrogen, between the light reactions and the CB cycle, and as a crucial evolutionary step for elevating C$_4$ efficiency, it is important to look at how the reallocation may have occurred and the larger-scale consequences. The modeling results indicate that a decrease of Rubisco content is favored in C$_4$, because overall nitrogen requirements decrease and such a reduction has minimal effects on net assimilation rate. Significantly lower $V_{\text{cmax}}$ in all of our C$_4$ species and lower Rubisco in previous studies confirmed the assertion (Brown, 1978; Ku et al., 1979; Sage and Pearcy, 1987; Sharwood et al., 2016). Any surplus nitrogen not invested in Rubisco could be distributed among three broad categories: 1) reallocated to the light reactions or 2) stored or used to construct new tissues or 3) simply not taken up from the growth environment, thus reducing total plant nitrogen requirements. Tissue et al. (1995) and Ghannoum et al. (2010) detected lower Rubisco content and higher chlorophyll and thylakoid content, supporting resource reallocation from RuBP carboxylation to electron transport within the leaf. Our measurements provide evidence that the coordination of $J_{\text{max}}/V_{\text{cmax}}$ resulted from a mix of hypotheses 1) and 3). The significantly higher $J_{\text{max}}$ and lower $V_{\text{cmax}}$ in C$_4$ than their closely-related C$_3$ species supports a reallocation of hypothesis 1). In addition,
hypothesis 3) likely occurred together with hypothesis 1) because C$_4$ species have significantly lower nitrogen content. Hypothesis 2), not exclusive to hypotheses 1) and 3), could be supported by evidence that C$_4$ plants maintain larger leaf areas (Ripley et al., 2008). These hypotheses are connected to potential ecological ramifications. Firstly, in a nitrogen-depleted habitat, C$_4$ could have a competitive advantage as confirmed by Ripley et al. (2008; although Sage & Pearcy, 1987, found no evidence for this). In habitats where nitrogen is not limiting, the excess nitrogen could be used to construct more leaf area (Sage & Pearcy, 1987; Anten et al., 1995; Ripley et al., 2008), and greater leaf area in the early stages of growth was indeed seen by Atkinson et al. (2016). On the other hand, the lack of nitrogen reallocation from the CB cycle to the light reactions may indicate physiological constraints in fertile habitats. For example, photorespiration in C$_3$ plants is proposed to enhance the nitrate metabolism (Oaks, 1994; Rachmilevitch et al., 2004; Bauwe et al., 2010; Bloom, 2015), therefore the formation of CCM, which inhibits photorespiration, may reduce overall plant-available nitrogen for C$_4$. In addition, the increase of $J_{\text{max}}$ in C$_4$ is due to an enhanced cyclic electron transport, while maintaining the linear electron transport at the same level of C$_3$. Elevating cyclic electron transport is therefore a potentially important step in engineering C$_4$ photosynthesis into C$_3$ crops.

Empirical evidence indicates adaptive plasticity (or acclimation) of C$_4$ photosynthetic coordination occurs under different environmental conditions, consistent with optimal predictions. Contrary to what Sage and McKown (2006) proposed, C$_4$ exhibited significant acclimation capability with varying CO$_2$ (Pinto et al. 2014; Pinto et al. 2016), water availability (Sharwood et al. 2014), light intensity (Sharwood et al. 2014; Pengelly et al. 2010; Sonawane, 2016) and temperature (Sonawane, 2016; Pittermann and Sage 2001; Kubien and Sage 2004; Serrano-Romero and Cousins, 2020) in both $J_{\text{max}}/V_{\text{cmax}}$ and
The varying trends in $J_{\text{max}}/V_{\text{cmax}}$ are generally consistent between the empirical measurements and optimal modeling predictions in the current study: higher resource allocation to electron transport under shade, elevated CO$_2$, high water availability and higher temperature. However, C$_3$ and C$_4$ species might respond differently to environmental variations, especially in response to CO$_2$ variation shown in the optimal modeling results. The $V_{\text{pmax}}/V_{\text{cmax}}$ exceeds the optimal prediction when acclimated to the current environmental condition, perhaps because $V_{\text{pmax}}/V_{\text{cmax}}$ is constraint by a legacy of acclimation to historical CO$_2$. These acclimation responses should be considered and included in ESMs and LSMs to predict responses of C$_3$ and C$_4$ ecosystems to future climate change (Rogers et al., 2017; Smith and Keenan, 2020).

Our improved estimates of $J_{\text{max}}/V_{\text{cmax}}$, $V_{\text{pmax}}/V_{\text{cmax}}$ and flr-ETR/$J_{\text{max}}$ for C$_4$ plants could directly benefit terrestrial biosphere models. Although $J_{\text{max}}$, $V_{\text{cmax}}$ and $V_{\text{pmax}}$ are key input parameters in global-scale models (Beerling & Quick, 1995; Zaehle et al., 2005; Bonan et al., 2011; Walker et al., 2014), it is difficult and perhaps not feasible to measure all parameters for numerous sites. If one the ratioed parameters described here, either $J_{\text{max}}/V_{\text{cmax}}$, $V_{\text{pmax}}/V_{\text{cmax}}$ and/or flr-ETR/$J_{\text{max}}$ is obtained then other parameters could be estimated. Using $J_{\text{max}}/V_{\text{cmax}}$, $V_{\text{pmax}}/V_{\text{cmax}}$ and flr-ETR/$J_{\text{max}}$ is especially crucial in C$_4$ species because in vivo estimation of $V_{\text{cmax}}$ and $V_{\text{pmax}}$ are more difficult and less reliable, and in vitro measurements are not easily performed over broad taxonomic or spatial scales. Our results suggest a value of around 5.5 for $J_{\text{max}}/V_{\text{cmax}}$ and a value of 2 for $V_{\text{pmax}}/V_{\text{cmax}}$ supported by optimality modeling and empirical measurements. With an empirical value of around 60% found in the current study, the measurement of flr-ETR could provide another tool to further estimate $J_{\text{max}}$, $V_{\text{cmax}}$ and $V_{\text{pmax}}$. Previous theoretical analysis also inferred values of flr-ETR/$J_{\text{max}}$ of 60% and 50% in C$_4$ (Yin et al., 2011; Yin & Struik, 2012; Yin & Struik, 2018).
The evolution of C₄ photosynthesis required the reorganization and coordination of the CB-cycle, the light reactions and the PEPC-based CCM. Strong divergence in \( J_{\text{max}}/V_{\text{cmax}} \) between C₄ and C₃ species indicates the resource allocation changes between light reactions and the CB cycle that were necessary to support the enhanced ATP requirement of the C₄ CCM (Osborn & Sack, 2012; Zhou et al., 2018). Observed \( J_{\text{max}}/V_{\text{cmax}} \) were within the predicted optimal zone suggesting that the resource reallocation between Rubisco carboxylation and electron transport are operating near optimality under current environmental conditions; however, the long tail exceeding the optimal \( J_{\text{max}}/V_{\text{cmax}} \) in empirical measurements indicates multiple species have over-allocated to electron transport, perhaps a legacy of native ecological conditions. The coordination between CB and C₄ cycles was in line with the optimal conditions under 200 ppm representing an over-allocation of resources for current environmental conditions, but there is little costs to assimilation rate due to this lack of optimality. Rapid coordination occurred early in C₄ evolution, but it appears that C₄ photosynthesis is still under selection for further optimization. The enhanced understanding of the evolution-based photosynthetic reorganization and coordination in C₄ photosynthesis, along with our ratio-based approach to obtain photosynthetic parameters can lead to a better parameterization of terrestrial biosphere models for C₄.

**Acknowledgements**

We sincerely thank Dr. Matteo Detto (Princeton University) for his help in building the optimal models for \( J_{\text{max}}/V_{\text{cmax}} \).

**Reference**
504 Alonso-Cantabrana H., Cousins A.B., Danila F., Ryan T., Sharwood R.E., von Caemmerer S., Furbank R.T. (2018). Diffusion of CO₂ across the mesophyll-bundle sheath cell interface in a C₄ plant with genetically reduced PEP carboxylase activity. Plant Physiology, 178, 72–81.

505 Anten N.P., Schieving F., Medina E., Werger M.J.A., Schuffelen P. (1995). Optimal leaf area indices in C₃ and C₄ mono-and dicotyledonous species at low and high nitrogen availability. Physiologia plantarum, 95, 541-550.

506 Atkinson R.R., Mockford E.J., Bennett C., Christin P.A., Spriggs E.L., Freckleton R.P., Thompson K., Rees M., Osborne C.P. (2016). C₄ photosynthesis boosts growth by altering physiology, allocation and size. Nature Plants, 2, 16038.

507 Bauwe H., Hagemann M., Fernie A.R. (2010). Photorespiration: players, partners and origin. Trends in plant science, 15, 330-336.

508 Beerling D.J., Quick W.P. (1995). A new technique for estimating rates of carboxylation and electron transport in leaves of C₃ plants for use in dynamic global vegetation models. Global Change Biology, 1, 289-294.

509 Bellasio C., Beerling D.J., Griffiths H. (2016). An Excel tool for deriving key photosynthetic parameters from combined gas exchange and chlorophyll fluorescence: theory and practice. Plant, cell & environment, 39, 1180-1197.

510 Bellasio C., Burgess S.J., Griffiths H., Hibberd J.M. (2014). A high throughput gas exchange screen for determining rates of photorespiration or regulation of C₄ activity. Journal of experimental botany, 65, 3769-3779.

511 Bloom A.J. (2015). Photorespiration and nitrate assimilation: a major intersection between plant carbon and nitrogen. Photosynthesis Research, 123, 117-128.

512 Bonan G.B., Lawrence P.J., Oleson K.W., Levis S., Jung M., Reichstein M., Lawrence D.M., Swenson S.C. (2011). Improving canopy processes in the Community Land Model version 4 (CLM4) using global flux fields empirically inferred from FLUXNET data. Journal of Geophysical Research: Biogeosciences, 116(G2).

513 Booth B.B., Jones C.D., Collins M., Totterdell I.J., Cox P.M., Sitch S., Huntingford C., Betts R.A., Harris G.R. and Lloyd J. (2012). High sensitivity of future global warming to land carbon cycle processes. Environmental Research Letters, 7, p.024002.

514 Brown R.H. (1978). A difference in N use efficiency in C₃ and C₄ plants and its implications in adaptation and evolution 1. Crop Science, 18, 93-98.

515 von Caemmerer S. (2000). Biochemical models of photosynthesis. In Techniques in Plant Sciences. CSIRO Publishing, Colingwood, Australia, pp 91–122.
Cavender-Bares J., Kozak K.H., Fine P.V., Kembel S.W. (2009). The merging of community ecology and phylogenetic biology. *Ecology letters*, 12, 693-715.

Christin P.A., Osborne C.P. (2014). The evolutionary ecology of C₄ plants. *New Phytologist*, 204, 765-781.

Croft H., Chen J.M., Luo X., Bartlett P., Chen B., Staebler R.M. (2017). Leaf chlorophyll content as a proxy for leaf photosynthetic capacity. *Global change biology*, 23, 3513-3524.

Edwards E.J. (2019). Evolutionary trajectories, accessibility and other metaphors: the case of C₄ and CAM photosynthesis. *New Phytologist*, 223, 1742-1755.

Edwards E.J., Osborne C.P., Strömberg C.A.E., Smith S.A., C₄ Grasses Consortium. (2010). The origins of C₄ grasslands: integrating evolutionary and ecosystem science. *Science*, 328, 587-591.

Edwards E.J., Still C.J., Donoghue M.J. (2007). The relevance of phylogeny to studies of global change. *Trends in Ecology & Evolution*, 22, 243-249.

Ehleringer J.R., Cerling T.E., Helliker B.R. (1997). C₄ photosynthesis, atmospheric CO₂, and climate. *Oecologia*, 112, 285-299.

Ehleringer J.R., Monson R.K. (1993). Evolutionary and ecological aspects of photosynthesis pathway variation. *Annual Review of Ecology and Systematics*, 24, 411-439.

Ehleringer J.R., Sage R.F., Flanagan L.B., Pearcy R.W. (1991). Climate change and the evolution of C₄ photosynthesis. *Trends in ecology & evolution*, 6, 95-99.

Evans J.R. (1989). Partitioning of nitrogen between and within leaves grown under different irradiances. *Functional Plant Biology*, 16, 533–548, doi:10.1071/PP9890533.

Evans J., Poorter H. (2001). Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area and nitrogen partitioning in maximizing carbon gain. *Plant, Cell & Environment*, 24, 755-767.

Farquhar G.D., Von Caenmerer S., Berry J.A. (1980). A biochemical model of photosynthetic carbon dioxide assimilation in leaves of 3-carbon pathway species. *Planta*, 149, 78–90.

Fujita N., Miwa T., Ishijima S., Izui K., Katsuki H. (1984). The primary structure of phosphoenolpyruvate carboxylase of Escherichia coli. Nucleotide sequence of the ppc gene and deduced amino acid sequence. *The Journal of Biochemistry*, 95, 909-916.

Genty B., Briantais J.M., Baker N.R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 990, 87-92.
Ghannoum O., Evans J.R., von Caemmerer S. (2010). Nitrogen and water use efficiency of 
C₄ plants. In: C₄ photosynthesis and related CO₂ concentrating mechanisms (eds. 
Raghavendra, A.S. & Sage, R.F.). Springer Science, The Netherlands, Dordrecht, pp. 
129–146.

Grass Phylogeny Working Group II. 2012. New grass phylogeny resolves deep evolutionary 
relationships and discovers C₄ origins. New Phytologist, 193, 304-312.

Griffith D.M., Osborne C.P., Edwards E.J., Bachle S., Beerling D.J., Bond W.J., Gallaher 
T.J., Helliker B.R., Lehmann C.E., Leatherman L. and Nippert J.B. (2020). Lineage- 
based functional types: characterising functional diversity to enhance the representation 
of ecological behaviour in Land Surface Models. New Phytologist, 228, 15-23.

Hatch M.D. (1987). C₄ photosynthesis: a unique blend of modified biochemistry, anatomy 
and ultrastructure. Biochimica et Biophysica Acta (BBA)-Reviews on Bioenergetics, 895, 
81-106.

Heyduk K., Moreno-Villena J.J., Gilman I.S., Christin P.A., and Edwards E.J. 2019. The 
genetics of convergent evolution: insights from plant photosynthesis. Nature Review 
Genetics, 20, 485-493.

Kromdijk J., Long S.P. (2016). One crop breeding cycle from starvation? How engineering 
crop photosynthesis for rising CO₂ and temperature could be one important route to 
alleviation. Proceedings of the Royal Society B: Biological Sciences, 283, p.20152578.

Ku M.S.B., Schmitt M.R., Edwards G.E. (1979). Quantitative determination of RuBP 
carboxylase-oxygenase protein in leaves of several C₃ and C₄ plants. Journal of 
experimental botany, 114, 89–98.

Kubien D.S., von Caemmerer S., Furbank R.T., Sage R.F. (2003). C₄ photosynthesis at low 
temperature. A study using transgenic plants with reduced amounts of Rubisco. Plant 
Physiology, 132, 1577-1585.

Kubien D.S., Sage R.F. (2004). Low-temperature photosynthetic performance of a C₄ grass 
and a co-occurring C₃ grass native to high latitudes. Plant, Cell & Environment, 27, 907- 
916.

Leakey A.D. (2009). Rising atmospheric carbon dioxide concentration and the future of C₄ 
crops for food and fuel. Proceedings of the Royal Society B: Biological Sciences, 276, 
2333-2343. doi: 10.1098/rspb.2008.1517

Lundgren M.R., Dunning L.T., Olofsson J.K., MorenoVillena J.J., Bouvier J.W., Sage T.L., 
Khoshravesh R., Sultmanis S., Stata M., Ripley B.S., Vorontsova M.S. (2019). C₄ 
anatomy can evolve via a single developmental change. Ecology letters, 22, 302-312.
Morgan J.A., LeCain D.R., Pendall E., Blumenthal D.M., Kimball B.A., Carrillo Y., Williams D.G., Heisler-White J., Dijkstra F.A., and West M. (2011). C₄ grasses prosper as carbon dioxide eliminates desiccation in warmed semi-arid grassland. *Nature*, 476, 202-206. doi: 10.1038/nature10274.

Munekage Y.N. (2016). Light harvesting and chloroplast electron transport in NADP-malic enzyme type C₄ plants. *Current opinion in plant biology*, 31, 9-15.

Munekage Y.N., Taniguchi Y.Y. (2016). Promotion of cyclic electron transport around photosystem I with the development of C₄ photosynthesis. *Plant and Cell Physiology*, 57, 897-903.

Nakamura N., Iwano M., Havaux M., Yokota A., Munekage Y.N. (2013). Promotion of cyclic electron transport around photosystem I during the evolution of NADP–malic enzyme-type C₄ photosynthesis in the genus Flaveria. *New Phytologist*, 199, 832-842.

Niinemets Ü., Tenhunen J.D. (1997). A model separating leaf structural and physiological effects on carbon gain along light gradients for the shade-tolerant species Acer saccharum. *Plant Cell & Environment*, 20, 845-866.

Oaks A. (1994). Efficiency of nitrogen utilization in C₃ and C₄ cereals. *Plant Physiology*, 106: p.407.

Ögren E., Evans J.R. (1993). Photosynthetic light-response curves. *Planta*, 189, 182-190.

Onoda Y., Hikosaka K., Hirose T. (2005). The balance between RuBP carboxylation and RuBP regeneration: a mechanism underlying the interspecific variation in acclimation of photosynthesis to seasonal change in temperature. *Functional Plant Biology*, 32, 903-910.

Osborne C.P., Sack L. (2012). Evolution of C₄ plants: a new hypothesis for an interaction of CO₂ and water relations mediated by plant hydraulics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367, 583-600.

Pengelly J.J., Sirault X.R., Tazoe Y., Evans J.R., Furbank R.T., von Caemmerer S. (2010). Growth of the C₄ dicot Flaveria bidentis: photosynthetic acclimation to low light through shifts in leaf anatomy and biochemistry. *Journal of experimental botany*, 61, 4109-4122.

Pignon C.P., Long S.P. (2020). Retrospective analysis of biochemical limitations to photosynthesis in 49 species: C₄ crops appear still adapted to pre-industrial atmospheric [CO₂]. *Plant, Cell & Environment*, 43, 2606-2622.

Pinto H., Powell J.R., Sharwood R.E., Tissue D.T., Ghannoum O. (2016). Variations in nitrogen use efficiency reflect the biochemical subtype while variations in water use...
efficiency reflect the evolutionary lineage of C₄ grasses at inter-glacial CO₂. *Plant, cell & environment*, 39, 514-526.

Pinto H., Sharwood R.E., Tissue D.T., Ghannoum O. (2014). Photosynthesis of C₃, C₃–C₄, and C₄ grasses at glacial CO₂. *Journal of Experimental Botany*, 65, 3669-3681.

Pittermann J., Sage R.F. (2001). The response of the high altitude C₄ grass *Muhlenbergia montana* (Nutt.) AS Hitchc. to long- and short-term chilling. *Journal of Experimental Botany*, 52, 829-838.

Porra R.J., Thompson W.A., Kriedemann P.E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 975, 384-394.

Quebbeman J.A., Ramirez J.A. (2016). Optimal allocation of leaf-level nitrogen: Implications for covariation of $V_{\text{cmax}}$ and $J_{\text{max}}$ and photosynthetic downregulation. *Journal of Geophysical Research: Biogeosciences*, 121, 2464-2475.

Rachmilevitch S., Cousins A.B., Bloom A.J. (2004). Nitrate assimilation in plant shoots depends on photorespiration. *Proceedings of the National Academy of Sciences*, 101, 11506-11510.

Raines C.A. (2011). Increasing photosynthetic carbon assimilation in C₃ plants to improve crop yield: current and future strategies. *Plant physiology*, 155, 36-42.

Reich P.B., Hobbie S.E., Lee T.D., Pastore M.A. (2018). Unexpected reversal of C₃ versus C₄ grass response to elevated CO₂ during a 20-year field experiment. *Science*, 360, 317-320. doi: 10.1126/science.aas9313

Ripley B.S., Abraham T.I., Osborne C.P. (2008). Consequences of C₄ photosynthesis for the partitioning of growth: a test using C₃ and C₄ subspecies of *Alloteropsis semialata* under nitrogen-limitation. *Journal of experimental botany*, 59, 1705-1714.

Rogers A., Medlyn B.E., Dukes J.S., Bonan G., Von Caemmerer S., Dietze M.C., Kattge J., Leakey A.D., Mercado L.M., Niinemets Ü., Prentice I.C. (2017). A roadmap for improving the representation of photosynthesis in Earth system models. *New Phytologist*, 213, 22-42. doi: 10.1111/nph.14283

Rodriguez-Calcerrada J., Reich P.B., Rosenqvist E., Pardos J.A., Cano F.J., Aranda I. (2008). Leaf physiological versus morphological acclimation to high-light exposure at different stages of foliar development in oak. *Tree physiology*, 28, 761-771.
Sage R.F. 2001. Environmental and evolutionary preconditions for the origin and diversification of the C₄ photosynthetic syndrome. *Plant Biology*, 3, 202–213.

Sage R.F. (2004). The evolution of C₄ photosynthesis. *New Phytologist*, 161, 341–370.

Sage R.F. (2016). Tracking the evolutionary rise of C₄ metabolism. *Journal of experimental botany*, 67, 2919-2922.

Sage R.F., McKown A.D. (2006). Is C₄ photosynthesis less phenotypically plastic than C₃ photosynthesis? *Journal of experimental botany*, 57, 303-317.

Sage R.F., Pearcy R.W. (1987). The nitrogen use efficiency of C₃ and C₄ plants: I. Leaf nitrogen, growth, and biomass partitioning in Chenopodium album (L.) and *Amaranthus retroflexus* (L.). *Plant Physiology*, 84, 954-958.

Schaefer K., Schwalm C.R., Williams C., Arain M.A., Barr A., Chen J.M., Davis K.J., Dimitrov D., Hilton T.W., Hollinger D.Y., Humphreys E. (2012). A model-data comparison of gross primary productivity: Results from the North American Carbon Program site synthesis. *Journal of Geophysical Research: Biogeosciences*, 117(G3).

Serrano-Romero, E.A., Cousins, A.B. (2020). Cold acclimation of mesophyll conductance, bundle-sheath conductance and leakiness in *Miscanthus* giganteus. *New Phytologist* 226, 1594-1606.

Sharwood R.E., Sonawane, B.V., Ghannoum O. (2014). Photosynthetic flexibility in maize exposed to salinity and shade. *Journal of Experimental Botany*, 65, 3715-3724.

Sharwood R.E., Ghannoum O., Whitney S.M. (2016). Prospects for improving CO₂ fixation in C₃-crops through understanding C₄-Rubisco biogenesis and catalytic diversity. *Current Opinion in Plant Biology*, 31, 135-142.

Smith N.G., Keenan T.F. (2020). Mechanisms underlying leaf photosynthetic acclimation to warming and elevated CO₂ as inferred from least-cost optimality theory. *Global Change Biology*, 26, 5202-5216.

Sonawane, B.V. (2016). Environmental regulation of CO₂ concentrating mechanisms in C₄ grasses with different biochemical subtypes (Doctoral dissertation, Western Sydney University (Australia)).

Spriggs, E.L., Christin, P.A. and Edwards, E.J. (2014). C₄ photosynthesis promoted species diversification during the Miocene grassland expansion. *Plos one*, 9, p.e97722.

Stata M., Sage T.L., Sage R.F. (2019). Mind the gap: the evolutionary engagement of the C₄ metabolic cycle in support of net carbon assimilation. *Current Opinion in Plant Biology*, 49, 27-34. doi: 10.1016/j.pbi.2019.04.008
Sundermann E., Lercher M. and Heckmann D. (2018). Modeling Cellular Resource Allocation Reveals Low Phenotypic Plasticity of C₄ Plants and Infers Environments of C₄ Photosynthesis Evolution. bioRxiv (Preprint). Available at doi: https://doi.org/10.1101/371096 (Accessed April 15, 2020).

Takabayashi A., Kishine M., Asada K., Endo T., Sato F. (2005). Differential use of two cyclic electron flows around photosystem I for driving CO₂-concentration mechanism in C₄ photosynthesis. Proceedings of the National Academy of Sciences, 102, 16898-16903.

Tissue D.T., Griffin K.L., Thomas R.B., Strain B.R. (1995). Effects of low and elevated CO₂ on C₃ and C₄ annuals. II. Photosynthesis and leaf biochemistry. Oecologia, 101, 21-28.

Walker A.P., Beckerman A.P., Gu L., Kattge J., Cernusak L.A., Domingues T.F., Scales J.C., Wohlfahrt G., Wullschleger S.D., Woodward F.I. (2014). The relationship of leaf photosynthetic traits—Vₖₗₐₓ and Jₖₗₐₓ—to leaf nitrogen, leaf phosphorus, and specific leaf area: a meta-analysis and modeling study. Ecology and evolution, 4, 3218-3235.

Walker B.J., Drewry D.T., Slattery R.A., VanLoocke A., Cho Y.B., Ort D.R. (2018). Chlorophyll can be reduced in crop canopies with little penalty to photosynthesis. Plant Physiology, 176, 1215-1232.

Wang S., Tholen D., and Zhu X.G. (2017). C₄ photosynthesis in C₃ rice: a theoretical analysis of biochemical and anatomical factors. Plant, cell & environment, 40, 80-94.

Ward J.K., Tissue D.T., Thomas R.B., Strain B.R. (1999). Comparative responses of model C₃ and C₄ plants to drought in low and elevated CO₂. Global Change Biology 5: 857-867.

Williams B.P., Johnston I.G., Covshoff S., Hibberd J.M. (2013). Phenotypic landscape inference reveals multiple evolutionary paths to C₄ photosynthesis. Elife, 2, p.e00961. doi: 10.7554/eLife.00961

Wittmer M.H., Auerswald K., Bai Y., Schaeufele R., Schnyder H. (2010). Changes in the abundance of C₃/C₄ species of Inner Mongolia grassland: evidence from isotopic composition of soil and vegetation. Global Change Biology, 16, 605-616. doi: 10.1111/j.1365-2486.2009.02033.x

Wullschleger S.D. (1993). Biochemical limitations to carbon assimilation in C₃ plants - A retrospective analysis of the A/C₄ curves from 109 species. Journal of Experimental Botany, 44, 907-920.

Yin X., Van Der Putten P.E., Driever S.M., Struijk P.C. (2016). Temperature response of bundle-sheath conductance in maize leaves. Journal of experimental botany, 67, 2699-2714.
Yin X., Schapendonk A.H., Struik P.C. (2018). Exploring the optimum nitrogen partitioning to predict the acclimation of C₃ leaf photosynthesis to varying growth conditions. *Journal of experimental botany*, 70, 2435-2447.

Yin X., Struik P.C. (2012). Mathematical review of the energy transduction stoichiometries of C₄ leaf photosynthesis under limiting light. *Plant, cell & environment*, 35, 1299-1312.

Yin X., Struik P.C. (2018). The energy budget in C₄ photosynthesis: insights from a cell-type-specific transport model. *New Phytologist*, 218, 986-998.

Yin X., Struik P.C., Romero P., Harbinson J., Evers J.B., Van Der Putten P.E., Vos J.A.N. (2009). Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C₃ photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (*Triticum aestivum*) canopy. *Plant, Cell & Environment*, 32, 448-464.

Yin X., Sun Z., Struik P.C., Van der Putten P.E., Van Ieperen W.I.M., Harbinson J. (2011). Using a biochemical C₄ photosynthesis model and combined gas exchange and chlorophyll fluorescence measurements to estimate bundle-sheath conductance of maize leaves differing in age and nitrogen content. *Plant, cell & environment*, 34, 2183-2199.

Zaehle S., Sitch S., Smith B., Hatterman F. (2005). Effects of parameter uncertainties on the modeling of terrestrial biosphere dynamics. *Global Biogeochemical Cycles* 19: GB3020.

Zhou H., Akçay E., Helliker BR. 2019. Estimating C₄ photosynthesis parameters by fitting intensive A/Cᵢ curves. *Photosynthesis research*, 141, 181-194.

Zhou H., Helliker B.R., Huber M., Dicks A., Akçay E. (2018). C₄ photosynthesis and climate through the lens of optimality. *Proceedings of the National Academy of Sciences*, 115, 12057-12062.

Zhou H., Akçay E., Edwards E., and Helliker B.R. (2020). The legacy of C₄ evolution in the hydraulics of C₃ and C₄ grasses. bioRxiv. doi: https://doi.org/10.1101/2020.05.14.097030.
### Table 1  Phylogenetic results of the best-fitted models and their parameters for photosynthesis parameters (summarizing Table S2-S10).

| Property          | Model       | Model type | AICw | Root | C3  | C4  |
|-------------------|-------------|------------|------|------|-----|-----|
| $J_{\text{max}}/V_{\text{cmax}}$ | Model 6*    | OU         | 1.000| 1.59 | 5.90|
| $V_{\text{cmax}}$ | Model 6*    | OU         | 0.769| 59.52| 23.03|
| $J_{\text{max}}$ | Model 6*    | OU         | 0.583| 88.94| 132.16|
| Total Chl         | Model 1     | BM         | 0.293| 0.36 |
| flr-ETR/$J_{\text{max}}$ | Model 5*    | OU         | 1.000| 1.08 | 0.64|
| Chl a/b           | Model 5     | OU         | 0.995| 3.40 | 4.61|
| flr-ETR           | Model 1     | BM         | 0.397| 88.94|
| $V_{\text{pmax}}$ | Model 4     | OU         | 0.854| 41.52|
| $V_{\text{pmax}}/V_{\text{cmax}}$ | Model 4    | OU         | 0.875| 1.97 |
| N                 | Model 5*    | OU         | 0.562| 3.62 | 2.53|

If the values for $C_3$ and $C_4$ were different, it meant there were significant different values for $C_3$ and $C_4$ species (the evolutionary model with two different values of the root fit significantly better than the evolutionary model with the similar root).
Figures

Fig. 1 Modeling results of assimilation rate with respect to maximal electron transport to maximal Rubisco carboxylation ($J_{\text{max}}/V_{\text{cmax}}$) and maximal PEP carboxylation to maximal Rubisco carboxylation ($V_{p\text{max}}/V_{\text{cmax}}$) under CO$_2$ concentration of 200, 400 and 600 ppm. Other environmental conditions are soil water potential ($\psi_s$)= -0.5 MPa, VPD=0.625, temperature of 25 °C and light intensity of 2000 μmol m$^{-2}$ s$^{-1}$. Left: 3D plot; right: corresponding contour plot.
Fig. 2 Empirical measurements (a) and optimal modeling results (b) of $J_{\text{max}}/V_{\text{cmax}}$ for C$_3$ and C$_4$ under $\psi_S$=-0.5 MPa, VPD=0.625, temperature of 25 °C and light intensity of 2000 μmol m$^{-2}$ s$^{-1}$, the cultivating environmental condition. In (b), the black line represents C$_3$, solid red line represents C$_4$ modeling results with controlling $V_{\text{pmax}}/V_{\text{cmax}}$ at the in vivo measurement level, dashed red line represents C$_4$ modeling results with controlling $V_{\text{pmax}}/V_{\text{cmax}}$ at the in vitro measurement level.
Fig. 3 Empirical measurements (a) and optimal modeling results (b) of $V_{p\max}/V_{c\max}$ for C4 under $\psi_S=-0.5$ MPa, VPD=0.625, temperature of 25 °C and light intensity of 2000 μmol m$^{-2}$ s$^{-1}$, the cultivating environmental condition. In (b), solid red line represents C4 modeling results with controlling $J_{\max}/V_{c\max}$ at the in vivo measurement level, dashed red line represents C4 modeling results with controlling $J_{\max}/V_{c\max}$ at the in vitro measurement level.
Fig. 4 Modeling results of assimilation rate with varying $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ for C$_4$ under different CO$_2$ concentration and $\psi_S$=-1MPa, VPD=1.25, temperature of 25 °C and light intensity of 2000 μmol m$^{-2}$ s$^{-1}$, the common grassland growth condition. Modeling results were obtained by controlling the other parameter at the in vivo measurement level.
**Fig. 5** Modeling results of changing $V_{cmax}$ on assimilation rates for C₃ (acd) and for C₄ (bdf) under different CO₂. Solid black line: the initial modeling condition of $V_{cmax}$ (a typical C₃ value of 69 μmol m⁻² s⁻¹); dashed black line: 90% of the initial $V_{cmax}$; dotted black line: 80% of the initial $V_{cmax}$; solid grey line: 70% of the initial $V_{cmax}$; dashed grey line: 60% of the initial $V_{cmax}$; dotted grey line: 50% of the initial $V_{cmax}$. C₃ and C₄ parameters shared similar parameters except for the carbon concentration mechanism and different hydraulic conductance to mimic the initial origin of C₄ (see Zhou et al (2020) for other parameters).
**Fig. 6** The regression for maximal assimilation rate ($A_{\text{max}}$), $J_{\text{max}}$, total chlorophyll (Total chl), $V_{\text{cmax}}$, $V_{\text{pmax}}$, nitrogen concentration, $J_{\text{max}}/V_{\text{cmax}}$, $V_{\text{pmax}}/V_{\text{cmax}}$ and fluorescence-estimated electron transport to $J_{\text{max}}$ (ETR/$J_{\text{max}}$) vs. the evolutionary age for the nine origins to show the evolutionary trend within $C_4$ (red) and within their closely-related $C_3$ species (black).
Fig. 7 Modeling results of optimal $J_{\text{max}}/V_{\text{cmax}}$ and $V_{\text{pmax}}/V_{\text{cmax}}$ for C$_3$ (black lines) and C$_4$ (red lines) under different environmental conditions. (a) Solid line: different CO$_2$; dashed line: different water limitation conditions (1: saturated water; 2: $\psi_S=\text{-0.5 MPa, VPD}=0.625$; 3: $\psi_S=\text{-1 MPa, VPD}=1.25$ MPa; 4: $\psi_S=\text{-1.5 MPa, VPD}=1.875$; 5: $\psi_S=\text{-2 MPa, VPD}=2.5$). (b) Solid line: different light intensities; dashed line: different temperature. Modeling results were obtained by controlling the other parameter at the in vivo measurement level.