Optimal reorganization of photosynthesis after the evolution of C₄

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Abstract

The evolution of C₄ photosynthesis evolved numerous times independently in the grasses over tens of millions of years, and each event required the development of distinct biochemistry and anatomy. Recent theoretical, anatomical and phylogenetic studies made great progress in reconstructing the evolutionary processes leading to the formation of the C₄ carbon concentration mechanism (CCM) in grasses. After the formation of the full CCM, C₄ physiology continued to diverge between C₃ and C₄ grasses, presumably as selection optimized photosynthetic function. In this study, we combine optimality modeling, physiological measurements and phylogenetic analysis to examine how various aspects of C₄ photosynthetic machinery were reorganized within a lineage and as compared to closely-related C₃ grasses. Both model and empirical results support a strong, and comparatively rapid, reorganization in resource allocation between the Calvin-Benson cycle and light reactions in C₄, as determined by a higher maximal electron transport to maximal Rubisco carboxylation rate ($J_{\max}/V_{\max}$). Our model, chlorophyll a/b ratios, and fluorescence-based electron transport measurements all suggest that linear electron transport represents a lower proportion (approximately 67%) of total electron transport in C₄, and that the impetus for increased cyclic-electron transport is to balance ATP:NADPH stoichiometry, as opposed to decreasing O₂ in the bundle sheath cells. Finally, the tight coordination between RuBP carboxylation and PEP carboxylation occurred coincidently with the evolution of the C₄ CCM, with a relatively constant maximal PEP carboxylation rate and $V_{\max}$ ($V_{\text{pmax}}/V_{\max}$).

Key words C₄ evolution, chlorophyll a/b, cyclic-electron transport, grass, optimal $J_{\max}/V_{\max}$, photosynthesis

Introduction

C₄ photosynthesis pathway evolved as a response to inefficiencies of C₃ photosynthesis that are exacerbated under certain environmental conditions (Ehleringer and Monson 1993; Ehleringer et al. 1997). Rubisco, the carboxylating enzyme of the Calvin-Benson (CB) cycle, not only assimilates CO₂, but also O₂, which is the first step in the photorespiratory pathway that reduces C₃ photosynthesis up to 30% (Ehleringer et al., 1991; Bauwe et al., 2010; Raines 2011). The C₄ pathway dramatically reduces Rubisco oxygenation, and consequently photorespiration, by concentrating CO₂ around Rubisco. This carbon concentrating mechanism (CCM) is achieved by segregating atmospheric CO₂ uptake by Phosphoenolpyruvate carboxylase (PEPc) and CB-cycle

CO₂ assimilation by Rubisco into two compartments within the leaf. In the mesophyll cells, carbonic anhydrase hydrates CO₂ and then PEPc catalyzes HCO₃⁻ and PEP into C₄ acids. The C₄ acids are transported to the bundle sheath cells that surround leaf veins, in a configuration called Kranz anatomy in most C₄ species. In the bundle sheath cells, C₄ acids are decarboxylated to create a high CO₂ environment for the Rubisco, and PEP is regenerated and translocated back to the mesophyll cells (Hatch & Osmond, 1976; Kellogg, 1999). The bundle sheath is often constructed in a manner that lessens the leakiness of CO₂ to the mesophyll, an anatomical phenomenon that enhances the efficiency of C₄ pathway (Brown and Byrd 1993; Ubierna et al. 2013; Kromdijk et al. 2014). Meanwhile, the regeneration of PEP requires ATP, so the C₄ CCM has an energetic cost that is not required by C₃ photosynthesis (Hatch, 1987).

The assembly of the full C₄ CCM is thought to have occurred through intermediate steps of selective advantage (Sage, 2001, 2004, 2018; Christin & Osborne, 2014; Lundgren, 2019). It is thought that the anatomical precursors of C₄ evolution were the enlargement of the vascular bundle sheath and a decrease of inter-veinal distance, termed the proto-Kranz condition (McKown & Dengler, 2007; Christin et al., 2013a; Griffiths et al., 2013). Then, a C₂ cycle evolved in which photorespired CO₂ is recovered and concentrated in the bundle sheath (Sage et al., 2012; Mallmann et al., 2014). Along with the evolution of the C₂ cycle, the mesophyll to bundle sheath ratio was reduced further. The enzymes related to the CCM function exist in the C₃ pathway and the citric-acid cycle, and spatially explicit expression of these enzymes led to the establishment of the full C₄ cycle (Aubry et al., 2011; Christin et al., 2013b). However, after the formation of the full CCM, C₄ physiology continued to diverge between C₃ and C₄ species, presumably as selection optimized the function of the CCM within a given ecological niche (Christin and Osborne, 2014; Sage, 2016). Zhou et al. (2018) and Zhou (2020) discussed the potential reorganization of hydraulic systems after C₄ evolved within a grass lineage, and here we examine how various aspects of C₄ photosynthetic machinery— nitrogen allocation between the Calvin-Benson cycle and the light reactions, electron transport, and the coordination of Rubisco, electron transport and PEP carboxylation— were reorganized within a C₄ lineage and as compared to closely-related C₃ grasses.

Resource allocation (mainly nitrogen) between the CB cycle and the light reactions would likely change between C₃ and C₄ due to the formation of the CCM. The CCM concentrates CO₂ around the CB-cycle, thus allowing for less investment in nitrogen-rich Rubisco as compared to a C₃

plant, but additional ATP from the light reactions is required to maintain the CCM (Hatch, 1987). Thus, the evolution of the full CCM changes the balance between nitrogen investment in the CB cycle versus the light reactions. The resource allocation between the CB cycle and the light reactions can be represented by the ratio of maximal electron transport to maximal Rubisco carboxylation rate ($J_{\max}/V_{\max}$). Although $J_{\max}/V_{\max}$ has been empirically measured in numerous C_3 species (Wullschlegel, 1993) and optimal $J_{\max}/V_{\max}$ has been modeled for C_3 (Walker et al., 2014; Kromdijk and Long, 2016; Quebbeman and Ramirez, 2016), there have been few measurements in C_4 , and we are not aware of any theoretical optimality predictions. For C_4 species, the assimilation rate would be mostly limited by the light reactions (Baker and Long, 1988; Percy and Ehleringer, 1984; Collatz et al., 1992) and there would be a redundancy in RuBP carboxylation if a C_3 -like $J_{\max}/V_{\max}$ was maintained (Zhou et al., 2018). Furthermore, Zhou et al. (2018) showed that if nitrogen is reallocated from the CB cycle to the light reactions, which results in a higher $J_{\max}/V_{\max}$, C_4 assimilation rates will increase significantly in almost all environmental conditions. In C_3 plants, $J_{\max}/V_{\max}$ should vary along with different environmental factors (temperature, CO_2 , light, and water availability; Onoda et al., 2005; Rodriguez-Calcerrada et al., 2008; Kromdijk and Long, 2016; Yin et al., 2018), and we expect the same for C_4 . How optimal $J_{\max}/V_{\max}$ varies with environment would be helpful in predicting the acclimation of C_3 and C_4 to different habitats and a changing climate, especially in biogeographical, bio-geochemical and vegetation models (Beerling and Quick, 1995; Quebbeman and Ramirez, 2016).

The C_4 CCM requires additional ATP; how and where this ATP is produced should affect the efficiency of the CCM. The primary electron transport process to generate ATP and NADPH necessary to run the CB cycle is the O_2 -producing, linear electron transport through both PSI and PSII. There are two potential reasons that the balance of linear electron transport in C_4 species would be altered. The first is to balance the stoichiometry of ATP and NADPH. The extra NADPH is not required for the CCM, and can be a harmful byproduct (Kramer and Evans, 2011; Yin and Struik, 2018). Second, the extra O_2 produced could lessen the efficiency of the CCM by increasing O_2 around Rubisco (Yin et al., 2011; von Caemmerer and Furbank, 2016). Thus, enhanced cyclic-electron transport, which produces ATP only, has been proposed to play a crucial role in driving the energy requirements in the bundle sheath cells (Takabayashi et al., 2005; Munekage, 2016; Munekage and Taniguchi, 2016; Yin and Struik, 2018). Changes in the balance of linear and cyclic electron transport would be accompanied with predictable variation

in other traits. These electron-transport pathways have different association with PSI and PSII, and PSI has a higher concentration of chlorophyll a compared to chlorophyll b than PSII. Therefore, C_4 species should have a higher chlorophyll a/b ratio (Takabayashi et al., 2005), if cyclic electron transport is elevated. Also, electron transport estimates based on fluorescence measures electron transport associated with PSII in C_4 species. We propose the ratio of electron transport estimated from fluorescence (ETR) to $J_{\max}$ should be relatively low in C_4 species as compared to C_3 species. Establishing this ratio will allow for better estimation of $J_{\max}$ from fluorescence measurements in C_4 species.

The operation of CB cycle, electron transport and the CCM should be coordinated to optimize the efficiency of C_4 photosynthesis (von Caemmerer and Furbank, 2016). The PEP carboxylation process and RuBP carboxylation work in tandem to capture the CO_2 . A separate aspect of C_4 coordination is that both RuBP regeneration and PEP regeneration use ATP from electron transport and provide reaction substrates for the CB cycle and PEP carboxylation, which provides. Such coordination among CB cycle, electron transport, and the CCM could be represented by the relationships among $V_{\max}$, $J_{\max}$ and maximal PEP carboxylation rate (V_{pmax}). As a relationship between $J_{\max}$ and $V_{\max}$ has been discussed above, to depict a complete picture of the functional coordination, maximal PEP carboxylation rate to $V_{\max}$ ($V_{\text{pmax}}/V_{\max}$) should be taken into consideration (Yin et al., 2016).

Because of the importance of testing physiological differences between species within a phylogenetic context (Edwards et al., 2007; Cavender-Bares et al., 2009), we use phylogenetic comparative methods and evolutionary models to examine the reorganization of the photosynthetic system after the evolution of C_4 across a phylogeny of C_3 and C_4 grasses from the PACMAD clade. We used both optimality modeling and physiological measurements to test the following hypotheses: (1) There will be a reorganization in resource allocation between the CB cycle and light reactions in C_4 ; specifically, C_4 will have a higher $J_{\max}/V_{\max}$ to maximize the assimilation rate. (2) The electron-transport rate to $J_{\max}$ ratio (ETR/ $J_{\max}$) is lower, and chlorophyll a/b ratios higher, in C_4 species. (3) The enhanced cyclic-electron transport aims to balance the stoichiometry of ATP and NADPH, as well as to decrease O_2 accumulation in the bundle sheath. (4) The coordination of RuBP carboxylation by Rubisco, electron transport and PEPc carboxylation of PEP result in relatively constant $V_{\text{pmax}}/V_{\max}$ to maximize assimilation

rate. Lastly, a better formal understanding of how $J_{\max}/V_{\max}$ and $V_{\max}/V_{\max}$ vary in C_4 species will provide important input parameters for large-scale models to predict the carbon/nitrogen cycle, vegetation dynamics and productivity.

Materials and Methods

Plant material

We cultivated 36 closely related species, including nine C_3 , 26 C_4 species in three C_4 subtypes and one intermediate species selected from Grass Phylogeny Working Group II, (2012). We, then, extracted the dated phylogenetic tree for the species from the dated phylogenetic trees (Spriggs et al., 2014). C_4 species belong to three C_4 subtypes and could be classified as nine closely-related C_3 and C_4 Clusters. Detailed cultivation and phylogeny information is the same as Zhou (2020).

Physiological Modeling

Optimal $J_{\max}/V_{\max}$ and $V_{\max}/V_{\max}$ We used the model of Zhou et al. (2018), which incorporates the soil-plant-air water continuum into traditional C_3 and C_4 photosynthesis models and optimizes stomatal resistance and leaf/fine-root allocation to maximize total carbon gain, to get predictions of the further evolution and reorganization of photosynthetic systems. Using such a framework, we can model the optimal $J_{\max}/V_{\max}$ and $V_{\max}/V_{\max}$ considering the following nitrogen stoichiometry:

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

$$N_{org} = N_P + N_E + N_R + N_S + N_O, \quad (1)$$

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

$$N_P + N_E = 0.079J_{max} + 0.0331\chi, \quad (2)$$

$$N_S = \nu J_{max}, \quad (3)$$

$$N_R = V_{cmax}/(6.25 \times V_{cr} \times \xi), \quad (4)$$

$$N_{PEP} = V_{pmax}/(6.72 \times V_{pr} \times \xi), \quad (5)$$

χ is the concentration of chlorophyll per unit area ($\mu\text{mol Chl m}^{-2}$), 0.079 is in $\text{mmol N s} (\mu\text{mol})^{-1}$, and 0.0331 is in $\text{mmol N} (\mu\text{mol Chl})^{-1}$, $\nu \approx 0.3 (\text{mmol N s} (\mu\text{mol})^{-1})$. V_{cr} is the specific activity of Rubisco (the maximum rate of RuBP carboxylation per unit Rubisco; $\approx 20.5 \mu\text{mol CO}_2 (\text{g Rubisco})^{-1}\text{s}^{-1}$) and 6.25 is grams RuBisCO per gram nitrogen in RuBisCO. V_{pr} is the specific activity of PEPc, that is, the maximum rate of RuBP carboxylation per unit PEPc ($\approx 181.7 \mu\text{mol CO}_2 (\text{g PEPc})^{-1}\text{s}^{-1}$) (Supplementary Material I), 6.72 is grams PEPc per gram nitrogen in PEPc (calculated from the amino acids composition of Fujita et al., 1984), and ξ is the mass in grams of one millimole of nitrogen equal to $0.014 \text{ g N} (\text{mmol N})^{-1}$.

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_{max} . We make this assumption for the light saturated condition and use the empirical equation of Croft et al. (2017) to equation (2)

$$\chi = \frac{1000J_{max}}{2.49} / \eta, \quad (6)$$

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

$$N_{org} - N_O = 0.079J_{max} + 0.0331\chi + \nu J_{max} + \frac{V_{cmax}}{6.25 \times V_{cr} \times \xi} + V_{pmax}/(6.72 \times V_{pr} \times \xi), \quad (7)$$

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

$$J = \frac{I_2 + J_{max} - \sqrt{(I_2 + J_{max})^2 - 4\Theta I_2 J_{max}}}{2\Theta}. \quad (8)$$

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

In the optimal modeling processes for C_4 pathway, we set $N_{org} - N_O$ as constant of $129 \text{ mmol N m}^{-2}$ (which yield a $V_{cmax} = 39 \mu\text{mol m}^{-2} \text{ s}^{-1}$, $J_{max} = 195 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and $V_{pmax} = 78 \mu\text{mol m}^{-2} \text{ s}^{-1}$, assuming $J_{max}/V_{cmax} = 5$ and $V_{pmax}/V_{cmax} = 2$) and model the assimilation rates with different J_{max}/V_{cmax} from 1 to 8 of 0.01 interval by maintaining $V_{pmax}/V_{cmax} = 2$, which is the empirically

measured average in our study. Then, we model the assimilation rates with different V_{pmax}/V_{cmax} from 0.5 to 6 of 0.01 interval under $J_{max}/V_{cmax}=5$, which is also the empirical average in our measurements. The corresponding J_{max}/V_{cmax} or J_{max}/V_{cmax} under the highest assimilation rates represent the optimal ratios. Using the model described above, we were able to model the optimal J_{max}/V_{cmax} and V_{pmax}/V_{cmax} under different environmental gradients: CO₂ of 200, 300, 400, 500 and 600 ppm; VPD and ψ_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 $\mu\text{molm}^{-2}\text{s}^{-1}$; temperature of 15, 20, 25, 30 and 35 °C. Since there is uncertainty for relationships, we performed sensitivity analysis for the total nitrogen (from 100% to 50% with 10% interval of the regular nitrogen) for optimal J_{max}/V_{cmax} and V_{pmax}/V_{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 $N_{org} - N_O$ is used, except that a simplified version of equation (7) is used as below (Quebbeman and Ramirez, 2016):

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

Effects of decreasing V_{cmax} Using the model, we also simulated the effect of decreasing V_{cmax} on the assimilation rate of 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.

Effects of O₂ evolution in bundle sheath We used the C₄ photosynthesis model to model the effects of different proportion of O₂ evolution (indicated by α) in bundle sheath on assimilation rate (von Caemmerer, 2000; Yin et al., 2011). The modeling conditions are $\alpha=0, 0.15, 0.5$ and 1. A detailed description of the model could be found Zhou et al. (2019).

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 $\mu\text{molm}^{-2}\text{s}^{-1}$. 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 $\mu\text{mol s}^{-1}$ for all the measurements. The cuvette was covered by Fun-Tak to lessen leakiness. The A/C_i curves were used to estimate V_{cmax} , J_{max} , and V_{pmax} using Zhou et al. (2019). Fluorescence results were used to calculate the ETR (Genty et al., 1989).

Chlorophyll measurements and leaf nitrogen

Chlorophyll a and b 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. Calculations of Chlorophyll a and b concentrations were calculated using the equation of Porra et al. (1989). Then 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_{cmax} , J_{max} , $J_{\text{max}}/V_{\text{cmax}}$, Total Chl, ETR/ J_{max} , Chl a/b, ETR, V_{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. Furthermore, we used SubtypeModels to test whether there are significant differences among C₄ subtypes (Table S1). The detailed description of the evolutionary models and phylogenetic analysis can be found in Zhou (2020).

Results

As a preliminary analysis for the raw data, we conducted ANOVA tests within each phylogenetic cluster (Table 1). ANOVA tests indicated all C₄ had higher $J_{\text{max}}/V_{\text{cmax}}$, and Chl a/b than their

corresponding C₃ in different clusters, while C₄ had the lower V_{cmax} and ETR/J. There are no consistent relationship for J_{max} , chlorophyll concentration, and ETR between C₃ and C₄ among clusters. For nitrogen content, C₃ have higher or equivalent values than corresponding C₄. We further performed phylogenetic analysis to test our hypotheses shown as below.

C₄ species have higher $J_{\text{max}}/V_{\text{cmax}}$ than C₃ species

The modeling results of the optimal $J_{\text{max}}/V_{\text{cmax}}$ and empirical measurements for $J_{\text{max}}/V_{\text{cmax}}$ are significantly higher for the C₄ species than those for the C₃ species (Fig. 1). The experimental measurements were relatively consistent with the modeling results, although measured values were slightly lower than modeled values in C₃ and C₄. C₄ species always had significantly higher $J_{\text{max}}/V_{\text{cmax}}$ than their closely-related C₃ species (Table 1); furthermore, phylogenetic analysis shows the $J_{\text{max}}/V_{\text{cmax}}$ follows the Ornstein-Uhlenbeck model with a higher root state for C₄ species and a lower root state for C₃ (Table 2; phylogeny is shown in Zhou (2019)). The evolutionary models differentiating subtypes did not fit significantly better than models only considering C₃ and C₄ photosynthesis types for $J_{\text{max}}/V_{\text{cmax}}$ (Table S2, S11), which means no significant differences of $J_{\text{max}}/V_{\text{cmax}}$ among subtypes were detected.

Table 1 The photosynthetic parameters of $J_{\text{max}}/V_{\text{cmax}}$, V_{cmax} , J_{max} , total Chl, ETR/ J_{max} , Chl a/b, ETR and nitrogen content between closely related C₃ and C₄ species. The different blocks show nine different clusters of closely-related C₃ and C₄ species. * indicate C₃ species, † indicate C₃-C₄ intermediate species, and the rest are C₄ species. Letters indicate significant differences for the species within each cluster. The data indicate average(standard error).

Species	$J_{\text{max}}/V_{\text{cmax}}$	J_{max}	V_{cmax}	Tchl	ETR/ J_{max}	ETR	Chl a/b	Nitrogen
<i>S. decipiens</i> †	1.5(0.1)a	71(4.3)a	54(3.8)a	0.36(0.03)a	1.2(0.02)a	82.4(6.4)a	3.4(0.1)ab	3.4(0.1)ab
<i>A. rufa</i>	4.9(0.1)b	94(6.4)b	20(1.2)b	0.37(0.03)a	0.6(0.02)b	64.6(3.7)ab	4.1(0.1)c	3.0(0.1)a
<i>A. villosa</i>	5.1(0.1)b	92(7.4)b	19(1.3)b	0.30(0.03)ab	0.6(0.02)b	59.2(4.9)b	4.2(0.1)c	3.5(0.1)b
<i>H. pernambucae</i>	1.5(0.1)a	59(3.3)bc	47(3.3)a	0.23(0.01)b	1.1(0.03)a	67.3(4.4)ab	3.3(0.1)b	3.6(0.1)b
<i>nse</i> *								
<i>S. laxum</i> *	1.4(0.1)a	80(4.7)c	56(3.4)a	0.29(0.03)ab	1.0(0.02)c	79.1(3.2)ab	3.5(0.1)a	4.6(0.11)c
<i>D. dinteri</i>	5.5(0.1)a	168(9.2)a	31(1.3)ad	0.38(0.02)a	0.6(0.01)a	100.7(5.9)a	4.8(0.1)a	3.5(0.2)a
<i>T. superba</i>	5.1(0.1)b	104(7.8)b	22(1.6)ab	0.37(0.02)a	0.6(0.01)a	67.8(5.9)bc	4.5(0.1)a	2.4(0.2)b
<i>L. simplex</i>	4.5(0.1)c	83(7.6)ab	19(1.3)b	0.38(0.04)a	0.6(0.03)a	48.5(4.3)b	4.0(0.1)b	2.2(0.1)b
<i>T. latifolia</i> *	1.5(0.1)d	77(6.6)bc	54(4.4)c	0.30(0.02)a	1.0(0.04)b	72.3(7.8)c	3.5(0.1)c	4.2(0.2)a

<i>C. latifolia</i> *	1.6(0.1)d	53(4.1)c	34(2.1)d	0.30(0.02)a	1.0(0.08)b	52.5(3.0)b	3.4(0.1)c	2.3(0.1)b
<i>Z. bulbosa</i>	4.3(0.2)a	88(9.2)a	20(1.2)a	0.21(0.01)a	0.6(0.01)a	46.3(4.7)a	3.9(0.1)a	2.9(0.1)a
<i>S. italica</i>	4.6(0.1)a	75(8.1)a	17(1.5)a	0.43(0.01)b	0.7(0.03)ab	50.9(3.4)a	4.0(0.1)a	3.1(0.2)a
<i>S. sphacelata</i>	5.3(0.1)b	102(8.0)ac	20(1.0)a	0.35(0.04)bc	0.6(0.03)ab	64.4(3.4)a	4.1(0.1)a	3.7(0.3)ab
<i>W. capillipes</i>	5.7(0.1)b	147(9.8)b	27(1.4)a	0.34(0.03)bc	0.8(0.01)b	110.9(7.8)b	4.1(0.1)a	3.5(0.1)a
<i>H. proluta</i> *	1.5(0.1)c	127(8.9)bc	84(5.5)b	0.30(0.03)c	1.1(0.03)c	136.2(7.4)c	3.1(0.1)b	4.3(0.1)b
<i>T. monachne</i>	5.5(0.2)a	160(13.8)a	30(2.9)a	0.31(0.01)ab	0.7(0.02)a	111.0(9.3)a	3.7(0.1)a	3.9(0.3)a
<i>E. meyeriana</i>	5.2(0.1)ab	135(7.6)ab	26(1.3)a	0.29(0.02)ab	0.6(0.01)b	81.7(7.5)ab	3.8(0.1)a	3.6(0.2)a
<i>M. maximus</i>	5.1(0.1)b	117(5.5)b	24(0.9)a	0.39(0.02)b	0.6(0.02)ab	80.5(5.1)b	3.5(0.1)a	4.5(0.3)a
<i>U. ruziziensis</i>	5.2(0.1)ab	121(6.0)ab	23(1.1)a	0.24(0.02)a	0.6(0.02)ab	76.6(3.2)b	3.5(0.0)a	3.9(0.2)a
<i>E. punctata</i>	5.0(0.1)b	115(8.4)b	23(1.3)a	0.27(0.01)a	0.7(0.01)ab	80.3(4.9)ab	3.6(0.1)a	3.9(0.2)a
<i>D. clandestinum</i> *	1.7(0.1)c	151(7.1)a	93(3.7)b	0.71(0.06)c	1.1(0.02)c	159.7(11.5)c	2.9(0.2)b	4.2(0.2)a
<i>P. coloratum</i>	5.1(0.1)a	105(7.0)ab	21(1.2)a	0.36(0.02)a	0.7(0.01)a	73.9(4.8)ab	4.3(0.1)a	3.7(0.2)a
<i>P. milicea</i>	4.9(0.1)a	134(8.9)a	27(1.3)a	0.33(0.01)a	0.7(0.01)a	85.7(5.6)ac	3.9(0.1)b	2.6(0.1)b
<i>P. queenslandicum</i>	5.0(0.1)a	86(9.1)b	17(1.6)a	0.24(0.02)b	0.7(0.03)a	59.0(4.7)b	3.9(0.0)b	2.3(0.1)b
<i>P. white</i>	4.8(0.1)a	115(7.7)ab	23(1.1)a	0.30(0.02)ab	0.7(0.01)a	77.6(4.7)ab	4.1(0.0)ab	2.2(0.3)b
<i>P. bisulcatum</i> *	1.6(0.1)b	93(7.0)b	64(4.4)b	0.47(0.01)c	1.1(0.04)b	100.1(4.3)c	3.2(0.0)c	4.9(0.3)c
<i>A. purpurea</i> Var. long	5.3(0.1)a	100(8.3)a	19(1.2)a	0.35(0.02)a	0.8(0.02)ab	118.7(8.8)a	4.9(0.1)a	2.5(0.2)a
<i>S. obtusa</i>	5.8(0.1)b	158(10.0)b	28(1.7)b	NA	0.7(0.02)a	82.0(5.9)b	NA	2.6(0.0)a
<i>S. ciliata</i>	5.4(0.1)ab	146(9.6)b	26(1.5)b	0.34(0.02)a	0.8(0.02)b	117.3(8.0)a	4.7(0.1)a	2.9(0.0)a
<i>E. obtusa</i>	5.8(0.1)a	191(13.3)a	33(2.1)a	0.46(0.04)a	0.8(0.02)a	138.6(6.2)a	4.1(0.0)a	2.6(0.2)a
<i>F. africana</i>	5.4(0.1)a	145(8.4)b	27(1.4)ab	0.52(0.02)a	0.7(0.02)a	108.0(3.2)b	3.9(0.1)a	2.5(0.2)a
<i>S. fimbriatus</i>	5.0(0.2)b	105(8.3)c	21(1.3)b	0.28(0.01)b	0.6(0.01)a	67.4(5.3)c	3.9(0.1)a	2.9(0.1)a
<i>C. sp.</i> *	1.6(0.1)c	96(5.7)c	63(4.9)c	0.61(0.07)a	1.2(0.05)b	114.8(2.7)b	3.0(0.2)b	2.7(0.2)a
<i>E. obtusa</i>	5.3(0.1)a	127(12.7)a	23(1.9)a	0.30(0.02)a	0.7(0.02)a	92.5(6.4)a	4.7(0.2)a	NA
<i>M. caerulea</i> *	1.9(0.1)b	61(3.7)b	32(3.1)b	0.32(0.02)a	1.0(0.04)b	57.7(3.3)b	3.6(0.1)b	3.6(0.2)
<i>E. frumentacea</i>	5.2(0.1)	85(7.2)	17(1.0)	0.26(0.03)	0.61(0.02)	49.2(3.0)	4.0(0.0)	4.1(0.2)

Table 2 Phylogenetic results of the best-fitted models and their parameters for photosynthesis parameters (summarizing Table S2-S10). If the values for C₃ and C₄ were different, it meant there were significant different values for C₃ and C₄ species (the evolutionary model with two different values of the root fit significantly better than the evolutionary model with the similar root).

Property	Model	AICw	Root
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		Model type		C ₃	C ₄
$J_{\max}/V_{\max}$	Model 5	OU	1.000	1.59	5.15
$V_{\max}$	Model 5	OU	0.902	59.46	23.03
$J_{\max}$	Model 5	OU	0.620	88.72	124.88
Total Chl	Model 1	BM	0.409	0.36	
ETR/ $J_{\max}$	Model 5	OU	1.000	1.08	0.68
Chl a/b	Model 5	OU	0.995	3.40	4.61
ETR	Model 1	BM	0.465	88.94	
V_{pmax}	Model 4	OU	0.954	46.55	
$V_{\text{pmax}}/V_{\max}$	Model 4	OU	0.977	1.97	
N	Model 5	OU	0.617	3.62	2.53

Fig. 1 Empirical measurements (a) and optimal modeling results (b) of $J_{\max}/V_{\max}$ for C₃ and C₄ under $\psi_s = -0.5$ MPa, VPD=0.625, temperature of 25 °C and light intensity of 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the approximate greenhouse growth conditions. In (b), the black line represents C₃ and grey line represents C₄.

Higher $J_{\max}/V_{\max}$ in C₄ is mainly driven by lower $V_{\max}$

We looked further into how such a higher $J_{\max}/V_{\max}$ in C₄ species is reached by comparing other empirical measurements. We found that although in some C₄ species $J_{\max}$ is higher, a lot of C₄ species did not have significantly higher $J_{\max}$ than their closely-related C₃ species, and measurements of total chlorophyll content were also similar (Table 1). However, our phylogenetic analysis detected a significant higher root state for $J_{\max}$ in C₄ than in C₃ (Table 2).

Conversely, V_{cmax} is significantly lower in nearly all C_4 species than in the closely-related C_3 species and phylogenetic analysis confirmed a higher V_{cmax} in C_3 (Table 1, 2). In order to look at the potential effects of decreasing V_{cmax} on assimilation rate, we held the J_{max} as constant and changed the V_{cmax} from 100% to 50% of the original C_3 parameter values in the C_3 and C_4 models. A decrease in V_{cmax} will significantly decrease the assimilation rates of C_3 species from 10 °C to 35 °C, while decreasing V_{cmax} has little effects on the assimilation rates of C_4 species (Fig. 2). V_{cmax} , J_{max} , and chlorophyll also did not show significant differences among subtypes (Table S3-5, S12-14).

Variation in optimal $J_{\text{max}}/V_{\text{cmax}}$ with environmental conditions

To understand how the $J_{\text{max}}/V_{\text{cmax}}$ is predicted to vary across environmental gradients (Fig. 3), we calculated its optimal value for different CO_2 concentration, water limitation, and light intensities. The optimal $J_{\text{max}}/V_{\text{cmax}}$ is predicted to increase linearly in both C_3 and C_4 species with increasing CO_2 concentration, but more steeply in C_3 species (Fig. 3a). The optimal $J_{\text{max}}/V_{\text{cmax}}$ decreases similarly in both C_3 and C_4 species along with increasing water limitation (Fig. 3a). The optimal $J_{\text{max}}/V_{\text{cmax}}$ is predicted to increase in both C_3 and C_4 species with decreasing light intensity and increasing temperatures (Fig. 3b). 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 $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature of 30 °C).

The ETR, calculated from fluorescence measurements, to J_{max} ratio was significantly higher ratio in C_3 species than that in their closely related C_4 species, with averages of 106% and 67.8% for C_3 and C_4 respectively (Table 1). Phylogenetic analysis indicated the root state of ETR itself is higher in C_4 species, but the differences are not significant (Table 2). Chlorophyll a/b is significantly higher in C_4 species than C_3 species, which is confirmed by both comparing closely-related C_4 and C_3 species within clusters (Fig 5c) and phylogenetic analysis (Table 2). Phylogenetic analysis indicates only Chlorophyll a/b is significantly different among subtypes (Table S7, S16). We also modeled the effects of O_2 accumulation on the C_4 assimilation rate, and there was very little change in assimilation when α ranged from 0 to 1 (Fig. S1). The value α indicates a change in the proportion of O_2 evolution in the bundle sheath where $\alpha=1$ represents all O_2 evolution emanating from electron transport occurring in the bundle sheath and $\alpha=0$ represent zero O_2 accumulation.

Fig. 2 Modeling results of changing V_{cmax} on assimilation rates (A_n) 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$); 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 shared similar parameters except for the carbon concentration mechanism and different hydraulic conductance to mimic the initial origin of C₄ (full model and other parameters are shown in Zhou et al. (2018)).

Fig. 3 Modeling results of optimal $J_{\text{max}}/V_{\text{cmax}}$ for C₃ (solid lines) and C₄ (dashed lines) under different environmental conditions. (a) Black lines depict different CO₂ concentrations (bottom axis); grey lines different water limitation conditions (top axis; 1: saturated water; 2: $\psi_s = -0.5$ MPa, VPD=0.625; 3: $\psi_s = -1$ MPa, VPD=1.25; 4: $\psi_s = -1.5$ MPa, VPD=1.875; 5: $\psi_s = -2$ MPa, VPD=2.5). (b) Black lines depict optimal $J_{\text{max}}/V_{\text{cmax}}$ different light intensities (bottom axis); grey lines for different temperatures (top axis). C₃ and C₄ parameters shared similar parameters except for the carbon concentration mechanism and different hydraulic conductance to mimic the initial origin of C₄ and most-closely related C₃ and C₄ species.

The empirical measurements and phylogenetic analysis indicate $V_{\text{pmax}}/V_{\text{cmax}}$ are under the stabilized selection for a stabilized optimum of 1.97 and a range from 1.35 to 2.47, although

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Since there is a great variation of total nitrogen content in the plants and there is uncertainty in the stoichiometry of PEPC, we performed sensitivity analysis for the optimal modeling of $J_{\max}/V_{\max}$ and $V_{\text{pmax}}/V_{\text{cmax}}$. For C_3 photosynthesis, optimal $J_{\max}/V_{\text{cmax}}$ increases slightly with decreasing total nitrogen (7.73% increase of $J_{\max}/V_{\text{cmax}}$ with 50% reduction in total nitrogen), while for C_4 , optimal $J_{\max}/V_{\text{cmax}}$ decreases with total nitrogen (20.34% increase of $J_{\max}/V_{\text{cmax}}$ with 50% reduction in total nitrogen) (Fig. S3a). The optimal $V_{\text{pmax}}/V_{\text{cmax}}$ increases somewhat with decreasing total nitrogen (15.3% increase of $V_{\text{pmax}}/V_{\text{cmax}}$ with 50% reduction in total nitrogen) (Fig. S3a). The optimal $J_{\max}/V_{\text{cmax}}$ 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). Also, the optimal $V_{\text{pmax}}/V_{\text{cmax}}$ is relatively constant with the variation of the $1/(6.72 \times V_{\text{pr}} \times \xi)$ term.

Discussion

The evolution of C_4 photosynthesis was likely associated with several important shifts in photosynthetic traits. Our results show that one of these shifts was the allocation of nitrogen resources between the CB cycle and the light reactions, resulting in a higher $J_{\max}/V_{\text{cmax}}$ for C_4

than C₃ species. An increase in $J_{\max}/V_{\max}$ due to reallocation of nitrogen is likely a crucial evolutionary step for C₄ evolution after the CCM evolved, and it is important to look at how it may have occurred. Zhou et al. (2018) proposed that if nitrogen is reallocated from the CB cycle (e.g., Rubisco) to the light reactions (an increase in $J_{\max}$, and a decrease in $V_{\max}$), the assimilation rate of C₄ would significantly increase because the extra ATP costs could be satisfied, and within the CCM Rubisco operates more efficiently, which means less of the enzyme is required. Our optimality model confirms this intuition, predicting higher $J_{\max}/V_{\max}$ for C₄ species than C₃. Our empirical measurements conform to this prediction qualitatively. All C₄ grasses have a lower $V_{\max}$ than the closely-related C₃ in the current study, which suggests C₄ photosynthesis does allow for less investment of nitrogen in Rubisco.

Previous empirical measurements of $J_{\max}/V_{\max}$ from previous studies reported greater variation than we found in our measurements and a range from 2 to 6 for C₄ species (Massad et al., 2007; Ye et al., 2013; Ge et al., 2014; Bellasio et al. 2016), although the average across these studies is close to our measurement of 5.1. The variation may result from the uncertainty of previous estimation methods for C₄ photosynthesis, uncertainties that we have assuaged with our work. Previous modeling work assumed a high $J_{\max}/V_{\max}$ for C₄, while considering similar nitrogen content in C₃ and C₄ (Vico and Porporato, 2008; Osborn and Sack, 2012). Our results validate these assumptions based on new empirical data and optimality modeling.

Similar results were either proposed or found by Brown (1978), Ku et al. (1979), Sage et al. (1987) and Sharwood et al. (2016). Tissue et al. (1995) and Ghannoum et al. (2010) detected lower Rubisco content and higher chlorophyll and thylakoid content, supporting reallocation within the leaf. Any surplus nitrogen could be distributed among three broad categories: 1) reallocated to the light reactions or 2) stored or used to construct new organs or 3) simply not taken up, thus reducing plant nitrogen requirements. Our empirical measurements provide evidence for all of these options, although we cannot differentiate between the second and third. Some of our C₄ species, e.g., *Danthoniopsis dinteri* and *Panicum milicea*, had a significantly higher $J_{\max}$ than closely-related C₃ which suggests nitrogen reallocation from the CB cycle to the light reactions. Phylogenetic analysis results also supports such a reallocation happened generally with a significant higher $J_{\max}$ in C₄. Several C₄ grasses we measured, however, had a $J_{\max}$ that was similar to closely related C₃ species, while maintaining a lower $V_{\max}$. Our modeling

results show that decreasing V_{max} by 50% has little effect on the assimilation rate. ANOVA tests showed that both hypothesis 2) and 3) could have happened together with hypothesis 1), because some C_4 species have significant lower nitrogen content, but some have equivalent values. However, phylogenetic analysis of nitrogen indicated that in general, C_4 species have a lower nitrogen content than C_3 species, which support option 3). It is worth considering the potential ecological and evolutionary ramifications. Firstly, in a nitrogen-depleted habitat, C_4 could have a competitive advantage as confirmed by Riley et al. (2008; although Sage and 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 and Pearcy, 1987; Anten et al., 1995; Riley 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 species.

The extra ATP required to run the C_4 CCM is generated primarily by cyclic electron transport, and this likely evolved to balance ATP:NADPH stoichiometry, as opposed to minimizing O_2 production. Aside from cyclic electron transport, all mechanisms that provide extra ATP are associated with the O_2 -producing PSII (Kramer and Evans, 2011), and would not lead to a significant increase of chl a/b as found here and in other studies (Ku et al., 1991). However, chl a/b differ among subtypes may indicate there could be different types or different magnitudes of electron transport pathways to support the CCM in different subtypes. Our model results show that a wide-range of O_2 concentration in the bundle sheath has minimal impact on photosynthetic rate, and therefore indicate that decreasing O_2 evolution is not the driving force behind increasing cyclic electron transport. Rather, our results support the alternative hypothesis cyclic electron transport balances the stoichiometry of NADPH and ATP demand (Nakamura et al., 2013; Ishikawa et al., 2016; Munekage and Taniguchi, 2016). The ETR/J_{max} indicated a relatively consistent proportion of PSII fluorescence to total electron transport across all C_4 species, which did not significantly differ among C_4 subtypes. The reason could be that although the stoichiometry of NADPH and ATP is different between mesophyll cells and bundle

sheath cells in NADP-ME and NAD-ME subtypes, the total stoichiometry is the same (Hatch et al., 1987; Kanai and Edwards, 1999; Takabayashi et al., 2005; Yin et al., 2018).

In C_3 species, the fluorescence-based ETR is widely used as an approximation of $J_{\max}$ (Baker *et al.* 2007; Sage and Kubien, 2007; Yin et al., 2009); however, the estimation method has not previously been suitable to C_4 pathway because the fluorescence-based ETR does not account for the contribution of cyclic-electron transport to $J_{\max}$. Because we found a relatively constant $ETR/J_{\max}$ for all C_4 species, we can assume a constant proportion of electron cycling and thus, we can now expand approaches that use combined gas exchange and fluorescence to estimate the $J_{\max}$ as well as other C_4 photosynthetic parameters. Yin et al. (2011) use an assumed value of 60% for $ETR/J_{\max}$, which assumed that all electron transport for PEP regeneration was not from linear electron transport, which is a little lower than our value of 67.8%. The theoretical modeling analysis of Yin and Struik (2012) and Yin and Struik (2018) predicted cyclic electron transport could account for 45% or 50% of total $J_{\max}$.

The tight coordination between RuBP carboxylation and PEP carboxylation likely occurred early in the evolution of the C_4 of CCM, leading to a relatively constant $V_{p\max}/V_{c\max}$. There were few $V_{p\max}/V_{c\max}$ values reported in previous studies, but our results are comparable with existing ones. Yin et al. (2011) and Yin et al., (2016) reported estimation results of 2.43 and 2.5 respectively for $V_{p\max}/V_{c\max}$. Estimates from biochemical measurements of Pengelly et al. (2010) and Kubien et al. (2003) were 2.1-2.5 and 3.1 respectively. Our empirical $V_{p\max}/V_{c\max}$ is in line with the optimal modeling results under 200-300 ppm, as opposed to CO_2 concentrations closer to current growth conditions. All extant C_4 species have gone through a low CO_2 bottleneck over the last 5 million years. It is possible that this bottleneck represented strong selection to increase the $V_{p\max}/V_{c\max}$ to maintain a high assimilation rate. As CO_2 has risen, $V_{p\max}/V_{c\max}$ has exceeded the optimal $V_{p\max}/V_{c\max}$, but the effects of a high $V_{p\max}/V_{c\max}$ on assimilation rate at elevated CO_2 were minimal.

Our improved estimates of $J_{\max}/V_{c\max}$ and $V_{p\max}/V_{c\max}$ for C_4 plants can aid predictions of net primary productivity by large-scale models. $J_{\max}/V_{c\max}$ is an important input parameter in biogeochemical cycle, biodiversity or productivity models (e.g., Biome-BGC, Community Land Model) (Beerling and Quick, 1995; Zaehle et al., 2005; Bonan et al., 2011; Walker et al., 2014),

however, there have been no reliable parameters reported for C₄ species. Our results suggests a value of around 5 supported both optimality modeling and empirical measurements. Our optimality modeling framework can also be used to adaptive plasticity (or acclimation) of $J_{\max}/V_{\max}$ under different environmental conditions that can be used to predict responses of ecosystems to future climate change. Previous modeling (Sage, 1990; Hikosaka and Terashima, 1995) and experimental studies (Evans, 1989b; Pons and Pearcy, 1994; Tissue, 1999; Onoda et al., 2005) indicated higher allocation to the light reactions (namely, higher $J_{\max}/V_{\max}$) under shade, elevated CO₂ and higher temperature for C₃ species, which is consistent with our predictions. However, C₃ and C₄ species could respond differently to environmental variation (Ward et al., 1999; Wand et al., 1999; Ripley et al., 2013), and our modeling suggests that the acclimation to CO₂ in C₄ species may be of a lesser extent than C₃ species (Sage and McKown, 2005). Additionally, $V_{\text{pmax}}/V_{\text{cmax}}$ may be a legacy of ancient environmental conditions, and its plasticity may be lower, but this requires further examination. Generally, not all species or physiological traits may be plastic along with environmental change (Herrick and Thomas, 2001; Kubiske et al., 2002); therefore, further exploring acclimation and genetic constraints will be important for predicting species responses to climate change.

In conclusion, here we examined the divergence of photosynthetic traits between C₃ and C₄ grasses after the evolution of the C₄ CCM. Our results contribute to our understanding of the ecological and evolutionary consequences of this important evolutionary innovation. Unlike the divergence and reorganization of hydraulic traits, which may follow an evolutionary trend (Zhou, 2020), we find that photosynthetic properties are under stabilizing selection. Such observations may indicate that photosynthetic properties were under much stronger selective pressures or evolved step by step along with the CCM, and once the CCM formed, photosynthetic properties quickly reach stable states, whereas hydraulic properties responded after the formation the CCM and the selective pressure was lower.

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Author Contribution

HZ, EA, and BH conceptualized the idea. HZ and BH designed the experiments and methods. HZ and EA built the optimal models. HZ performed the modeling work, performed empirical measurements and analyzed the data. HZ wrote the first draft, BH and EA contributed substantially to revisions.

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Supporting information

Fig. S1 Modeling results to show the effect of O₂ evolution on the assimilation rate of C₄ pathway.

Fig. S2 Modeling results of optimal $V_{\text{pmax}}/V_{\text{cmax}}$ for C₄ under different environmental conditions.

Fig. S3 Sensitivity analysis of total nitrogen and PEPC stoichiometry on optimal $J_{\text{max}}/V_{\text{cmax}}$ for C₃ and $V_{\text{pmax}}/V_{\text{cmax}}$ C₄.

Table S1 The evolutionary models used for the phylogenetic analysis for photosynthetic properties evolutionary differences between C₃ and C₄ species.

Table S2 Phylogenetic analysis results for $J_{\text{max}}/V_{\text{cmax}}$ for C₃ and C₄ species.

Table S3 Phylogenetic analysis results for V_{cmax} for C₃ and C₄ species.

Table S4 Phylogenetic analysis results for J_{max} for C₃ and C₄ species.

Table S5 Phylogenetic analysis results for total chlorophyll for C₃ and C₄ species.

Table S6 Phylogenetic analysis results for ETR/J for C₃ and C₄ species.

Table S7 Phylogenetic analysis results for chlorophyll a/chlorophyll b for C₃ and C₄ species.

Table S8 Phylogenetic analysis results for ETR for C₃ and C₄ species.

Table S9 Phylogenetic analysis results of evolutionary model fitting for V_{pmax} for C₄ species.

Table S10 Phylogenetic analysis results of evolutionary model fitting for V_{pmax}/V_{cmax} for C_4 , C_3 .

Table S11 Phylogenetic analysis results of evolutionary model fitting for J_{max}/V_{cmax} for C_3 and three C_4 subtypes.

Table S12 Phylogenetic analysis results of evolutionary model fitting for V_{cmax} for C_3 and three C_4 subtypes.

Table S13 Phylogenetic analysis results of evolutionary model fitting for J_{max} for C_3 and three C_4 subtypes.

Table S14 Phylogenetic analysis results of evolutionary model fitting for total chlorophyll for C_3 and three C_4 subtypes.

Table S15 Phylogenetic analysis results of evolutionary model fitting for ETR/J for C_3 and three C_4 subtypes.

Table S16 Phylogenetic analysis results of evolutionary model fitting for chlorophyll a/chlorophyll b for C_3 and three C_4 subtypes.

Table S17 Phylogenetic analysis results of evolutionary model fitting for ETR for C_3 and three C_4 subtypes.

Table S18 Phylogenetic analysis results of evolutionary model fitting for V_{pmax} for C_3 and three C_4 subtypes.

Table S19 Phylogenetic analysis results of evolutionary model fitting for V_{pmax}/V_{cmax} for C_3 and three C_4 subtypes.