



# C<sub>4</sub> photosynthesis and climate through the lens of optimality

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CO<sub>2</sub>, temperature, water availability, and light intensity were all potential selective pressures that determined the competitive advantage and expansion of the C<sub>4</sub> photosynthetic carbon-concentrating mechanism over the last ~30 My. To tease apart how selective pressures varied along the ecological trajectory of C<sub>4</sub> expansion and dominance, we coupled hydraulics to photosynthesis models while optimizing photosynthesis over stomatal resistance and leaf/fine-root allocation. We further examined the importance of nitrogen reallocation from the dark to the light reactions. We show here that the primary selective pressures favoring C<sub>4</sub> dominance changed through the course of C<sub>4</sub> evolution. The higher stomatal resistance and leaf-to-root ratios enabled by C<sub>4</sub> led to an advantage without any initial difference in hydraulic properties. We further predict a reorganization of the hydraulic system leading to higher turgor-loss points and possibly lower hydraulic conductance. Selection on nitrogen reallocation varied with CO<sub>2</sub> concentration. Through paleoclimate model simulations, we find that water limitation was the primary driver for a C<sub>4</sub> advantage, with atmospheric CO<sub>2</sub> as high as 600 ppm, thus confirming molecular-based estimates for C<sub>4</sub> evolution in the Oligocene. Under these high-CO<sub>2</sub> conditions, nitrogen reallocation was necessary. Low CO<sub>2</sub> and high light, but not nitrogen reallocation, were the primary drivers for the mid- to late-Miocene global expansion of C<sub>4</sub>. We also predicted the timing and spatial distribution for origins of C<sub>4</sub> ecological dominance. The predicted origins are broadly consistent with prior estimates, but expand upon them to include a center of origin in northwest Africa and a Miocene-long origin in Australia.

C<sub>4</sub> evolution | optimal stomatal conductance | resource allocation | water limitation | dark/light reaction

The evolution of the C<sub>4</sub> photosynthetic pathway enabled the concentration of CO<sub>2</sub> around Rubisco, the enzyme responsible for the first major step of carbon fixation in the C<sub>3</sub> photosynthetic pathway, thus reducing photorespiration. C<sub>3</sub> photosynthesis is present in all plants, but within C<sub>4</sub> plants, the C<sub>3</sub> pathway is typically ensconced within specialized bundle sheath cells that surround leaf veins. CO<sub>2</sub> that diffuses into a leaf is shuttled from adjacent mesophyll cells to the bundle sheath via a four-carbon pump, the energetic cost of which is remunerated by ATP derived from the light reactions (1, 2). As a whole, the C<sub>4</sub> pathway reduces photorespiration, a process that can dramatically reduce photosynthesis and begins with the assimilation of O<sub>2</sub>, instead of CO<sub>2</sub>, by Rubisco. Over the last 30 My, the reduction in C<sub>3</sub> photosynthesis by photorespiration was large and broad enough to select for the independent evolution of the C<sub>4</sub> pathway more than 60 times across the terrestrial plants (3). The diversity of plant families with C<sub>4</sub> is greatest in the eudicots (1,200 species) and the Poaceae, the monocot family containing the grasses (4,500 species) (2), which accounts for nearly 25% of terrestrial plant productivity and several important agricultural species (4).

While increased photorespiration was central to the evolution of the C<sub>4</sub> carbon concentrating mechanism (CCM), the relative ecological importance of different environmental drivers of the photorespiratory increase is not as clear (5, 6). Lower CO<sub>2</sub> and higher temperature lead to higher rates of photorespiration, which selected for the evolution of C<sub>3</sub>-C<sub>4</sub> intermediates and ultimately C<sub>4</sub>. Past

physiological models, therefore, focused on temperature and CO<sub>2</sub> concentration as selective pressures for C<sub>4</sub> evolution and expansion (7, 8). Under warmer temperatures and low CO<sub>2</sub>, C<sub>4</sub> photosynthesis has greater carbon gain than C<sub>3</sub>, but under cooler temperatures and high CO<sub>2</sub>, the metabolic costs of the C<sub>4</sub> pathway and lower photorespiration in C<sub>3</sub> leads to greater carbon gain in C<sub>3</sub>. Alternatively, water availability has been proposed as the impetus for C<sub>4</sub> evolution in eudicots (2), and recent phylogenetic analyses have suggested the same in grasses (6). Water availability should have an impact on C<sub>4</sub> evolution that could work independently or in concert with changes in CO<sub>2</sub> and temperature. First, water deficits indirectly increase photorespiration in C<sub>3</sub> plants by forcing stomatal closure to reduce leaf water loss, consequently decreasing the flux of CO<sub>2</sub> into the leaf and the availability of CO<sub>2</sub> for Rubisco (9). Second, the C<sub>4</sub> CCM allows for the maintenance of lower stomatal conductance, and therefore lower water loss, for a given assimilation rate, leading to a higher water-use efficiency (WUE) than C<sub>3</sub> (10).

The different environmental drivers of the photorespiratory increase in C<sub>4</sub> progenitors—atmospheric CO<sub>2</sub> concentration, temperature, and water availability—changed dramatically over the period of C<sub>4</sub> diversification and expansion. Although there is uncertainty of CO<sub>2</sub> concentration from different proxies (11), atmospheric CO<sub>2</sub> generally decreased from the mid-Oligocene (~600 ppm) to the ~400 ppm in the midearly Miocene (12, 13) but with significant variability (±100 ppm; refs. 13 and 14), after which it reduced to values of less than ~300 ppm in the Pliocene (13). Physiological models that focused on temperature and CO<sub>2</sub> implied that C<sub>4</sub> evolved, in both grasses and eudicots, at the low

## Significance

We use a coupled photosynthesis–hydraulic optimal physiology model in conjunction with paleoclimate modeling to examine the primary selective pressures along the ecological trajectory of C<sub>4</sub> photosynthesis and to confirm and revise likely geographical points of dominance and expansion. Water limitation was the primary driver for the initial ecological advantage of C<sub>4</sub> over C<sub>3</sub> in the mid-Oligocene until CO<sub>2</sub> became low enough to, along with light intensity, drive the global expansion of C<sub>4</sub> in the Miocene. Our integrated modeling framework also predicts C<sub>4</sub> evolution should be followed by a decrease in hydraulic conductance, an increase in the leaf-turgor-loss point, and CO<sub>2</sub>-dependent reallocation of nitrogen between dark and light reactions.

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end of this CO<sub>2</sub> range in the mid-Miocene to the Pliocene (2, 7, 8, 15). Isotopic and fossil evidence shows that C<sub>4</sub> grasses became a major component of grassland biomes—in terms of biomass, C<sub>4</sub> lineage diversity, or herbivore dietary components—in the mid-Miocene, but molecular evidence suggests that C<sub>4</sub> photosynthesis may have arisen in the grasses as early as the mid-Oligocene, more than 30 Mya (11). Similarly, phylogenetic reconstructions provide evidence that some eudicots evolved C<sub>4</sub> as early as the monocots and also saw the greatest rate of C<sub>4</sub> diversification and expansion in the late Miocene (16, 17). The error associated with these molecular dating techniques is large, however, and the uncertainty range for even the oldest C<sub>4</sub> lineages overlaps with the mid-Miocene estimates for C<sub>4</sub> evolution and expansion. Along with CO<sub>2</sub>, precipitation declined over the period of C<sub>4</sub> diversification and expansion, leading to vast terrestrial areas where low or highly seasonal precipitation inputs led to the loss of forests and, consequently, the evolution of the world's first grasslands (18). The spread of grasslands indicates a habitat change with larger surface radiation loads, higher surface temperatures, and increased potential for plant water loss (5, 19). Therefore, if the early evolution of C<sub>4</sub> suggested by molecular-dating approaches are correct, then water availability played an important role for both C<sub>4</sub> grasses and eudicots, while CO<sub>2</sub> was still relatively high (5, 16, 19, 20). The potentially interacting roles of water availability, changes in radiation, and CO<sub>2</sub> along the ecological trajectory of C<sub>4</sub> photosynthesis have not been fully investigated within comprehensive physiological and paleoclimate models.

A related but largely unstudied physiological change during the divergence of C<sub>4</sub> photosynthesis from C<sub>3</sub> is the allocation of nitrogen between the dark reactions and the light reactions. C<sub>4</sub> plants might allocate a greater proportion of N to light reactions than to dark reactions compared with C<sub>3</sub> because of the extra ATP cost of the CCM (21, 22). We propose that the reallocation of N between dark and light reactions provides a further advantage for C<sub>4</sub> above the CCM alone and that different environmental conditions can select for a shift in the degree of reallocation both through evolutionary time and across species in extant plants.

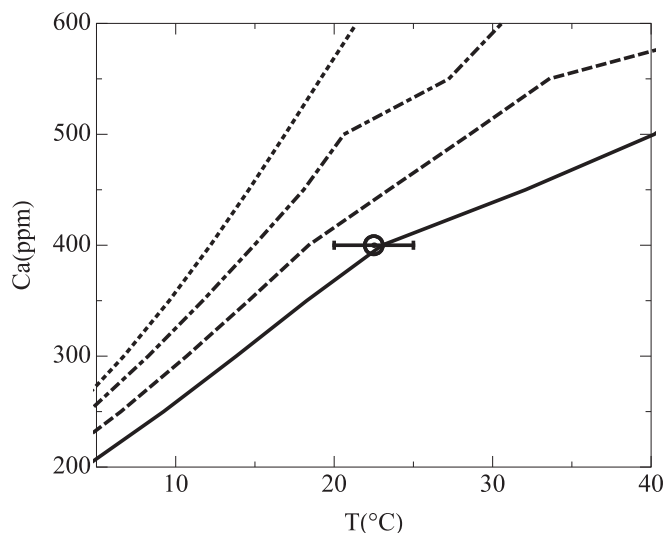
Our goal is to integrate several ecologically relevant selective pressures that determined the competitive advantage and expansion of the C<sub>4</sub> pathway from the mid-Oligocene through to the late Miocene. C<sub>4</sub> evolved via C<sub>3</sub>–C<sub>4</sub> intermediates that display a number of successive biochemical and anatomical traits that reduce photorespiration compared with C<sub>3</sub> plants, but further reductions in photorespiration, enhanced WUE and nitrogen-use efficiency, and increases in ecological niche space did not occur until the evolution of the full C<sub>4</sub> CCM (23, 24). We therefore assume that C<sub>3</sub> plants, and not C<sub>3</sub>–C<sub>4</sub> intermediates, were the major ecological competitors of C<sub>4</sub> plants. We examine how changes in selective pressures augmented the relative advantage of these two evolutionarily stable states within the framework of an optimality model in which the plant makes allocation “decisions” to maximize photosynthetic assimilation rate. We advance our understanding of C<sub>4</sub> photosynthesis in five ways. First, we revisit the temperature–CO<sub>2</sub> crossover approach and integrate the effects of water limitation, light, optimal allocation decisions, and the interactions between these in a single model. Second, we formalize the hypothesis that C<sub>4</sub> photosynthesis has a higher WUE than C<sub>3</sub>, using an optimality argument to balance carbon gain and water loss. Specifically, we let both stomatal conductance and leaf/fine-root allocation emerge endogenously, rather than assuming a priori that C<sub>4</sub> grasses have lower stomatal conductance. This allows us to elucidate the previously unexplored role of optimal stomatal conductance (but see ref. 15) and resource allocation in mediating ecological success due to water limitation and to predict further divergence of hydraulic properties. Third, we explicitly include the additional ATP cost of the C<sub>4</sub> pathway with a mechanistic model (1, 25), which previous modeling analysis did not explicitly consider (7, 8, 19). Fourth, we consider reallocation of nitrogen from the dark reactions to the light reactions, which can change tradeoffs

between photosynthesis and water use by C<sub>4</sub>. Finally, we drive the optimality model under three CO<sub>2</sub> scenarios with outputs from a fully coupled general circulation model for Miocene/Oligocene climate to examine regions and timing of C<sub>4</sub> ecological advantage as a proxy for potential evolutionary origins.

## Results

We validated our optimality model through comparisons with previous models and empirical data from closely related C<sub>3</sub> and C<sub>4</sub> species measured under similar conditions (26) (SI Appendix, Fig. S1). Model outputs were consistent with observed patterns of C<sub>3</sub> versus C<sub>4</sub> for stomatal resistance, biomass allocation, photosynthesis, and leaf water potential. Leaf water potential predictions matched observed values, while predicted values for other measures were slightly higher. We incorporated our stomatal resistance and biomass outputs into a Penman–Monteith model to determine if we could replicate the observed ecosystem-level water balance of C<sub>3</sub>–C<sub>4</sub> mixed grasslands (27) (SI Appendix, Supporting Information S13). Our model confirmed that increasing the C<sub>4</sub> grass component reduces desiccation under higher temperatures and CO<sub>2</sub> (SI Appendix, Fig. S2 and Table S3). We further predicted that local desiccation would occur in pure C<sub>3</sub> grasslands due to warming, even with CO<sub>2</sub> increasing from 400 ppm to 600 ppm (SI Appendix, Fig. S3). In contrast, local desiccation would be mitigated in pure C<sub>4</sub> grasslands.

Assimilation-based crossover temperatures, defined as the temperature at which assimilation by the C<sub>4</sub> pathway exceeds that of the C<sub>3</sub> pathway, decrease as water limitation increases and light intensity increases across all CO<sub>2</sub> concentrations (Fig. 1 and SI Appendix, Fig. S4). Without water stress (solid black line in Fig. 1), our model predicts a C<sub>3</sub>/C<sub>4</sub> crossover temperature of 23 °C under 380 ppm, a result similar to previous data and/or models (7, 8). The model results in Fig. 1 were all under the light intensity of 1,400 μmol·m<sup>-2</sup>·s<sup>-1</sup> and with a C<sub>4</sub> J<sub>max</sub>/V<sub>cmax</sub> ratio of 4.5, which corresponds to a reallocation of nitrogen from dark to light reactions. Model results for a C<sub>4</sub> J<sub>max</sub>/V<sub>cmax</sub> ratio of 2.1 (no reallocation) were similar (SI Appendix, Fig. S4A), with the exception of low CO<sub>2</sub> and low water availability. Crossover temperatures are higher with J<sub>max</sub>/V<sub>cmax</sub> = 4.5, showing that nitrogen



**Fig. 1.** Crossover temperatures of photosynthesis for C<sub>3</sub> and C<sub>4</sub> with the change of CO<sub>2</sub> concentration under different water conditions. Light intensity was 1,400 μmol·m<sup>-2</sup>·s<sup>-1</sup> for all model runs. J<sub>max</sub>/V<sub>cmax</sub> = 2.1 for C<sub>3</sub> and J<sub>max</sub>/V<sub>cmax</sub> = 4.5 for C<sub>4</sub>. Solid black line: VPD = 0.1 kPa, ψ<sub>s</sub> = 0 MPa; dashed black line: VPD = 0.625 kPa, ψ<sub>s</sub> = -0.5 MPa; dot-dashed black line: VPD = 1.25 kPa, ψ<sub>s</sub> = -1 MPa; dotted black line: VPD = 1.875 kPa, ψ<sub>s</sub> = -1.5 MPa. The circle and error bars indicated the average and confidence intervals of crossover temperature in Collatz (8).

reallocation decreases the  $C_4$  advantage under water limitation and low  $CO_2$ . Under saturated soil and low vapour pressure deficit (VPD), crossover temperatures decrease along with increasing light intensity (SI Appendix, Fig. S4 C and D). An increase in light intensity provides a larger relative benefit for  $C_4$  at low  $CO_2$ , because  $C_3$  photosynthesis remains  $CO_2$ -limited throughout, while  $C_4$  light limitations lessen as light increases. Photosynthetic limitation states were examined under multiple environmental scenarios, using  $J_{max}/V_{cmax} = 2.1$  or 4.5 for  $C_4$ . With  $J_{max}/V_{cmax} = 2.1$ ,  $C_4$  is light-limited in most conditions (SI Appendix, Fig. S5 A and C). With  $J_{max}/V_{cmax} = 4.5$ , or when  $CO_2$  decreases to 200 ppm,  $C_4$  becomes limited by  $CO_2$  under low temperatures and by light under high temperatures (SI Appendix, Fig. S5 B and D-F).

To provide a more quantitative measure of  $C_4$  advantage, we calculated the net assimilation rate difference between  $C_4$  and  $C_3$ ,  $\Delta A_n$  (net assimilation of  $C_4$  minus that of  $C_3$ ), through all environmental variations (Fig. 2 and SI Appendix, Fig. S6). The positive contour space ( $\Delta A_n > 0$ ) means that  $C_4$  outcompetes  $C_3$  within given environmental dimensions, and the higher the  $\Delta A_n$ , the greater the advantage of  $C_4$ . In Fig. 2, the light intensity of  $1,400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  is fixed for all model runs. Under  $CO_2 = 200$  ppm,  $\Delta A_n$  is higher under moist conditions than water-limited conditions (Fig. 2 A and B). In contrast, under higher  $CO_2$  (400 and 600 ppm),  $C_4$  has the greatest advantage only in water-limited conditions, leaving a relatively small environmental envelope for  $C_4$  (Fig. 2 C-F). This is because  $C_3$  photosynthesis has a greater proportional increase in assimilation from 200 to 400 or 600 ppm  $CO_2$ . Across all scenarios, increasing  $J_{max}/V_{cmax}$  increases both the  $\Delta A_n$  and space for  $C_4$  advantage (Fig. 2 B, D, and F). Light responses were

examined under saturated soils (SI Appendix, Fig. S6) and at low  $CO_2$ .  $\Delta A_n$  increases strongly as light increases, whereas there is a much smaller light effect at 400 ppm  $CO_2$  and higher, and a high  $J_{max}/V_{cmax}$  was required for a  $C_4$  advantage ( $\Delta A_n > 0$ ).

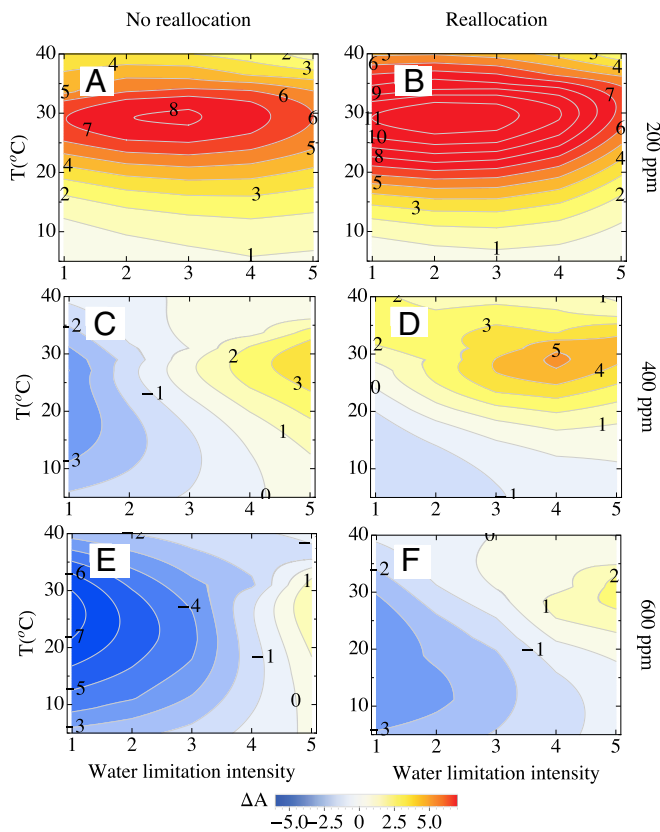
By driving the optimality model with outputs from the paleoclimate model, we can predict the geographic centers for  $C_4$  ecological dominance as a proxy for  $C_4$  origins. Areas of central Asia, southwest Asia, and northern Africa/Arabia would strongly select for  $C_4$  at 600 ppm  $CO_2$  because of the warm temperatures and arid conditions simulated there (Fig. 3A). Southwestern Australia also has a significant land area that would support  $C_4$ , and to a lesser extent, so does southwestern North America. As  $CO_2$  decreased to 400 and 270 ppm, the areas mentioned above expanded to strongly support a  $C_4$  ecological advantage with the addition of southern Africa and southern South America (Fig. 3 B and C). As  $CO_2$  decreased,  $C_4$  favorability maintained a foothold in the semiarid sites and moved into wetter regions, while still requiring warm temperatures for an advantage. At both 400 and 600 ppm, a higher  $J_{max}/V_{cmax}$  ratio was required for  $C_4$  to maintain a higher advantage over  $C_3$  (Fig. 3 A and B and SI Appendix, Fig. S7 A and B). At 270 ppm,  $C_4$  had a broad advantage over  $C_3$  with a lower  $J_{max}/V_{cmax}$  ratio (Fig. 3 C and SI Appendix, Fig. S7 C).

We calculated the photosynthesis rates of the two pathways by only varying the  $J_{max}/V_{cmax}$  for  $C_4$  to further examine the pure effect of nitrogen reallocation (Fig. 4). With  $J_{max}/V_{cmax} = 2.1$  for both  $C_3$  (solid black line) and  $C_4$  (dashed line), the  $C_4$  assimilation rate is rarely higher than  $C_3$ , which indicates  $C_4$  does not have an obvious advantage under current  $CO_2$ . However, with  $J_{max}/V_{cmax} = 4.5$  for  $C_4$  (dotted line),  $C_4$  has an advantage over  $C_3$  at higher temperatures.

Under all environmental and nitrogen allocation scenarios, optimal stomatal resistance ( $r_s$ ) and leaf biomass/total biomass of leaf and fine-root allocation ( $f$ ) are higher in  $C_4$  plants than  $C_3$  plants, and response patterns were similar across  $CO_2$  concentrations (SI Appendix, Fig. S8). In addition,  $f$  decreases and  $r_s$  increases as the intensity of water limitation increases. Results are consistent for  $C_4$  with a  $J_{max}/V_{cmax}$  of 2.1 and  $J_{max}/V_{cmax}$  of 4.5. The higher  $r_s$  in  $C_4$  plants led to a consistently higher water potential than  $C_3$  plants in all simulated conditions (SI Appendix, Fig. S1B). We also predicted that  $C_4$  plants should have a higher leaf-turgor-loss point than closely related  $C_3$  plants, and we found empirical support for this prediction across four closely related  $C_3$ - $C_4$  clusters (Fig. 5).

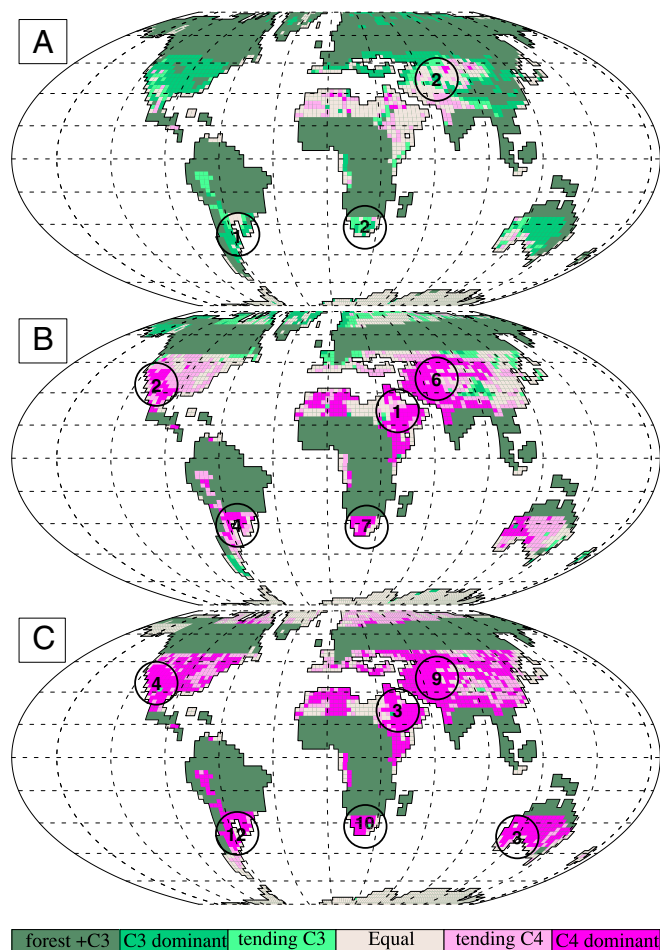
## Discussion

Based on the conditions under which  $C_4$  plants have the ecological advantage over  $C_3$ , our results offer physiological and climatological support for a potential Oligocene ecological dominance of  $C_4$ . This finding is in concert with the early ranges of  $C_4$  evolution from molecular-based approaches (16, 17), and we use this ecological dominance as a proxy to identify the regions where  $C_4$  would likely emerge. Isotopic and fossil evidence suggest that  $C_4$  photosynthesis first arose in the mid-Miocene, whereas molecular and phylogenetic approaches suggest that  $C_4$  first arose anywhere from the mid-Miocene to mid-Oligocene (11). Our paleoclimate model broadly represents the environmental conditions for Oligocene to mid-Miocene (12, 28, 29), with high  $CO_2$  conditions representing the mid-Oligocene, and low  $CO_2$  mid-Miocene. We find that environmental conditions favored  $C_4$  plants during the mid-Oligocene (~30 Mya) at warm, arid sites where water limitation acted as the primary selective pressure to increase photorespiration when  $CO_2$  was as high as 600 ppm. The geographic origins predicted by our model and those proposed by others (23) tend to agree, which lends general support to our approach. At the same time, there are important differences that impact both the location and potential age for the evolution of  $C_4$  (Fig. 3). Notably, we find a greatly expanded region of potential origin in northern Africa. Under Oligocene/Miocene climate, northern Africa was arid, but the Tethys sea had not yet closed, and the northwest and the northeast were



**Fig. 2.** The total difference in  $CO_2$  assimilation between  $C_4$  and  $C_3$  [ $A_n(C_4) - A_n(C_3)$ ] under various  $CO_2$  (200 ppm, 400 ppm, and 600 ppm) and water conditions under light intensity ( $1,400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ).  $J_{max}/V_{cmax} = 2.1$  for  $C_3$  and  $C_4$  (A, C, and E) and  $J_{max}/V_{cmax} = 2.1$  for  $C_3$  and  $J_{max}/V_{cmax} = 4.5$  for  $C_4$  (B, D, and F). Water limitation intensity is as follows: 1, VPD = 0.1 kPa,  $\psi_s = 0$  MPa; 2, 0.625 kPa, -0.5 MPa; 3, 1.25 kPa, and -1 MPa; 4, 1.875 kPa, -1.5 MPa; 5, 2.5 kPa, and -2 MPa.





**Fig. 3.** The regional distributions of  $C_3$  or  $C_4$  ecological dominance under Oligocene/Miocene climate and different  $CO_2$ . Dominance is determined by the assimilation difference [ $A_n(C_4) - A_n(C_3)$ ;  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ] with the thresholds as follows:  $>3$ ,  $C_4$  dominant; between 1 and 3,  $C_4$  slightly dominant; between  $-1$  and 1, equal dominance; between  $-3$  and  $-1$ ,  $C_3$  slightly dominant;  $< -3$ ,  $C_3$  dominant. For each grid cell, the optimality model was driven with outputs from the Community Land Model (CLM4.5) in the CESM: (A) 600 ppm  $CO_2$  and (B) 400 ppm  $CO_2$ , both with  $C_3 J_{\text{max}}/V_{\text{cmax}}$  ratio = 2.1 and  $C_4 J_{\text{max}}/V_{\text{cmax}}$  ratio = 4.5, (C) 270 ppm  $CO_2$ ,  $C_3 J_{\text{max}}/V_{\text{cmax}}$  ratio = 2.1 and  $C_4 J_{\text{max}}/V_{\text{cmax}}$  ratio = 2.1. Circles superimposed on figures indicate evolutionary origins from previous studies (23) and numbers within the circles indicate cumulative lineages within which  $C_4$  evolved by a given time period for (A) late Oligocene/early Miocene, (B) mid-Miocene, and (C) late Miocene/Pliocene.

consequently just wet enough to ecologically favor  $C_4$  over  $C_3$  plants. Likewise, Australia is thought to have developed conditions favorable for the evolution and expansion of  $C_4$  only within the last 9 Mya (23), yet we show it slightly favoring  $C_4$  under Oligocene  $CO_2$  and strongly favoring  $C_4$  by the mid-Miocene. Climate simulations suggest that both northern Africa and southwestern Australia had wetter summers than the current Mediterranean-type climate.

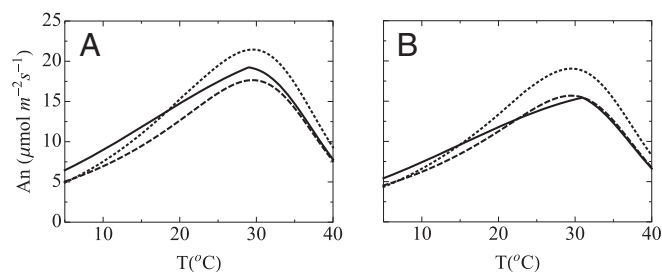
As  $CO_2$  decreased through the Miocene, warm temperatures remained a strong selective force, but the primary selective force for a  $C_4$  advantage over  $C_3$  shifted from water limitation to low  $CO_2$  and, to a lesser extent, light intensity. However, as increased light intensity alone could not lead to an advantage of  $C_4$  under high  $CO_2$  (SI Appendix, Fig. S6C), it seems likely that  $C_4$  could not dominate except in locally arid areas while  $CO_2$  was high. Thus, after its emergence,  $C_4$  radiation likely idled in small pockets of selective favorability as  $CO_2$  concentrations declined through the Miocene (13), similar to the “edaphic ghetto” hypothesis (30). Furthermore, given that  $CO_2$  may have been

rapidly cycling on orbital time scales between 500 ppm and 300 ppm (14), the transition to widespread  $C_4$  could have exhibited hysteresis and occurred through fits and starts. Such shifts in primary selective pressures on  $C_4$  photosynthesis over evolutionary time are consistent with the isotopic evidence (31, 32).

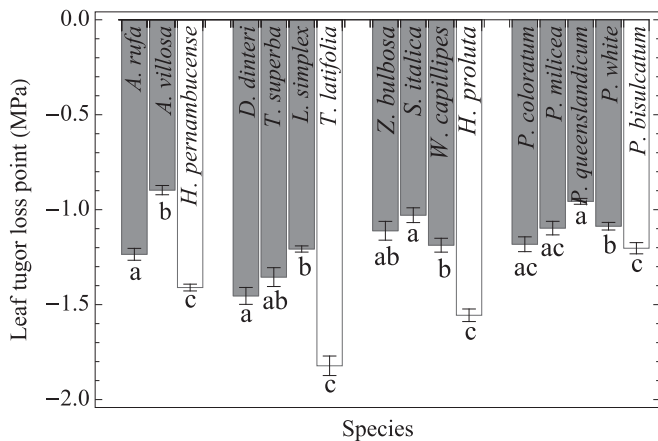
Consistent with previous studies, our model predicts that low  $CO_2$  (200–300 ppm) strongly favors  $C_4$  over  $C_3$  photosynthesis (e.g., refs. 7 and 15). We further show that low  $CO_2$  provides a clear  $C_4$  advantage under a large range of water availability and light intensity regimes. Under low  $CO_2$ , the greatest  $C_4$  advantage occurs in relatively moist and mildly water-limited conditions, opposite to that which is seen under high  $CO_2$ . Under low  $CO_2$ , new  $C_4$  species evolved in multiple lineages and together with the earlier  $C_4$  species started to increase their biomass to occupy open sites (11). The environmental conditions that led to the largest  $C_4$  advantage within our model, therefore, parallel those documented in extant  $C_4$ -dominated grasslands: highly seasonal precipitation that occurs chiefly within a warm growing season (33, 34). These are also similar to the conditions that led to the large-scale expansion of  $C_4$  grasslands in the Miocene—for example, the onset of summer monsoons and subsequent  $C_4$  grassland expansion in the Indian subcontinent (35).

The role of water limitation in  $C_4$  grass evolution has sparked interest in grass hydraulics and the anatomical shifts in  $C_3$  grasses that were prerequisites to  $C_4$  evolution (19, 20), and we further propose that the evolution of  $C_4$  photosynthesis leads to a reorganization of the hydraulic system. A lower leaf-turgor-loss point is typically a strong indicator of drought tolerance across species (36). On the contrary, we predict that the higher stomatal resistance of the  $C_4$  CCM leads to a higher leaf water potential than  $C_3$  in all water-limited conditions; thus, there is no need for  $C_4$  to maintain a lower leaf-turgor-loss point. We confirmed this prediction in four closely related  $C_3$ – $C_4$  clusters (Fig. 5). It is thought that the higher vein density of  $C_4$  grasses should lead to greater hydraulic conductance (19, 20), but we found a clear  $C_4$  advantage solely by allowing for optimal leaf: fine-root allocation and stomatal conductance. We also find that increasing hydraulic conductance had little impact on the  $C_4$  advantage (SI Appendix, Fig. S9), indicating that the  $C_4$  CCM itself is enough to result in greater carbon gain under water stress. These results do not contradict the idea that larger bundle sheaths and smaller interveinal distance—which were clear prerequisites for  $C_4$  evolution (20, 37)—led to greater hydraulic conductance and drought tolerance among  $C_4$  progenitors (20). They do, however, suggest that greater hydraulic conductance is not necessary to give  $C_4$  plants an advantage once the CCM evolved. We hypothesize that once  $C_4$  evolves in a lineage, selection on increased hydraulic conductance would not only lessen but invert, leading to the development of even narrower xylem conduits and greater drought resistance. There is empirical support for such a prediction in eudicots (38).

Different environmental conditions can select for a shift in the degree of nitrogen allocation across the light and dark reactions



**Fig. 4.** Assimilation rates of  $C_3$  with  $J_{\text{max}}/V_{\text{cmax}} = 2.1$  (solid black line),  $C_4$  with  $J_{\text{max}}/V_{\text{cmax}} = 2.1$  (dashed black line), and  $C_4$  with  $J_{\text{max}}/V_{\text{cmax}} = 4.5$  (dotted black line) (other parameters are maintained the same for  $C_3$  and  $C_4$ ) under light intensity of  $1,400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $CO_2$  of 400 ppm, and different water limitation conditions. (A) VPD = 0.625 kPa,  $\psi_s = -0.5$  MPa; (B) 1.25 kPa,  $-1$  MPa.



**Fig. 5.** Measured leaf turgor loss points in four closely related groups of C<sub>3</sub> and C<sub>4</sub> species (white bars: C<sub>3</sub> species; gray bars: C<sub>4</sub> species). Error bars show SEs. Different letters denote a significant difference within a group.

separately from the C<sub>4</sub> CCM (assessed here by a change in  $J_{max}/V_{cmax}$ ). In general, CCMs allow for less investment in nitrogen-rich Rubisco (39), and the nitrogen not used for Rubisco could be either reinvested in light-harvesting machinery or simply not used at all, thus reducing the total nitrogen requirement. Modeling studies have long assumed a high  $J_{max}/V_{cmax}$  for C<sub>4</sub> photosynthesis (19, 40), and measurements show lower Rubisco content and higher chlorophyll and thylakoid content, giving evidence of reallocation in extant C<sub>4</sub> species (21, 22). Empirical estimates of  $J_{max}/V_{cmax}$  in C<sub>4</sub> plants, are more variable, ranging from 2 to above 6, with a mean of around 4.5 (41–43), which is higher than the mean  $J_{max}/V_{cmax}$  estimates for C<sub>3</sub> plants of 2.1 (44). Increasing  $J_{max}/V_{cmax}$  almost always increases the photosynthesis rate of C<sub>4</sub> grasses (Fig. 4 and *SI Appendix, Fig. S10*) and therefore could lead to a competitive advantage over C<sub>3</sub> grasses as well as C<sub>4</sub> grasses that do not reallocate. Assuming there is little cost or no genetic constraints for reallocation, the selection pressure to reallocate would have been strongest when CO<sub>2</sub> was high because the CCM alone does not give C<sub>4</sub> a large advantage. When CO<sub>2</sub> was low during the late Miocene C<sub>4</sub> expansion, however, the CCM alone would give C<sub>4</sub> an advantage and reallocation would not change the competitive balance between C<sub>3</sub> and C<sub>4</sub>. As CO<sub>2</sub> remained low through to the Pleistocene, selection for nitrogen reallocation to the light reactions would lessen further, especially during the CO<sub>2</sub> minima of the Pleistocene glacial periods (~180 ppm).

Each evolutionary origin of C<sub>4</sub> photosynthesis represents both different selective pressures and taxonomic (genetic) constraints as climate and CO<sub>2</sub> changed. Taking the Chloridoideae as an example, we can use our model to develop hypotheses along the ecological trajectory of C<sub>4</sub> in this grass subfamily. The ecological advantage of C<sub>4</sub> photosynthesis in the Oligocene, while CO<sub>2</sub> was high, was driven by aridity, acting to decrease stomatal conductance that increased photorespiration in C<sub>4</sub> progenitors initially, and led to higher WUE upon the evolution of the CCM. There would have been enough of a reduction in water use that the turgor-loss point would increase and selection for increased hydraulic conductance would relax, allowing for the development of more resilient—and less conductive—xylem. There would have been strong selection for reallocation of nitrogen from the dark reactions to the light reactions. The large radiation of C<sub>4</sub> within the Chloridoideae occurring in the mid- to late Oligocene was likely driven by low CO<sub>2</sub> and high light, and the previously evolved hydraulic resilience would perhaps relegate this subfamily to being the dry-site specialists observed in current-day distributions (45). There would have been much less selective pressure to reallocate N during the large radiation, but such a reorganization was likely already in place within the clade. In contrast, for the lineages that evolved C<sub>4</sub> in the late Miocene

(e.g., *Stipagrostis*, *Eriachne*, *Neurachne*), CO<sub>2</sub> would have been the primary impetus for C<sub>4</sub> evolution, but for these lineages, there would have been little selection to reallocate nitrogen, and we predict that they would have greater hydraulic conductance and lower turgor-loss points than those of the Chloridoideae.

By optimizing carbon gain over water loss, we developed a plausible physiological explanation for the ecological advantage of C<sub>4</sub> through time and further proposed hypotheses about how a variety of traits that accompany the C<sub>4</sub> CCM developed in concert with the climate changes that occurred through this ecological trajectory (46). There are obvious caveats with our interpretations, because we focus solely on physiology and assume that competitive outcomes or selective pressures are decided primarily by photosynthetic rates. We also do not consider how larger ecological processes like disturbance can undermine physiology-based projections of plant distributions (47). However, by examining extant species within select lineages in both controlled and natural environments, these hypotheses can be examined empirically together with our physiological model, ultimately providing an integrative view of the selection pressures that led to the current physiologies and distribution of C<sub>4</sub> plants.

## Materials and Methods

**Overview of the Plant Model.** We first assume that the CCM is the only difference between C<sub>3</sub> and C<sub>4</sub>, corresponding to two closely related species whose other traits have not yet diverged. We then allow for divergence through shifts in nitrogen allocation between the light and dark reactions of C<sub>4</sub>. Our model incorporates the soil–plant–air–water continuum into traditional C<sub>3</sub> (48) and C<sub>4</sub> (25) photosynthesis models and assumes that plants optimize stomatal resistance and leaf/fine-root allocation to balance carbon gain and water loss (49). The rate of water loss through transpiration equals the rate of water absorption by the roots, at equilibrium (49). Stomatal resistance ( $r_s$ ) controls transpiration and photosynthesis. The leaf/fine-root ( $f$ ) ratio, defined as the ratio of biomass for leaves to the sum of biomasses for leaves and fine roots, controls the biomass allocated to leaf area for transpiration and photosynthesis. The lowering of leaf water potential through transpiration water loss and/or environmental factors (VPD and soil water potential) leads to a lowering of the photosynthetic rate via Weibull-type vulnerability curves (40). A full model description is in *SI Appendix, Supporting Information S11* with *SI Appendix, Table S1* for parameter abbreviations and *SI Appendix, Table S2* for input parameters. The model derivation and methods for numerical solutions using Mathematica (Wolfram Research, Inc.)/R are in *SI Appendix, Mathematica-S1* and *R* package.

**Optimal Stomatal Resistance and Allocation of Energy Between Leaves and Fine Roots.** We assume that the plant adjusts the  $r_s$  and  $f$  to optimize the total carbon gain

$$A_{total} = \frac{fNA_p}{\rho}$$

where  $\rho$  is the leaf mass density ( $g \cdot m^{-2}$ ), and for simplicity, we assume that  $N$  and  $\rho$  are fixed (49). This amounts to considering the optimization problem faced by the plant in a given instance during growth, where size is a constant. We treat the instantaneous optimization problem as a proxy for the optimal growth path as the growth rate is maximized at any given time. We regard  $\rho$  as a species-specific trait that changes at a slower time scale than  $r_s$  and  $f$ .

**Allocation of Nitrogen.** The ratio  $J_{max}/V_{cmax}$  was used as a proxy for nitrogen allocation between RuBP carboxylation and regeneration. The initial condition for  $J_{max}/V_{cmax}$  was 2.1 (44) for both C<sub>3</sub> and C<sub>4</sub>. For the reallocation, the value for C<sub>4</sub> is  $J_{max}/V_{cmax} = 4.5$  (19, 40). We used a simple stoichiometry for  $J_{max}$  and  $V_{cmax}$  by considering the sum of  $J_{max}$  and  $V_{cmax}$  as a constant representing total available nitrogen for photosynthesis; such a stoichiometry was drawn from the existing modeling work (19, 40). Two assumptions underlie this stoichiometry: (i) Investing one molecule of N to the dark reactions increases  $V_{cmax}$  to the same degree as investing one molecule of N to the light reactions increases  $J_{max}$ , and (ii) nitrogen allocation to enzymes involved in photorespiration (C<sub>3</sub>) and the CCM (C<sub>4</sub>) offset each other. These simplified assumptions are meant to represent an initial analysis of the effect of reallocation; they can be further adjusted when more detailed stoichiometry is available.

**Modeling Scenarios.** Photosynthesis was modeled over the following ranges of environmental conditions: 10 °C to 40 °C with 0.125 °C intervals; CO<sub>2</sub> 200 ppm to 600 ppm with 50 ppm intervals; water conditions VPD = 0.1, 0.625, 1.25, 1.875, and 2.5 kPa, with corresponding soil water potential ( $\psi_s$ ) = 0, -0.5, -1, -1.5, and -2 MPa and light intensities 1,400, 1,000, 600, 200, and 100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . We consider VPD = 0.1 kPa and  $\psi_s$  = 0 MPa as saturated and light intensity of 1,400  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  as an average light intensity of a day in open habitat. Environmental factors are intended to reflect growing-season averages.

**Paleoclimate Modeling of Geographic Centers of Evolution.** Building on existing boundary conditions and simulations using earlier versions of the National Center for Atmospheric Research (NCAR) coupled model (The Community Climate System Model, versions 3 and 4), we implement mid-Miocene simulations in Community Earth System Model (CESM) 1.0.5 (50) incorporating slightly updated boundary conditions (51) within CESM incorporating the Community Atmosphere Model, version 5 atmospheric component (52) and the CLM4 land surface model (53) (*SI Appendix, Supporting Information S12*).

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To drive the vegetation model, growth-season means of atmospheric incident solar radiation, 2 m relative humidity, soil water potential (upper six layers), and daily maximum of average 2 m temperature were generated from 30-year climatological monthly means of CLM output. These fields were masked to include grid cells in the growing season (temperature > 10 °C) and for “open” settings—that is, for grid cells made up of >20% of grassland, shrub-land, woodland, and desert based on the distributions in Herold et al. (51), thus filtering out closed-canopy forests and cold regions. Coding was performed in the NCAR Command Language (NCL); the source code is available from the Purdue University Research Repository <https://purr.purdue.edu>.

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