# $C_4$ photosynthesis and climate through the lens of optimality

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**Abstract**

$CO_2$, temperature, water availability and light intensity were all potential selective pressures to propel the initial evolution and global expansion of $C_4$ photosynthesis over the last 30 million years. To tease apart how the primary selective pressures varied along this evolutionary trajectory, we coupled photosynthesis and hydraulics models while optimizing photosynthesis over stomatal resistance and leaf/fine-root allocation. We further examined the importance of resource (e.g. nitrogen) reallocation from the dark to the light reactions during and after the initial formation of $C_4$ syndrome. We show here that the primary selective pressures— all acting upon photorespiration in $C_3$ progenitors— changed through the course of $C_4$ evolution. The higher stomatal resistance and leaf-to-root allocation ratio enabled by the $C_4$ carbon-concentrating mechanism led to a $C_4$ advantage without any change in hydraulic properties, but selection on nitrogen reallocation varied. Water limitation was the primary driver for the initial evolution of $C_4$ 25-32 million years ago, and could positively select for $C_4$ evolution with atmospheric $CO_2$ as high as 600 ppm. Under these high $CO_2$ conditions, nitrogen reallocation was necessary. Low $CO_2$ and light intensity, but not nitrogen reallocation, were the primary drivers during the global radiation of $C_4$ 5-10 MYA. Finally, our results suggest that identifying the predominate selective pressures at the time $C_4$ first evolved within a lineage should help explain current biogeographical distributions.

***Keywords*** $C_4$ evolution, optimal stomatal conductance, resource allocation, water limitation, selective pressure, dark/light reaction

**Significance Statement**

$C_4$ photosynthesis pathway had evolved more than 60 times independently across the terrestrial plants through mid-Oligocene (~30 MYA) and diversified at late Miocene (5 to 10 MYA). We use an optimal physiology model to examine the primary selective pressures along the evolutionary history. Water limitation was the primary driver for $C_4$ evolution from the initial evolutionary events 25 -32 MYA until $CO_2$ became low enough to, along with light intensity,

drive the global radiation of $C_4$ 5-10 MYA. This modeling framework can be used to investigate evolution of other physiological traits (e.g. N reallocation, hydraulics) after the initial formation of $C_4$ syndrome, which contributed to further increasing productivity of $C_4$ in historical and current environmental conditions.

## Introduction

The evolution of the $C_4$ photosynthetic pathway enabled the concentration of $CO_2$ around Rubisco, the enzyme responsible for the first major step of carbon fixation in the $C_3$ photosynthetic pathway, thus eliminating photorespiration. $C_3$ photosynthesis is present in all plants, and within $C_4$ plants the $C_3$ pathway is typically ensconced within specialized bundle sheath cells that surround leaf veins. $CO_2$ 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 $C_4$ pathway eliminates photorespiration, a process that can dramatically reduce photosynthesis and begins with the assimilation of $O_2$, instead of $CO_2$, by Rubisco. Over the last 30 million years, the reduction in $C_3$ photosynthesis by photorespiration was large and broad enough to select for the independent evolution of the $C_4$ pathway more than 60 times across the terrestrial plants (3). The diversity of plant families with $C_4$ is greatest in the eudicots (1200 species) and the Poaceae, the monocot family containing the grasses (4500 species) (2), account for nearly 25% of terrestrial plant productivity and several important agricultural species (4).

While increased photorespiration in $C_3$ progenitors was central to the evolution of the $C_4$ carbon concentrating mechanism (CCM), the relative importance of different environmental drivers of the photorespiratory increase has been the subject of much debate (5, 6, 7, 8). Lower $CO_2$ leads to higher rates of photorespiration, as does higher temperature. Past physiological models therefore focused on examining temperature and $CO_2$ concentration as selective pressures for $C_4$ evolution and expansion (5, 9, 10). Under warmer temperatures and low $CO_2$, $C_4$ photosynthesis has greater carbon gain than $C_3$, but under cooler temperatures and high $CO_2$, the metabolic costs of the $C_4$ pathway and lower photorespiration in $C_3$ leads to greater carbon gain in $C_3$. Alternatively, water availability has been proposed as the impetus for $C_4$ evolution in eudicots (2), and recent phylogenetic analyses have suggested the same in grasses (7, 13). Water availability should have an impact on $C_4$ evolution that could work independently or in concert

with changes in $CO_2$ and temperature. First, water deficits indirectly increase photorespiration in $C_3$ plants by forcing stomatal closure to reduce leaf water loss; consequently decreasing the flux of $CO_2$ into the leaf and the availability of $CO_2$ for Rubisco. Second, the $C_4$ carbon concentrating mechanism 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 $C_3$ (11, 12).

The different environmental drivers of the photorespiratory increase in $C_3$ plants—atmospheric $CO_2$ concentration, temperature and water availability— have changed dramatically as $C_4$ photosynthesis has evolved over the last 30 million years. Atmospheric $CO_2$ decreased monotonically from the mid-Oligocene (680 ± 200 ppm) to the early Miocene (357 ± 108 ppm) down to the Pleistocene minima, where $CO_2$ oscillated between approximately 180 and 280 ppm through glacial/interglacial cycles (14). Physiological models that focused on temperature and $CO_2$ implied that $C_4$ evolved, in both grasses and eudicots, at the low end of this $CO_2$ range in the mid- Miocene to the Pleistocene (2, 5, 9, 10, 15). $C_4$ grasses did become a major component of grassland biomes— in terms of biomass, $C_4$ lineage diversity, or herbivore dietary components— in the late Miocene (5 to 10 MYA), but molecular evidence suggests that $C_4$ photosynthesis arose in the grasses in the mid-Oligocene (~30 MYA) (16). Similarly, phylogenetic reconstructions provide evidence that eudicots of the Chenopodiaceae evolved $C_4$ as early as the monocots, but saw the greatest rate of $C_4$ evolution and diversification in the late Miocene (17, 18, 19). Along with $CO_2$, precipitation declined over the period of $C_4$ evolution and diversification, 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 (20, 21). The spread of grasslands indicate a habitat change with larger surface radiation loads, higher surface temperatures and increased potential for plant water loss (6, 22). Therefore, the early evolution of $C_4$ suggested by molecular phylogenies indicates that water availability played an important role for both $C_4$ grasses and eudicots while $CO_2$ was still relatively high (6, 18, 23, 24,

22, 25). The potentially interacting roles of water availability, changes in radiation and $CO_2$ along the evolutionary trajectory of $C_4$ photosynthesis have not been fully investigated within a comprehensive physiological model.

A related but largely unstudied evolutionary change during the divergence of $C_4$ photosynthesis from $C_3$ is the allocation of nutrients/resources (e.g. N considering enzymes and proteins) between the dark reactions and the light reactions. $C_4$ plants might allocate a greater proportion of N to light reactions than to dark reactions as compared to $C_3$ because of the extra ATP costs of the CCM (26, 27). We propose that the reallocation of N between dark and light reactions provides a further advantage for $C_4$ 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.

Changes in the environmental controls on photorespiration suggest that multiple environmental drivers interacted to differing degrees along the trajectory of $C_4$ evolution. Our goal here is to tease apart the selective pressures that led to the evolution of $C_4$ photosynthesis initially and the global expansion 5-10 MYA through to the current day. We use the framework of an optimality model in which the plant makes allocation "decisions" in order to maximize photosynthetic assimilation rate. Our approach advances our understanding of $C_4$ evolution in four important ways. First, we revisit the temperature-$CO_2$ crossover approach and integrate the effects of water limitation, light, optimal allocation decisions, and the interactions between these in a single model. Second, the hypothesis that $C_4$ photosynthesis has a higher WUE than $C_3$ implicitly relies on an optimality argument to balance carbon gain and water loss (28), yet the role of optimal stomatal conductance in mediating selective pressures due to water limitation during the evolution of $C_4$ plants remains largely unexplored (but see 15). Most previous models assume *a priori* that $C_4$ grasses have lower stomatal conductance. Instead, we let both stomatal conductance and leaf/fine-root allocation emerge endogenously from the model. Third, we use

the $C_4$ model including cost of the $C_4$ pathway in the light reactions (2 additional ATP per $CO_2$ fixed; 1, 29), which previous modeling analysis did not explicitly consider (9, 22, 30, 31). Finally, we consider reallocation of nitrogen from the dark reactions to the light reactions, which can change the tradeoffs between photosynthesis and water use by $C_4$ grasses.

## Results

Assimilation-based crossover temperatures, defined as the temperature at which assimilation by the $C_4$ pathway exceeds that of the $C_3$ pathway, decrease as water limitation increases and light intensity increases across all $CO_2$ concentrations (Fig. 1, Fig. S1). Without water stress (solid black line in Fig 1), our model predicts a $C_3/C_4$ crossover temperature of 23°C under 380 ppm; a result similar to previous data and/or models that did not explicitly account for water stress (9, 10, 32). The model results in Fig. 1 were all under saturated light and with a $C_4$ $J_{max}/V_{cmax}$ ratio of 4.5, which corresponds to a full reallocation of nitrogen from light to dark reactions. Model results for a $C_4$ $J_{max}/V_{cmax}$ ratio of 2.1 (corresponding to no reallocation) were similar (Fig. S1a) with the primary exception being that under low $CO_2$ and low water availability (e.g. $CO_2$=300 ppm, VPD = 3 kPa and $\Psi_S$ = -1.5 MPa or all $CO_2$ concentrations with higher VPD and lower $\Psi_S$), crossover temperatures are higher with $J_{max}/V_{cmax}$= 4.5, showing that nitrogen reallocation decreases the $C_4$ advantage under water limitation and low $CO_2$. Under saturated soil and low VPD, crossover temperatures decrease along with increasing light intensity (Fig. S1c, 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.

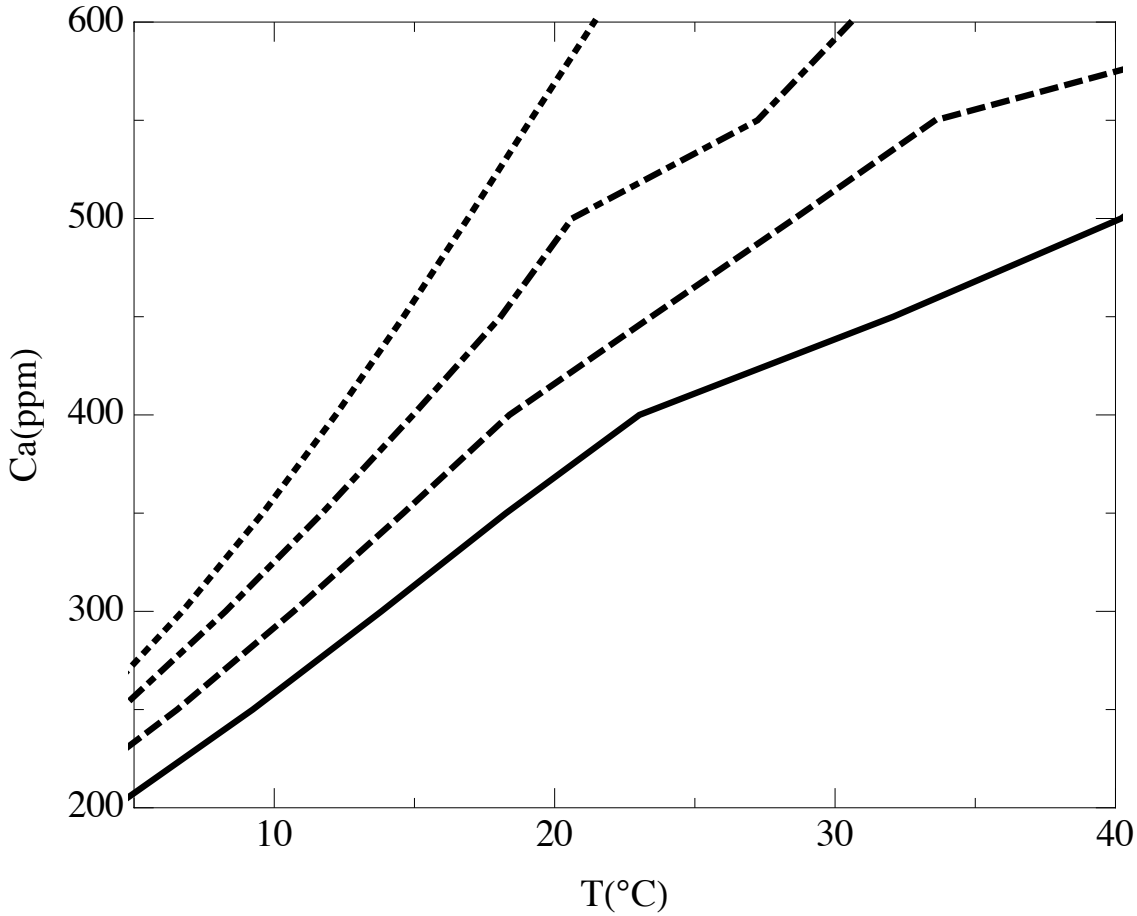


Fig. 1. Crossover temperatures of photosynthesis for $C_3$ and $C_4$ with the change of $CO_2$ concentration under different water conditions. Light intensity was 1400 μmol photons $m^{-2}s^{-1}$ for all model runs. $J_{max}/V_{cmax}$=2.1 for $C_3$ and $J_{max}/V_{cmax}$=4.5 for $C_4$. Solid black line: VPD=0.15kPa, $\Psi_S$=0 MPa; dashed black line: VPD=1kPa, $\Psi_S$=-0.5 MPa; dot-dashed black line: VPD=2kPa, $\Psi_S$=-1 MPa; dotted black line: VPD=3 kPa, $\Psi_S$=-1.5 MPa.

While crossover temperatures allow for a clear diagnostic of comparative assimilation, they do not demonstrate the degree of $C_4$ photosynthetic advantage. To this end, we calculated the net assimilation rate difference between $C_4$ and $C_3$, $\Delta A_n$ (net assimilation of $C_4$ minus that of $C_3$), to comprehensively examine the whole suite of environmental conditions (Fig. 2, 3). 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 $C_4$. Under a $CO_2$ concentration of 200 ppm

and saturated light, $\Delta A_n$ is higher under moist conditions than water-limited conditions (Fig. 2a, b). In contrast, under higher $CO_2$ concentrations (400 and 600 ppm), $C_4$ has the greatest advantage only in water-limited conditions, which leaves a relatively small environmental envelope for $C_4$ to evolve (areas where $\Delta A_n > 0$ in Fig. 2c-f). This result is due to the fact that $C_3$ photosynthesis has a greater proportional increase in assimilation from 200 to 400 and 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, f). At 200 ppm and saturated soils, $\Delta A_n$ is highest under saturated light, and decreases as light intensity decreases (Fig. 3a, b). At 400 ppm $CO_2$ and higher, $\Delta A_n$ indicated a $C_4$ advantage ($\Delta A_n > 0$) only when light intensities were above 1000 mmol m-2 s-1, temperature was above 30 °C and $J_{max}/V_{cmax}$ was high (Fig. 3 c, d). Finally, we calculated the photosynthesis rates of the two pathways under conditions often encountered in today's grasslands to look at the 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 $CO_2$ from saturated soils down to VPD = 2 kPa and $\Psi_S$ = -1 MPa. However, with $J_{max}/V_{cmax}$ =4.5 for $C_4$ (dotted line), $C_4$ does have an advantage over $C_3$ at temperatures above 25 $^{o}$C.

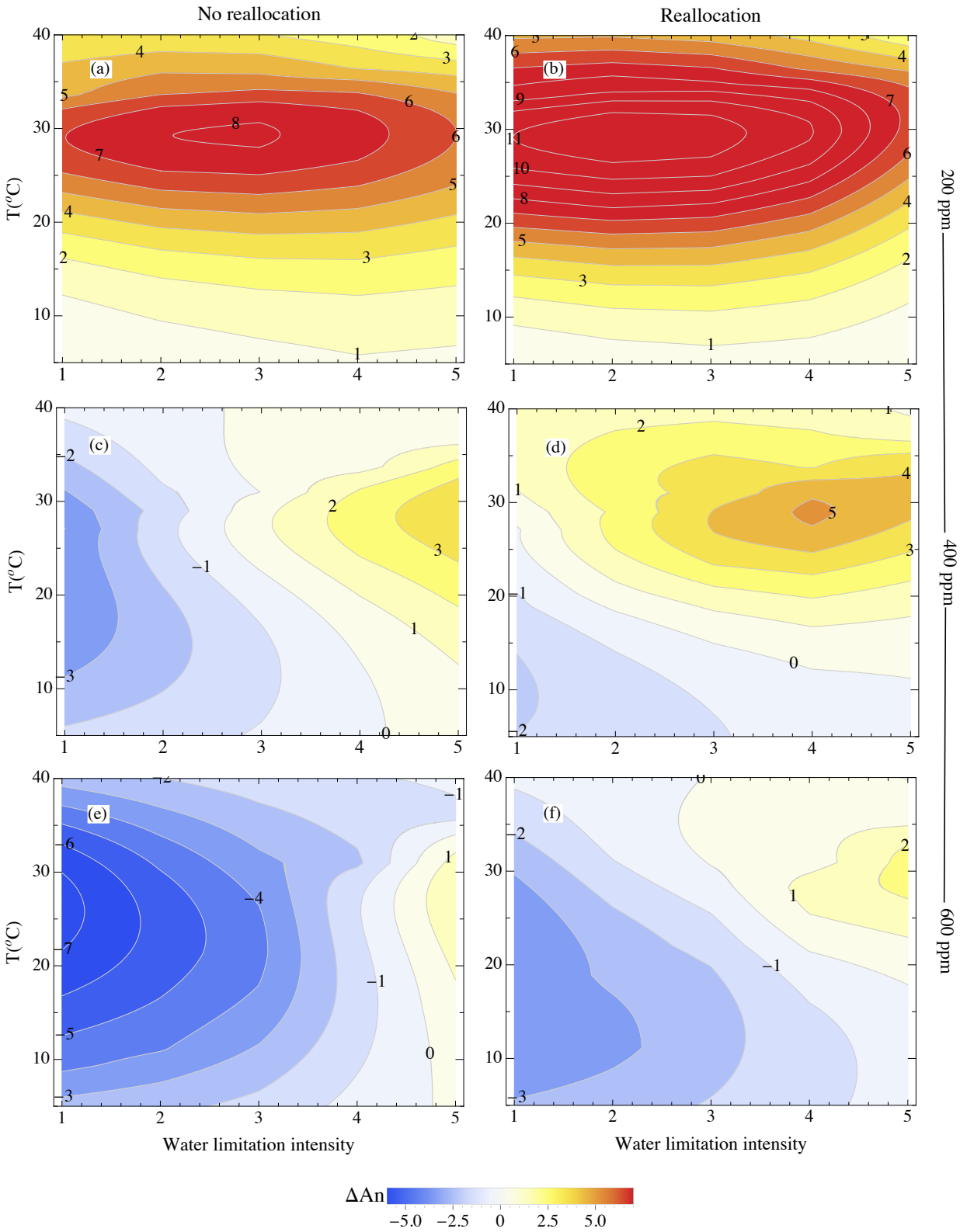


Fig. 2. The total difference in $CO_2$ assimilation between $C_4$ and $C_3$ ($A_n(C_4)$-$A_n(C_3)$) under various $CO_2$

(200 ppm, 400 ppm and 600 ppm) and water conditions under saturated light intensity (1400 µmol photons $m^{-2}s^{-1}$). $J_{max}/V_{cmax}$=2.1 for $C_3$ and $C_4$ (a, c, e) and $J_{max}/V_{cmax}$=2.1 for $C_3$ and $J_{max}/V_{cmax}$=4.5 for $C_4$ (b, d, f). Water limitation intensity is: 1: VPD =0.15 kPa, $\Psi_S$=0 MPa; 2: 1.5 kPa, -0.5MPa; 3: 2kPa, -1 MPa; 4: 3kPa, -1.5 MPa; 5: 4kPa, -2 MPa.

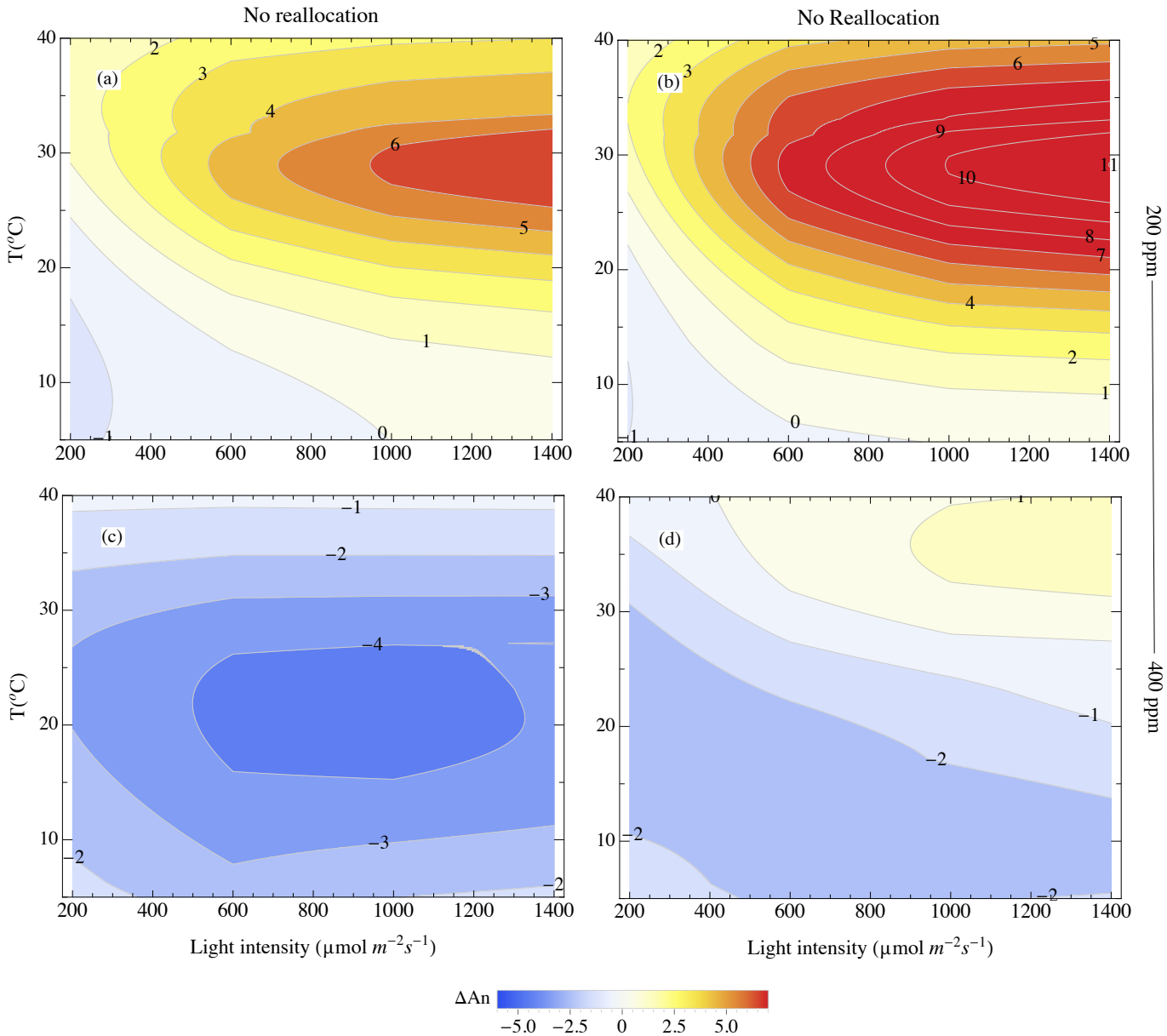

Fig. 3. The total difference in $CO_2$ assimilation between $C_4$ and $C_3$ ($A_n(C_4)$-$A_n(C_3)$) with $J_{max}/V_{cmax}$=2.1 for $C_3$ and $C_4$ under various $CO_2$ (200 ppm, 400 ppm) and different light intensities (from 200 to 1400 µmol photons $m^{-2}s^{-1}$) with saturated water condition (VPD=0.15kPa, $\Psi_S$=0 MPa) (a, c) and with $J_{max}/V_{cmax}$=2.1 for $C_3$ and $J_{max}/V_{cmax}$=4.5 for $C_4$ (b, d).

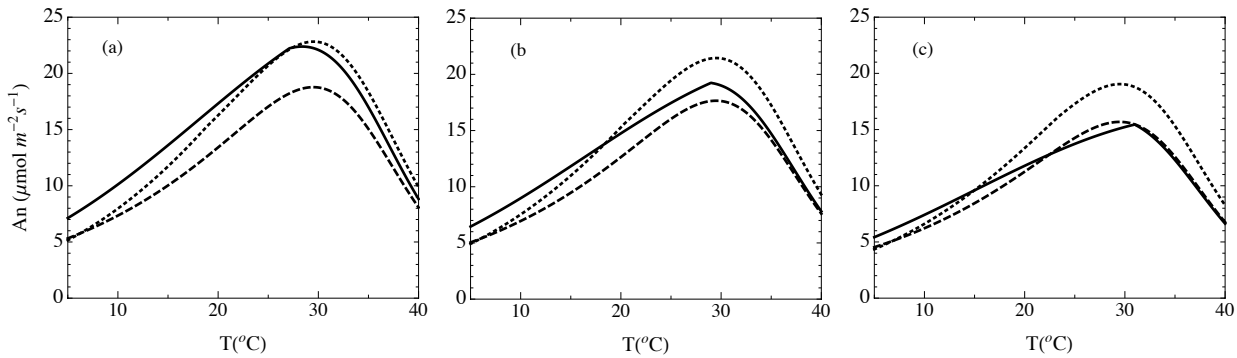


Fig. 4. Assimilation rates of $C_3$ with $J_{max}/V_{cmax}$=2.1 (solid black line), C4 with $J_{max}/V_{cmax}$=2.1 (dashed black line) and $C_4$ with $J_{max}/V_{cmax}$=4.5 (dotted black line) under light intensity of 1400 µmol photons $m^{-2}s^{-1}$, $CO_2$ of 400 ppm and different water limitation conditions. (a) saturated soils; (b) VPD=1kPa and $\Psi_S$=-0.5 MPa; (c) VPD=2kPa and $\Psi_S$=-1 MPa.

Under all environmental and nitrogen allocation scenarios, optimal stomatal resistance ($r_s$) and leaf biomass/total biomass allocation (f) are higher in $C_4$ plants than $C_3$ plants. The general pattern of response was similar across $CO_2$ concentrations, so only 400 ppm is presented with both highest and lowest water availability (Fig. 5 and Fig. S2). Optimal $f$ and $r_s$ for $C_3$ is always lower than that for $C_4$ under different water availability and $CO_2$ (Fig. 5). In addition, $f$ decreases and $r_s$ *increases* as intensity of water limitation increases. Results are the consistent for $C_4$ with a $J_{max}/V_{cmax}$ of 2.1 and $J_{max}/V_{cmax}$ of 4.5. The jumps in $r_s$ and $f$ in Fig. 5 correspond to the transition from RuBP carboxylation limited assimilation to RuBP regeneration limited assimilation ($A_j$). The transition temperature decreases as $CO_2$ increases. In the Fig. S3, the various limitation states are plotted together under multiple environmental scenarios, using both $J_{max}/V_{cmax}$=2.1 and 4.5 for $C_4$. With $J_{max}/V_{cmax}$=2.1, $C_4$ is light limited in most of the environmental conditions. With

$J_{max}/V_{cmax}$=4.5, $C_4$ starts to be limited by $CO_2$ under low temperatures and to be limited by light under high temperatures.

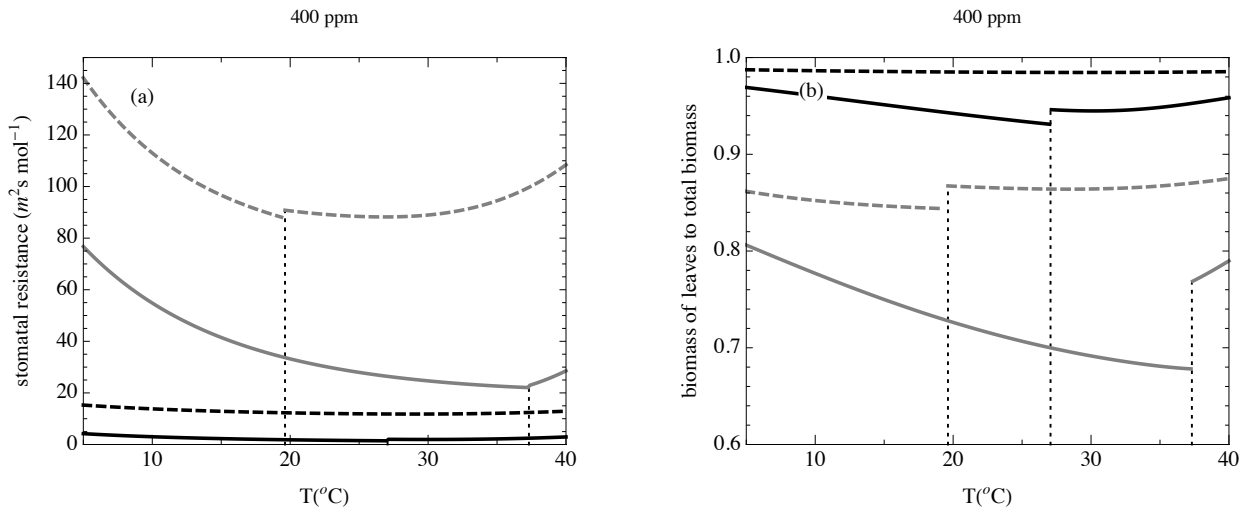


Fig. 5. Stomatal resistance ($r_s$) and leaf/fine-root allocation (f) as a function of temperature, with $J_{max}/V_{cmax}$=2.1 for both $C_3$ and $C_4$ with saturated light under $CO_2$ of 400 ppm and different water conditions. Solid black line: $C_3$ with VPD=0.15kPa, $\Psi_S$=0 MPa; dashed black line: $C_4$ with VPD=0.15kPa, $\Psi_S$=0; solid grey line: $C_3$ with VPD=4 kPa, $\Psi_S$=-2 MPa; dashed grey line: $C_4$ with VPD=4 kPa, $\Psi_S$=-2 MPa. Vertical lines indicate transition from RuBP carboxylation limited condition to RuBP regeneration limited condition for $C_3$ and $C_4$.

## Discussion

Our modeling results imply that the environmental selection for $C_4$ evolution was in place during the mid-Oligocene at warm, arid sites as water limitation acted as the primary selective pressure to increase photorespiration when $CO_2$ is above 400 ppm, and even up to 600 ppm. Under saturated water conditions, there was little room for $C_4$ to evolve 20-30 MYA as $CO_2$ was likely above 400 ppm (14, 33), and the ATP costs of the $C_4$ mechanism is too high and photorespiration in $C_3$ plants too low. As water becomes more limited, however, the $C_4$ advantage becomes

increasingly larger. Enhanced carbon gain under water limited conditions has been believed to be the selective force behind the evolution of $C_4$ in dicotyledonous plants (2) and more recently, in accordance with phylogenetic evidence, in grasses (7, 16). Molecular-based evidence supports a mid-Oligocene evolution of $C_4$ in some grass and eudicot lineages, and our results suggest the same (8, 18, 34). Lineages that evolved in $C_4$ during this period may have been predisposed to exist as arid-land, saline, or disturbed-site specialists as we see today in most $C_4$ eudicots and the earliest known grass subfamily in which $C_4$ evolved, the Chloridoideae (8, 35).

As $CO_2$ decreased through the Miocene, warm temperatures remained a strong selective force, but the main selective force for $C_4$ evolution shifted from water limitation to low $CO_2$ and, to a lesser extent, light intensity. Since increased light intensity alone could not lead to advantage of $C_4$ under high $CO_2$ (Fig. 3c), it seems likely that $C_4$ grasses could not dominate open grasslands, except in locally arid areas, while $CO_2$ was still high. So between the initial evolutionary events leading to the emergence of $C_4$ and the large-scale expansion within the grasses 5-10 MYA, $C_4$ grass radiation likely idled in small pockets of selective favorability as $CO_2$ concentrations declined through the Miocene (2, 8). As $CO_2$ declined, the high light levels inherent to grassland systems gave $C_4$ photosynthesis an increasing advantage, leading to broader geographic and evolutionary radiation. Our results are consistent with previous studies showing that low $CO_2$ (200-300 ppm) strongly favors $C_4$ over $C_3$ photosynthesis (e.g. 9, 15). And we further show that low $CO_2$ provides a clear $C_4$ advantage under a large range of water availability and light intensity regimes. The greatest $C_4$ advantage occurs, however, in relatively moist and mildly water-limited conditions; opposite to that which is seen under high $CO_2$ (Fig. 2c). The environmental conditions that lead to the largest $C_4$ advantage within our model therefore perfectly parallels those documented in the 20$^{th}$ century, $C_4$-dominated grasslands: highly seasonal precipitation that occurs chiefly within a warm growing season (36, 37, 38). 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 (39).

The potential role of water limitation to play a central role $C_4$ grass evolution has sparked interest in grass hydraulics and the anatomical shifts in $C_3$ grasses that were prerequisites to $C_4$ evolution (24, 22, 40). The modeling effort of Osborne and Sack (22) suggests a hydrological underpinning to the evolution of $C_4$ grasses, but found a much smaller environmental window for $C_4$ evolution than we did. At 400 ppm $CO_2$ and soil water potential of -1 MPa— a common occurrence in grasslands (41)— they showed that $C_4$ hydraulic conductance must be twice that of $C_3$ grasses for $C_4$ grasses to achieve slightly greater carbon uptake. In contrast, we find a clear $C_4$ advantage under these, and even drier, conditions by allowing for optimal solutions of stomatal conductance and leaf/fine-root ratio to maximize photosynthesis. Plant hydraulic conductance was kept equal across $C_3$ and $C_4$ throughout simulations, and increasing hydraulic conductance had little impact on our major results and conclusion (Fig. S4, S5), the implication being that the $C_4$ pathway itself is enough to result in greater carbon gain under water stress without any required increase in hydraulic conductance. These results do not necessarily contradict the idea that larger bundle sheaths and smaller interveinal distance— which were clear prerequisites for $C_4$ evolution (24, 42) — led to greater hydraulic conductance and drought tolerance among $C_3$ grass progenitors (24), but they do suggest that greater hydraulic conductance is not necessary to give $C_4$ plants an advantage once the carbon-concentrating mechanism evolved. In fact, 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 some empirical support for such a prediction in eudicots (43).

We assumed that during the early evolution of $C_4$, both $C_3$ and $C_4$ plants had a similar balance of nitrogen across the light and dark reactions, and that the allocation of nitrogen could be treated

separately from the evolution of the $C_4$ CCM as a target of selection. We propose that different environmental conditions can select for a shift in the degree of reallocation (assessed here by a change in $J_{max}/V_{cmax}$) both through evolutionary time and across species in extant plants. In general, CCMs allow for less investment in nitrogen-rich Rubisco (44), and the nitrogen not used for Rubisco could be either reinvested in light harvesting machinery, or simply not used at all; thus reducing the plant nitrogen requirement. Modeling studies have long assumed a high $J_{max}/V_{cmax}$ for $C_4$ photosynthesis (22, 31, 45) and measurements show lower Rubisco content and higher chlorophyll and thylakoid content, giving evidence of reallocation in extant $C_4$ species (26, 27, 46). Empirical estimates of $J_{max}/V_{cmax}$, in $C_4$ plants paint a more variable picture, ranging from 2 to above 6, with a mean of around 4.5 (47, 48, 49, 50, 51, 52), which is higher than the mean $J_{max}/V_{cmax}$ estimates for $C_3$ plants of 2.1 (53). Increasing $J_{max}/V_{cmax}$ almost always increases the photosynthesis rate of $C_4$ grasses (Fig. 4, Fig. S6), and therefore could lead to a competitive advantage over $C_3$ grasses as well as $C_4$ 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 $CO_2$ was high, e.g. during the initial evolutionary events in the Oligocene/Miocene, when the CCM alone does not give $C_4$ a large advantage (Fig. 2 c, e, Fig. 3 c). When $CO_2$ was low during the $C_4$ radiation 5-10 MYA, however, the CCM alone would give $C_4$ an advantage and reallocation would not change the competitive balance between $C_3$ and $C_4$ (Fig. 2 a and Fig. 3a). As $CO_2$ remained low through to the Pleistocene, selection for nitrogen reallocation to the light reactions would lessen further, especially during the $CO_2$ minima of the Pleistocene glacial periods (~ 180 ppm). In this context, an interesting question is how the evolutionary picture of $J_{max}$ and $V_{cmax}$ allocation was coordinated with the formation of $C_4$ pathway in response to the high $CO_2$ in Oligocene, to the $CO_2$ decrease through Pleistocene and, further, to the increase of $CO_2$ in the last 150 years.

$C_4$ photosynthesis first evolved 25 – 32 MYA, and many subsequent and independent evolutionary origins occurred through to the Pleistocene 2.8 MYA. Each evolutionary origin

represents both different selective pressures and taxonomic (genetic) constraints as climate and $CO_2$ changed. Taking the Chloridoideae as an example, we can use our model to develop hypotheses along the evolutionary trajectory of $C_4$ in this grass subfamily. The initial evolution of $C_4$ photosynthesis 25 – 32 MYA while $CO_2$ was high was driven by aridity, acting to decrease stomatal conductance that increased photorespiration in $C_3$ progenitors initially, and led to higher water use efficiency upon the evolution of the CCM. There would have been enough of a reduction in water use that selection for increased hydraulic conductance would relax, allowing for the development of more resilient— and less conductive— xylem. Also at this point, there would have been strong selection for reallocation of nitrogen from the dark reactions to the light reactions. The large radiation of $C_4$ within the Chloridoideae that occurred 5 – 10 MYA was likely driven by low $CO_2$ and high light, and the previously-evolved hydraulic resilience would lead to this subfamily becoming dry-site specialists observed in current-day distributions (35). 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 first evolved $C_4$ in the late Miocene (e.g. *Stipagrostis*, *Eriachne*, *Neurachne*), $CO_2$ would have been the primary impetus for $C_4$ evolution, but for these lineages there would have been little selection to reallocate nitrogen until the dawn of the industrial revolution. We would also expect these more recently evolved lineages to have greater hydraulic conductance than those of the Chloridoideae. By optimizing carbon gain over water loss, we developed a plausible physiological explanation for the early evolution of $C_4$ and further proposed hypotheses about how the variety of traits that comprise the $C_4$ syndrome developed in concert with the climate changes that occurred through the evolutionary trajectory (54). By selecting extant species within select lineages, these hypotheses can be examined empirically, ultimately providing an integrative view of the selection pressures that led to the current physiologies and distribution of $C_4$ plants.

## Materials and Methods

***Overview of the model***

We used different modeling scenarios to examine the advantage of $C_4$ photosynthesis for the initial origin, expansion and current distribution. Initially, we assume that the CCM is the only difference between $C_3$ and $C_4$. This comparison corresponds to two closely related species whose other traits have not had time to diverge in response to differential selection pressures. We then examine shifts in N allocation between the light and dark reactions of $C_4$, which may have happened in subsequent divergence of $C_3$ and $C_4$ after the CCM evolved.

The soil-plant-air water continuum was incorporated in $C_3$ photosynthesis models (55) and $C_4$ models (29) to examine interactions of $CO_2$, water availability, light and temperature. We used the optimality approach of Givnish (1986) (56), where $C_3$ and $C_4$ plants optimize stomatal resistance and leaf/fine-root allocation to balance carbon gain and water loss. A full model description is in SI in supporting information with Table S1 for parameter abbreviation and Table S2 for input parameters. The model derivation using Mathematica (Wolfram Research, Inc.) and methods for numerical solutions are in SII.

***Photosynthesis model***

We are using the traditional $C_3$ photosynthesis models (55) and $C_4$ models (29) for the photosynthesis modeling (SI in supporting information).

***Hydraulic system***

At equilibrium, the rate of water loss through transpiration equals the rate of water absorption by the roots (56):

$$\frac{EfN}{\rho} = k(1-f)N(\Psi_l - \Psi_s) \qquad (1)$$

where $\Psi_s$ is soil water potential, $k$ is the effective root hydraulic conductivity, $N$ is the total biomass of fine root and leaves, $\rho$ is the leaf mass density ($gcm^{-2}$) and $E$ is the transpiration rate per leaf area. $E$ could be written as $\delta/r_s$, where $\delta$ is the water partial pressure deficit between

saturated leaf surface and the atmosphere. Thus, leaf water potential ($\Psi_l$) is a function of $r_s$ and leaf/fine-root allocation ($f$, defined as investment into leaves/total investment in leaves and fine root)).

$$\Psi_l = \Psi_s - \frac{f\delta}{\rho k r_s (1-f)} \qquad (2)$$

***Inhibition of photosynthesis by water stress***

Reduced leaf water potential inhibits photosynthesis (57, 58, 59). We model this cost of transpiration as Weibull-type vulnerability curves relating leaf $\Psi_l$ and photosynthetic parameters (45):

$$V_{cmax,\Psi_l} = V_{cmax} e^{-(\frac{\Psi_l}{d_v})^{b_v}} \qquad (3)$$

$$J_{max,\Psi_l} = J_{max} e^{-(\frac{\Psi_l}{d_j})^{b_j}} \qquad (4)$$

$$V_{pmax,\Psi_l} = V_{pmax} e^{-(\frac{\Psi_l}{d_p})^{b_p}}, \qquad (5)$$

where $b$ and $d$ are curve fitting parameters. Since $\Psi_l$ is a function of $r_s$ and $f$, all those parameters are functions of $r_s$ and $f$.

***Optimal stomatal resistance and optimal 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 by

$$A_{total} = \frac{f N A_n}{\rho} \qquad (6)$$

where $\rho$ is the leaf mass density (g cm$^{-2}$). As a simplifying assumption, we assume $N$ and $\rho$ are fixed (similar to 56). Effectively, we consider the optimization problem faced by the plant in a given instance during its growth, where its size (of which $N$ is a proxy) can be regarded as a constant. Clearly, during plant growth, the assimilate will be turned into plant biomass, but the instantaneous optimization problem will still yield the optimal growth path as the growth rate is maximized at any given time. Finally, we regard $\rho$ as a species-specific trait that changes at a slower time-scale than $r_s$ and $f$. The first order optimality conditions for $r_s$ and $f$ are given by (56):

$$\frac{\partial(fA_n)}{\partial r_s} = f\frac{\partial A_n}{\partial r_s} = 0 \qquad (7)$$

$$\frac{\partial(fA_n)}{\partial f} = A_n + f\frac{\partial A_n}{\partial f} = 0. \qquad (8)$$

We checked the second order derivative to ensure that the numerical solutions to the first order conditions were maxima.

***Allocation of nitrogen***

We examine how nitrogen allocation between RuBP carboxylation and RuBP regeneration in $C_4$ grasses affect competitive advantage over $C_3$ grasses. Despite great variation in $V_{cmax}$ and $J_{max}$ based on the total leaf nitrogen content within $C_3$ plants, Wullschleger (1993) (53) found a mean of $J_{max}/V_{cmax}$ =2.1 across 109 $C_3$ species, which we use as a baseline for $C_3$ and $C_4$ pathways in analyzing the initial evolution of $C_4$. Then, we used $J_{max}/V_{cmax}$ =4.5 for $C_4$ (22, 45) to analyze the role that nitrogen reallocation played in the evolutionary trajectory of $C_4$ plants. In determining the values of $J_{max}$ and $V_{cmax}$, we used a simplified stoichiometry: we consider the total of $J_{max}$ and $V_{cmax}$ as a constant to hold nitrogen concentration constant (22, 45). Two assumptions are underlying this simplified stoichiometry: (1) investing one molecule of nitrogen to the dark reactions will increase of $V_{cmax}$ equal to the increase of $J_{max}$ by investing one molecule of nitrogen to the light reactions; (2) nitrogen allocation to enzymes involved in photorespiration and the CCM balanced each other.

***Modeling scenarios***

We modeled the photosynthesis rates of $C_3$ and $C_4$ under temperature range from 10 °C to 40 °C with an interval of 5 °C, under $CO_2$ mixing ratios ranging from 200 ppm to 600 ppm with an interval of 50 ppm, under different water conditions (VPD=0.001, 1, 2, 3, 4kPa corresponding to soil water potential ($\Psi_S$) =0, -0.5, -1, -1.5, -2 MPa) and under different light intensities (1400, 1000, 600, 200, 100 μmol photons $m^{-2}s^{-1}$). We consider VPD=0.001 kPa and $\Psi_S$ =0 MPa as saturated water condition and light intensity of 1400 μmol photons $m^{-2}$ $s^{-1}$ as an average saturated light intensity of a day.

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# Figure Legends

Fig. 1. Crossover temperatures of photosynthesis for $C_3$ and $C_4$ with the change of $CO_2$ concentration under different water conditions. Light intensity was 1400 μmol photons $m^{-2}s^{-1}$ for all model runs. $J_{max}/V_{cmax}$=2.1 for $C_3$ and $J_{max}/V_{cmax}$=4.5 for $C_4$. Solid black line: VPD=0.15kPa, $\Psi_S$=0 MPa; dashed black line: VPD=1kPa, $\Psi_S$=-0.5 MPa; dot-dashed black line: VPD=2kPa, $\Psi_S$=-1 MPa; dotted black line: VPD=3 kPa, $\Psi_S$=-1.5 MPa.

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 saturated light intensity (1400 μmol photons $m^{-2}s^{-1}$). $J_{max}/V_{cmax}$=2.1 for $C_3$ and $C_4$ (a, c, e) and $J_{max}/V_{cmax}$=2.1 for $C_3$ and $J_{max}/V_{cmax}$=4.5 for $C_4$ (b, d, f). Water limitation intensity is: 1: VPD =0.15 kPa, $\Psi_S$=0 MPa; 2: 1.5 kPa, -0.5MPa; 3: 2kPa, -1 MPa; 4: 3kPa, -1.5 MPa; 5: 4kPa, -2 MPa.

Fig. 3. The total difference in $CO_2$ assimilation between $C_4$ and $C_3$ ($A_n(C_4)$-$A_n(C_3)$) with $J_{max}/V_{cmax}$=2.1 for $C_3$ and $C_4$ under various $CO_2$ (200 ppm, 400 ppm) and different light intensities (from 200 to 1400 μmol photons $m^{-2}s^{-1}$) with saturated water condition (VPD=0.15kPa, $\Psi_S$=0 MPa) (a, c) and with $J_{max}/V_{cmax}$=2.1 for $C_3$ and $J_{max}/V_{cmax}$=4.5 for $C_4$ (b, d).

Fig. 4. Assimilation rates of $C_3$ with $J_{max}/V_{cmax}$=2.1 (solid black line), C4 with $J_{max}/V_{cmax}$=2.1 (dashed black line) and $C_4$ with $J_{max}/V_{cmax}$=4.5 (dotted black line) under light intensity of 1400 μmol photons $m^{-2}s^{-1}$, $CO_2$ of 400 ppm and different water limitation conditions. (a) saturated soils; (b) VPD=1kPa and $\Psi_S$=-0.5 MPa; (c) VPD=2kPa and $\Psi_S$=-1 MPa.

Fig. 5. Stomatal resistance ($r_s$) and leaf/fine-root allocation (f) as a function of temperature, with $J_{max}/V_{cmax}$=2.1 for both $C_3$ and $C_4$ with saturated light under $CO_2$ of 400 ppm and different water conditions. Solid black line: $C_3$ with VPD=0.15kPa, $\Psi_S$=0 MPa; dashed black line: $C_4$ with

VPD=0.15kPa, $\Psi_S$=0; solid grey line: $C_3$ with VPD=4 kPa, $\Psi_S$=-2 MPa; dashed grey line: $C_4$ with VPD=4 kPa, $\Psi_S$=-2 MPa. Vertical lines indicate transition from RuBP carboxylation limited condition to RuBP regeneration limited condition for $C_3$ and $C_4$.