

C₄ photosynthesis and climate through the lens of optimality

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1 **Abstract**

2 CO₂, temperature, water availability and light intensity were all potential selective pressures to
3 propel the initial evolution and global expansion of C₄ photosynthesis over the last 30 million
4 years. To tease apart how the primary selective pressures varied along this evolutionary
5 trajectory, we coupled photosynthesis and hydraulics models while optimizing photosynthesis
6 over stomatal resistance and leaf/fine-root allocation. We further examined the importance of
7 resource (e.g. nitrogen) reallocation from the dark to the light reactions during and after the
8 initial formation of C₄ syndrome. We show here that the primary selective pressures— all acting
9 upon photorespiration in C₃ progenitors— changed through the course of C₄ evolution. The
10 higher stomatal resistance and leaf-to-root allocation ratio enabled by the C₄
11 carbon-concentrating mechanism led to a C₄ advantage without any change in hydraulic
12 properties, but selection on nitrogen reallocation varied. Water limitation was the primary driver
13 for the initial evolution of C₄ 25-32 million years ago, and could positively select for C₄
14 evolution with atmospheric CO₂ as high as 600 ppm. Under these high CO₂ conditions, nitrogen
15 reallocation was necessary. Low CO₂ and light intensity, but not nitrogen reallocation, were the
16 primary drivers during the global radiation of C₄ 5-10 MYA. Finally, our results suggest that
17 identifying the predominate selective pressures at the time C₄ first evolved within a lineage
18 should help explain current biogeographical distributions.

19 **Keywords** C₄ evolution, optimal stomatal conductance, resource allocation, water limitation,
20 selective pressure, dark/light reaction

21 22 **Significance Statement**

23 C₄ photosynthesis pathway had evolved more than 60 times independently across the terrestrial
24 plants through mid-Oligocene (~30 MYA) and diversified at late Miocene (5 to 10 MYA). We
25 use an optimal physiology model to examine the primary selective pressures along the
26 evolutionary history. Water limitation was the primary driver for C₄ evolution from the initial
27 evolutionary events 25 -32 MYA until CO₂ became low enough to, along with light intensity,

- 1 drive the global radiation of C₄ 5-10 MYA. This modeling framework can be used to investigate
- 2 evolution of other physiological traits (e.g. N reallocation, hydraulics) after the initial formation
- 3 of C₄ syndrome, which contributed to further increasing productivity of C₄ in historical and
- 4 current environmental conditions.

1 **Introduction**

2 The evolution of the C₄ photosynthetic pathway enabled the concentration of CO₂ around
3 Rubisco, the enzyme responsible for the first major step of carbon fixation in the C₃
4 photosynthetic pathway, thus eliminating photorespiration. C₃ photosynthesis is present in all
5 plants, and within C₄ plants the C₃ pathway is typically ensconced within specialized bundle
6 sheath cells that surround leaf veins. CO₂ that diffuses into a leaf is shuttled from adjacent
7 mesophyll cells to the bundle sheath via a four-carbon pump, the energetic cost of which is
8 remunerated by ATP derived from the light reactions (1, 2). As a whole, the C₄ pathway
9 eliminates photorespiration, a process that can dramatically reduce photosynthesis and begins
10 with the assimilation of O₂, instead of CO₂, by Rubisco. Over the last 30 million years, the
11 reduction in C₃ photosynthesis by photorespiration was large and broad enough to select for the
12 independent evolution of the C₄ pathway more than 60 times across the terrestrial plants (3). The
13 diversity of plant families with C₄ is greatest in the eudicots (1200 species) and the Poaceae, the
14 monocot family containing the grasses (4500 species) (2), account for nearly 25% of terrestrial
15 plant productivity and several important agricultural species (4).

16
17 While increased photorespiration in C₃ progenitors was central to the evolution of the C₄ carbon
18 concentrating mechanism (CCM), the relative importance of different environmental drivers of
19 the photorespiratory increase has been the subject of much debate (5, 6, 7, 8). Lower CO₂ leads
20 to higher rates of photorespiration, as does higher temperature. Past physiological models
21 therefore focused on examining temperature and CO₂ concentration as selective pressures for C₄
22 evolution and expansion (5, 9, 10). Under warmer temperatures and low CO₂, C₄ photosynthesis
23 has greater carbon gain than C₃, but under cooler temperatures and high CO₂, the metabolic costs
24 of the C₄ pathway and lower photorespiration in C₃ leads to greater carbon gain in C₃.
25 Alternatively, water availability has been proposed as the impetus for C₄ evolution in eudicots
26 (2), and recent phylogenetic analyses have suggested the same in grasses (7, 13). Water
27 availability should have an impact on C₄ evolution that could work independently or in concert

1 with changes in CO₂ and temperature. First, water deficits indirectly increase photorespiration in
2 C₃ plants by forcing stomatal closure to reduce leaf water loss; consequently decreasing the flux
3 of CO₂ into the leaf and the availability of CO₂ for Rubisco. Second, the C₄ carbon concentrating
4 mechanism allows for the maintenance of lower stomatal conductance, and therefore lower water
5 loss, for a given assimilation rate; leading to a higher water-use-efficiency (WUE) than C₃ (11,
6 12).

7
8 The different environmental drivers of the photorespiratory increase in C₃ plants—atmospheric
9 CO₂ concentration, temperature and water availability— have changed dramatically as C₄
10 photosynthesis has evolved over the last 30 million years. Atmospheric CO₂ decreased
11 monotonically from the mid-Oligocene (680 ± 200 ppm) to the early Miocene (357 ± 108 ppm)
12 down to the Pleistocene minima, where CO₂ oscillated between approximately 180 and 280 ppm
13 through glacial/interglacial cycles (14). Physiological models that focused on temperature and
14 CO₂ implied that C₄ evolved, in both grasses and eudicots, at the low end of this CO₂ range in
15 the mid- Miocene to the Pleistocene (2, 5, 9, 10, 15). C₄ grasses did become a major component
16 of grassland biomes— in terms of biomass, C₄ lineage diversity, or herbivore dietary
17 components— in the late Miocene (5 to 10 MYA), but molecular evidence suggests that C₄
18 photosynthesis arose in the grasses in the mid-Oligocene (~30 MYA) (16). Similarly,
19 phylogenetic reconstructions provide evidence that eudicots of the Chenopodiaceae evolved C₄
20 as early as the monocots, but saw the greatest rate of C₄ evolution and diversification in the late
21 Miocene (17, 18, 19). Along with CO₂, precipitation declined over the period of C₄ evolution and
22 diversification, leading to vast terrestrial areas where low or highly seasonal precipitation inputs
23 led to the loss of forests and consequently, the evolution of the world's first grasslands (20, 21).
24 The spread of grasslands indicate a habitat change with larger surface radiation loads, higher
25 surface temperatures and increased potential for plant water loss (6, 22). Therefore, the early
26 evolution of C₄ suggested by molecular phylogenies indicates that water availability played an
27 important role for both C₄ grasses and eudicots while CO₂ was still relatively high (6, 18, 23, 24,

1 22, 25). The potentially interacting roles of water availability, changes in radiation and CO₂
2 along the evolutionary trajectory of C₄ photosynthesis have not been fully investigated within a
3 comprehensive physiological model.

4
5 A related but largely unstudied evolutionary change during the divergence of C₄ photosynthesis
6 from C₃ is the allocation of nutrients/resources (e.g. N considering enzymes and proteins)
7 between the dark reactions and the light reactions. C₄ plants might allocate a greater proportion
8 of N to light reactions than to dark reactions as compared to C₃ because of the extra ATP costs of
9 the CCM (26, 27). We propose that the reallocation of N between dark and light reactions
10 provides a further advantage for C₄ above the CCM alone, and that different environmental
11 conditions can select for a shift in the degree of reallocation both through evolutionary time and
12 across species in extant plants.

13
14 Changes in the environmental controls on photorespiration suggest that multiple environmental
15 drivers interacted to differing degrees along the trajectory of C₄ evolution. Our goal here is to
16 tease apart the selective pressures that led to the evolution of C₄ photosynthesis initially and the
17 global expansion 5-10 MYA through to the current day. We use the framework of an optimality
18 model in which the plant makes allocation “decisions” in order to maximize photosynthetic
19 assimilation rate. Our approach advances our understanding of C₄ evolution in four important
20 ways. First, we revisit the temperature-CO₂ crossover approach and integrate the effects of water
21 limitation, light, optimal allocation decisions, and the interactions between these in a single
22 model. Second, the hypothesis that C₄ photosynthesis has a higher WUE than C₃ implicitly relies
23 on an optimality argument to balance carbon gain and water loss (28), yet the role of optimal
24 stomatal conductance in mediating selective pressures due to water limitation during the
25 evolution of C₄ plants remains largely unexplored (but see 15). Most previous models assume *a*
26 *priori* that C₄ grasses have lower stomatal conductance. Instead, we let both stomatal
27 conductance and leaf/fine-root allocation emerge endogenously from the model. Third, we use

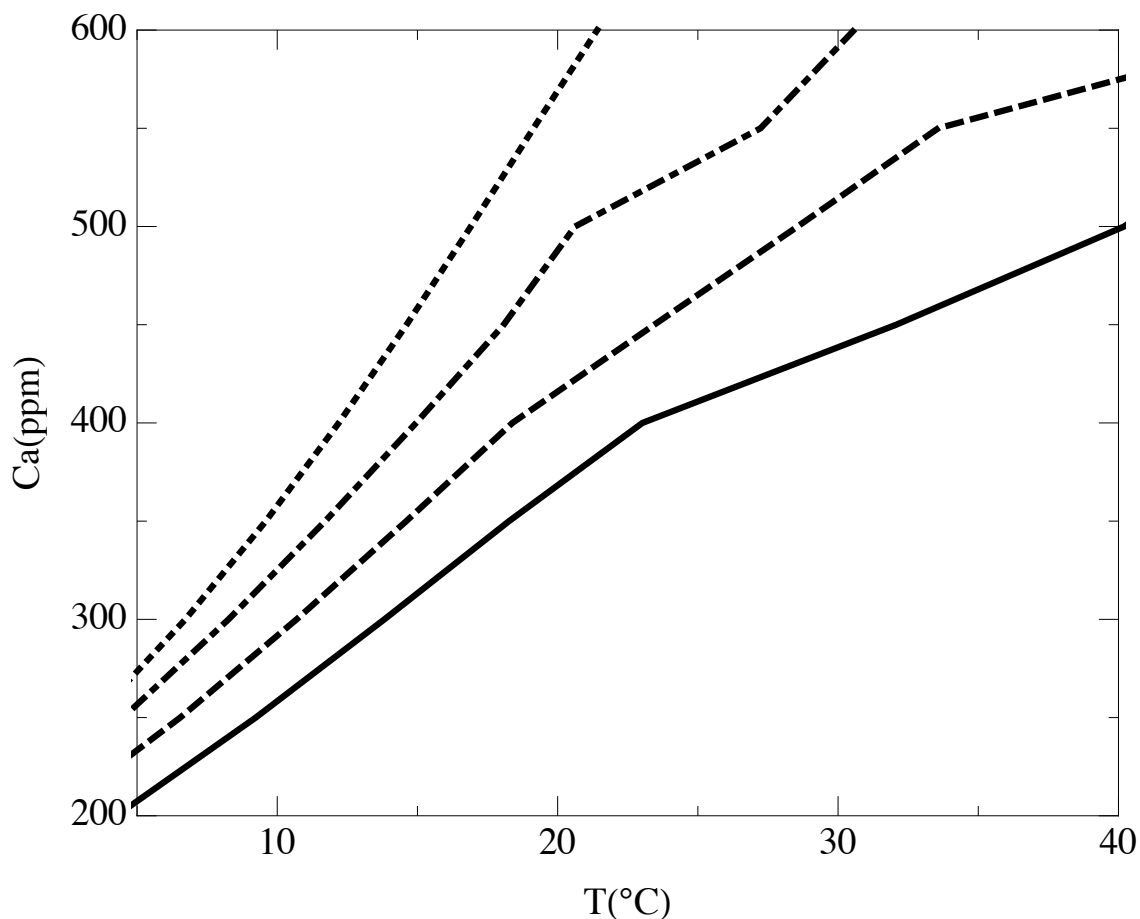
1 the C₄ model including cost of the C₄ pathway in the light reactions (2 additional ATP per CO₂
2 fixed; 1, 29), which previous modeling analysis did not explicitly consider (9, 22, 30, 31).
3 Finally, we consider reallocation of nitrogen from the dark reactions to the light reactions, which
4 can change the tradeoffs between photosynthesis and water use by C₄ grasses.

5

6 **Results**

7 Assimilation-based crossover temperatures, defined as the temperature at which assimilation by
8 the C₄ pathway exceeds that of the C₃ pathway, decrease as water limitation increases and light
9 intensity increases across all CO₂ concentrations (Fig. 1, Fig. S1). Without water stress (solid
10 black line in Fig 1), our model predicts a C₃/C₄ crossover temperature of 23°C under 380 ppm; a
11 result similar to previous data and/or models that did not explicitly account for water stress (9,
12 10, 32). The model results in Fig. 1 were all under saturated light and with a C₄ J_{max}/V_{cmax} ratio of
13 4.5, which corresponds to a full reallocation of nitrogen from light to dark reactions. Model
14 results for a C₄ J_{max}/V_{cmax} ratio of 2.1 (corresponding to no reallocation) were similar (Fig. S1a)
15 with the primary exception being that under low CO₂ and low water availability (e.g. CO₂=300
16 ppm, VPD = 3 kPa and $\Psi_s = -1.5$ MPa or all CO₂ concentrations with higher VPD and lower
17 Ψ_s), crossover temperatures are higher with $J_{max}/V_{cmax} = 4.5$, showing that nitrogen reallocation
18 decreases the C₄ advantage under water limitation and low CO₂. Under saturated soil and low
19 VPD, crossover temperatures decrease along with increasing light intensity (Fig. S1c, d). An
20 increase in light intensity provides a larger relative benefit for C₄ at low CO₂, because C₃
21 photosynthesis remains CO₂ limited throughout while C₄ light limitations lessen as light
22 increases.

23



1
2 Fig. 1. Crossover temperatures of photosynthesis for C₃ and C₄ with the change of CO₂ concentration
3 under different water conditions. Light intensity was 1400 μmol photons m⁻²s⁻¹ for all model runs.
4 $J_{\max}/V_{\text{cmax}}=2.1$ for C₃ and $J_{\max}/V_{\text{cmax}}=4.5$ for C₄. Solid black line: VPD=0.15kPa, $\Psi_s=0$ MPa; dashed black
5 line: VPD=1kPa, $\Psi_s=-0.5$ MPa; dot-dashed black line: VPD=2kPa, $\Psi_s=-1$ MPa; dotted black line:
6 VPD=3 kPa, $\Psi_s=-1.5$ MPa.

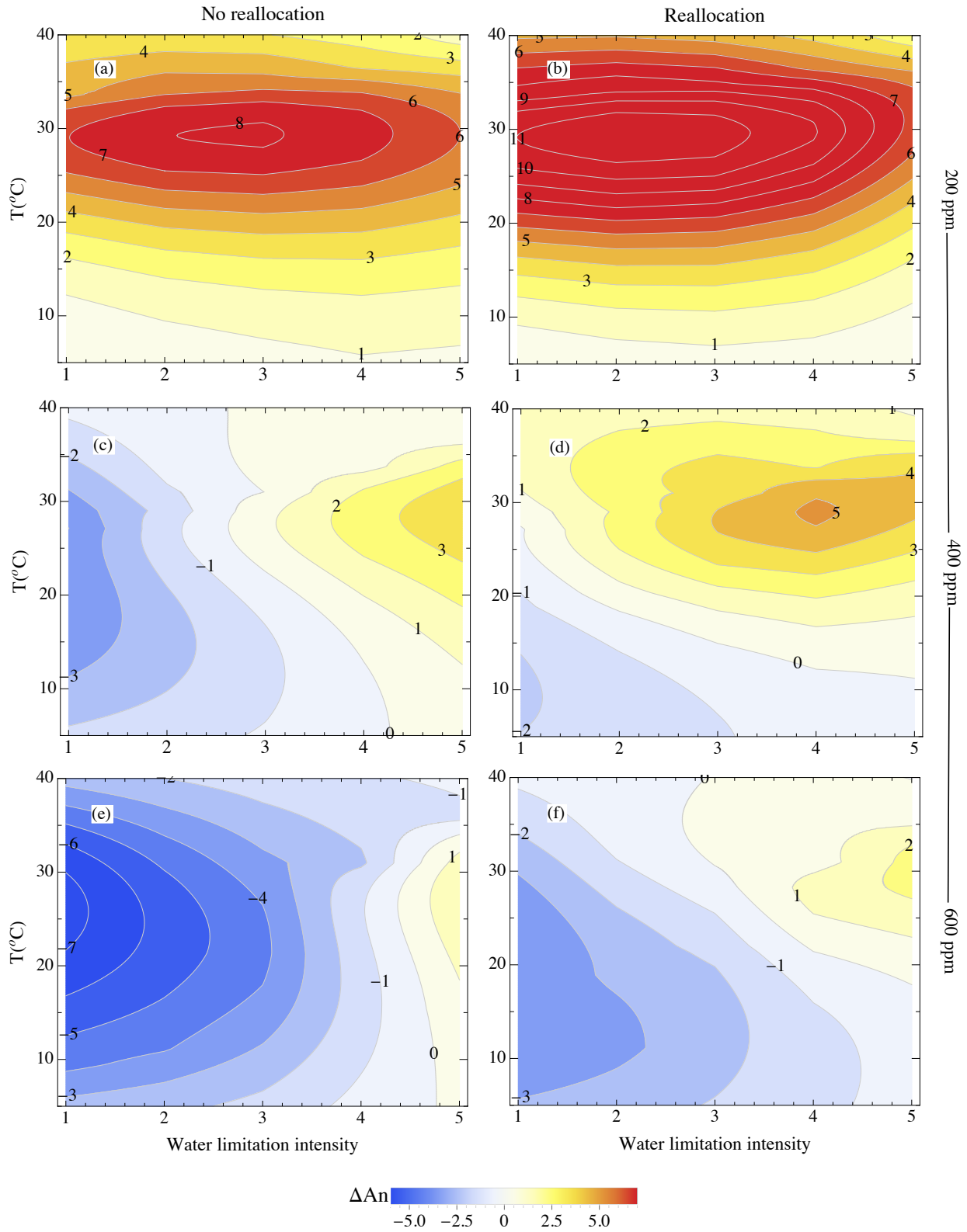
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8 While crossover temperatures allow for a clear diagnostic of comparative assimilation, they do
9 not demonstrate the degree of C₄ photosynthetic advantage. To this end, we calculated the net
10 assimilation rate difference between C₄ and C₃, ΔA_n (net assimilation of C₄ minus that of C₃), to
11 comprehensively examine the whole suite of environmental conditions (Fig. 2, 3). The positive
12 contour space ($\Delta A_n > 0$) means that C₄ outcompetes C₃ within given environmental dimensions,
13 and the higher the ΔA_n , the greater the advantage of C₄. Under a CO₂ concentration of 200 ppm

1 and saturated light, ΔA_n is higher under moist conditions than water-limited conditions (Fig. 2a,
2 b). In contrast, under higher CO₂ concentrations (400 and 600 ppm), C₄ has the greatest
3 advantage only in water-limited conditions, which leaves a relatively small environmental
4 envelope for C₄ to evolve (areas where $\Delta A_n > 0$ in Fig. 2c-f). This result is due to the fact that C₃
5 photosynthesis has a greater proportional increase in assimilation from 200 to 400 and 600 ppm
6 CO₂. Across all scenarios, increasing J_{max}/V_{cmax} increases both the ΔA_n and space for C₄
7 advantage (Fig. 2 b, d, f). At 200 ppm and saturated soils, ΔA_n is highest under saturated light,
8 and decreases as light intensity decreases (Fig. 3a, b). At 400 ppm CO₂ and higher, ΔA_n
9 indicated a C₄ advantage ($\Delta A_n > 0$) only when light intensities were above 1000 mmol m⁻² s⁻¹,
10 temperature was above 30 °C and J_{max}/V_{cmax} was high (Fig. 3 c, d). Finally, we calculated the
11 photosynthesis rates of the two pathways under conditions often encountered in today's
12 grasslands to look at the effect of nitrogen reallocation (Fig. 4). With $J_{max}/V_{cmax} = 2.1$ for both C₃
13 (solid black line) and C₄ (dashed line), the C₄ assimilation rate is rarely higher than C₃, which
14 indicates C₄ does not have an obvious advantage under current CO₂ from saturated soils down to
15 VPD = 2 kPa and $\Psi_s = -1$ MPa. However, with $J_{max}/V_{cmax} = 4.5$ for C₄ (dotted line), C₄ does have
16 an advantage over C₃ at temperatures above 25 °C.

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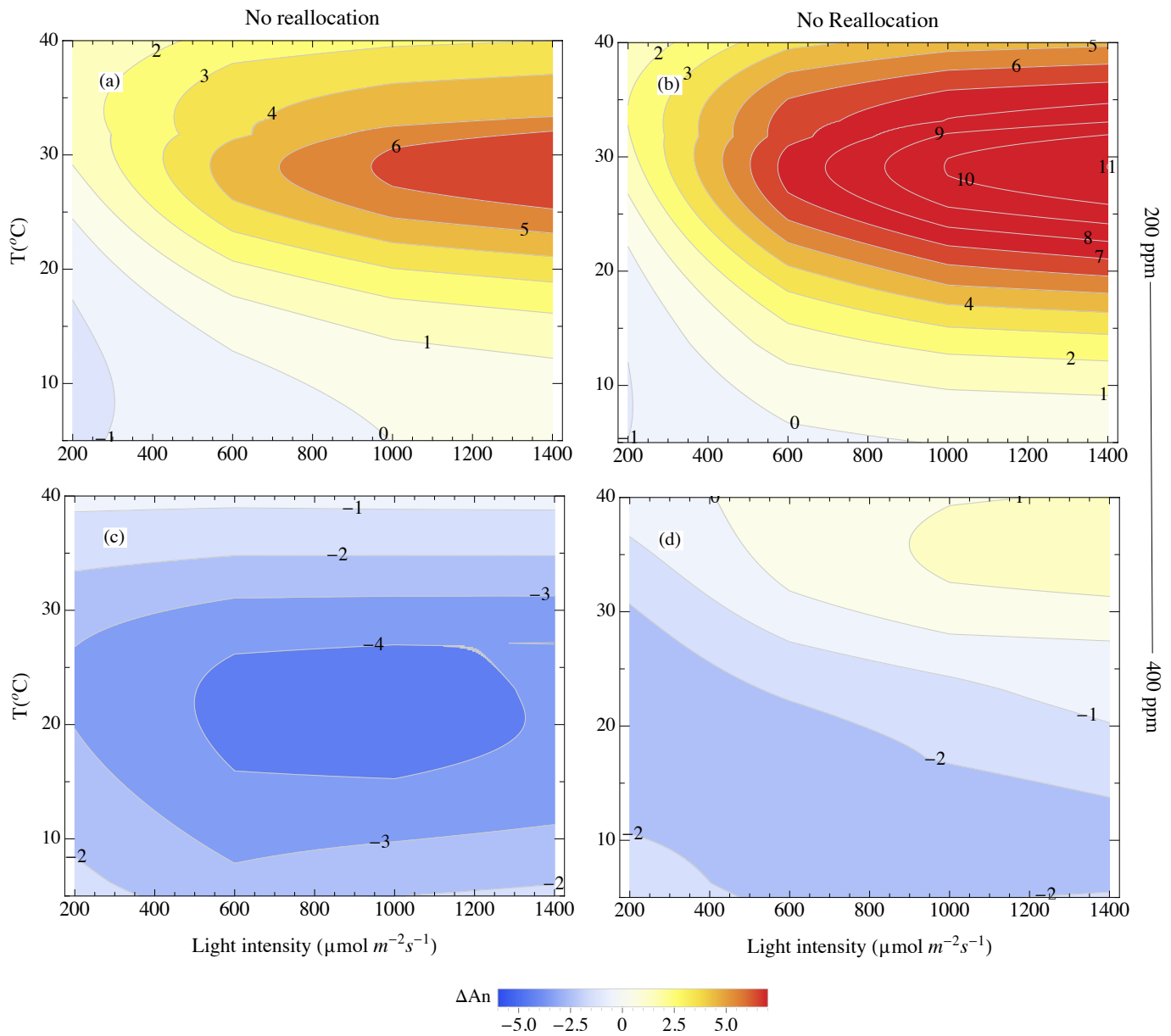


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2 Fig. 2. The total difference in CO₂ assimilation between C₄ and C₃ ($A_n(C_4) - A_n(C_3)$) under various CO₂

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

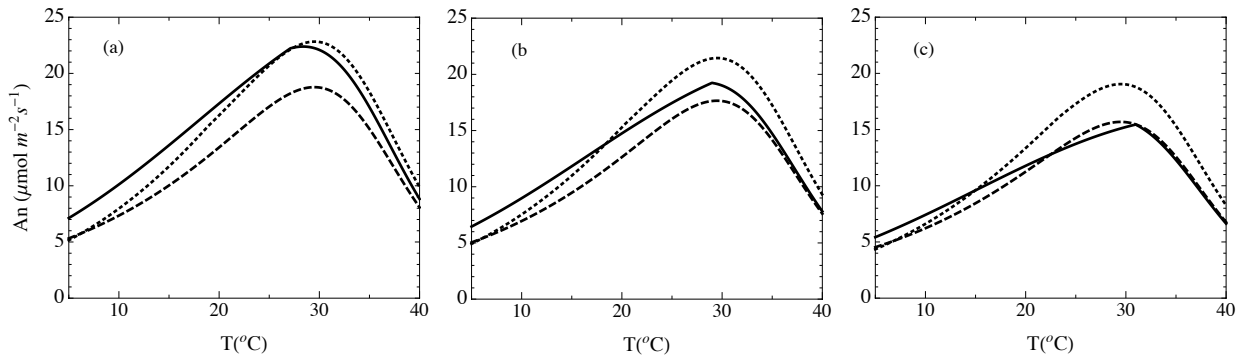
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1 Fig. 3. The total difference in CO₂ assimilation between C₄ and C₃ ($A_n(C_4)-A_n(C_3)$) with $J_{max}/V_{cmax}=2.1$ for
 2 C₃ and C₄ under various CO₂ (200 ppm, 400 ppm) and different light intensities (from 200 to 1400 μmol
 3 photons $\text{m}^{-2}\text{s}^{-1}$) with saturated water condition (VPD=0.15kPa, $\Psi_s=0$ MPa) (a, c) and with $J_{max}/V_{cmax}=2.1$
 4 for C₃ and $J_{max}/V_{cmax}=4.5$ for C₄ (b, d).

5



6

7 Fig. 4. Assimilation rates of C₃ with $J_{max}/V_{cmax}=2.1$ (solid black line), C₄ with $J_{max}/V_{cmax}=2.1$ (dashed
 8 black line) and C₄ with $J_{max}/V_{cmax}=4.5$ (dotted black line) under light intensity of 1400 μmol photons
 9 $\text{m}^{-2}\text{s}^{-1}$, CO₂ of 400 ppm and different water limitation conditions. (a) saturated soils; (b) VPD=1kPa and
 10 $\Psi_s=-0.5$ MPa; (c) VPD=2kPa and $\Psi_s=-1$ MPa.

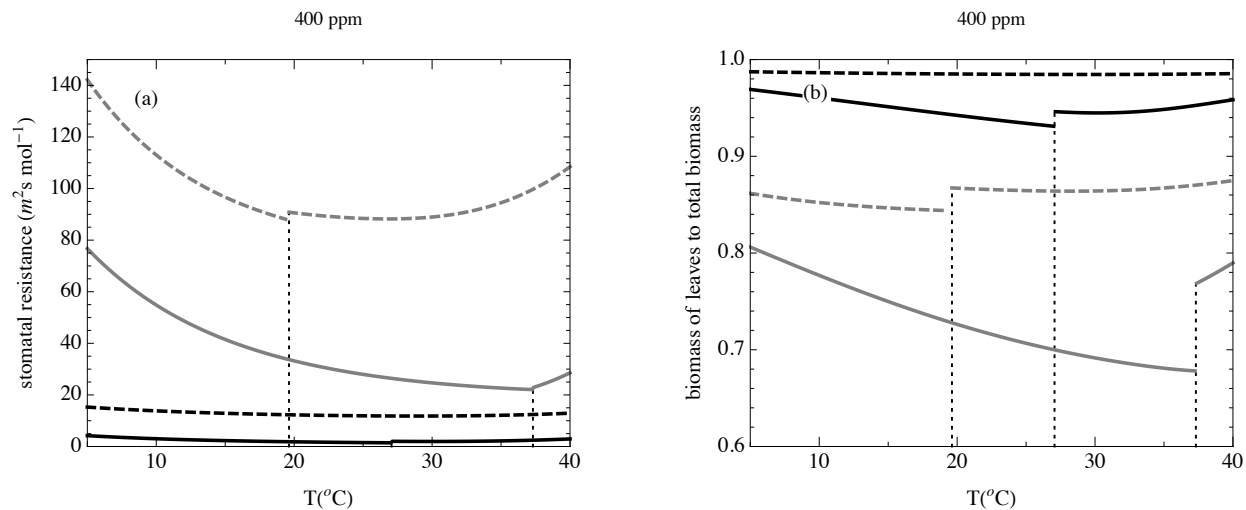
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12 Under all environmental and nitrogen allocation scenarios, optimal stomatal resistance (r_s) and
 13 leaf biomass/total biomass allocation (f) are higher in C₄ plants than C₃ plants. The general
 14 pattern of response was similar across CO₂ concentrations, so only 400 ppm is presented with
 15 both highest and lowest water availability (Fig. 5 and Fig. S2). Optimal f and r_s for C₃ is always
 16 lower than that for C₄ under different water availability and CO₂ (Fig. 5). In addition, f decreases
 17 and r_s increases as intensity of water limitation increases. Results are the consistent for C₄ with a
 18 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
 19 from RuBP carboxylation limited assimilation to RuBP regeneration limited assimilation (A_j).
 20 The transition temperature decreases as CO₂ increases. In the Fig. S3, the various limitation
 21 states are plotted together under multiple environmental scenarios, using both $J_{max}/V_{cmax}=2.1$ and
 22 4.5 for C₄. With $J_{max}/V_{cmax}=2.1$, C₄ is light limited in most of the environmental conditions. With

1 $J_{max}/V_{cmax}=4.5$, C_4 starts to be limited by CO_2 under low temperatures and to be limited by light
 2 under high temperatures.

3

4



5

6 Fig. 5. Stomatal resistance (r_s) and leaf/fine-root allocation (f) as a function of temperature, with
 7 $J_{max}/V_{cmax}=2.1$ for both C_3 and C_4 with saturated light under CO_2 of 400 ppm and different water
 8 conditions. Solid black line: C_3 with VPD=0.15kPa, $\Psi_S=0$ MPa; dashed black line: C_4 with
 9 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
 10 kPa, $\Psi_S=-2$ MPa. Vertical lines indicate transition from RuBP carboxylation limited condition to RuBP
 11 regeneration limited condition for C_3 and C_4 .

12

13 Discussion

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

1 increasingly larger. Enhanced carbon gain under water limited conditions has been believed to be
2 the selective force behind the evolution of C₄ in dicotyledonous plants (2) and more recently, in
3 accordance with phylogenetic evidence, in grasses (7, 16). Molecular-based evidence supports a
4 mid-Oligocene evolution of C₄ in some grass and eudicot lineages, and our results suggest the
5 same (8, 18, 34). Lineages that evolved in C₄ during this period may have been predisposed to
6 exist as arid-land, saline, or disturbed-site specialists as we see today in most C₄ eudicots and the
7 earliest known grass subfamily in which C₄ evolved, the Chloridoideae (8, 35).

8
9 As CO₂ decreased through the Miocene, warm temperatures remained a strong selective force,
10 but the main selective force for C₄ evolution shifted from water limitation to low CO₂ and, to a
11 lesser extent, light intensity. Since increased light intensity alone could not lead to advantage of
12 C₄ under high CO₂ (Fig. 3c), it seems likely that C₄ grasses could not dominate open grasslands,
13 except in locally arid areas, while CO₂ was still high. So between the initial evolutionary events
14 leading to the emergence of C₄ and the large-scale expansion within the grasses 5-10 MYA, C₄
15 grass radiation likely idled in small pockets of selective favorability as CO₂ concentrations
16 declined through the Miocene (2, 8). As CO₂ declined, the high light levels inherent to grassland
17 systems gave C₄ photosynthesis an increasing advantage, leading to broader geographic and
18 evolutionary radiation. Our results are consistent with previous studies showing that low CO₂
19 (200-300 ppm) strongly favors C₄ over C₃ photosynthesis (e.g. 9, 15). And we further show that
20 low CO₂ provides a clear C₄ advantage under a large range of water availability and light
21 intensity regimes. The greatest C₄ advantage occurs, however, in relatively moist and mildly
22 water-limited conditions; opposite to that which is seen under high CO₂ (Fig. 2c). The
23 environmental conditions that lead to the largest C₄ advantage within our model therefore
24 perfectly parallels those documented in the 20th century, C₄-dominated grasslands: highly
25 seasonal precipitation that occurs chiefly within a warm growing season (36, 37, 38). These are
26 also similar to the conditions that led to the large-scale expansion of C₄ grasslands in the

1 Miocene, for example the onset of summer monsoons and subsequent C₄ grassland expansion in
2 the Indian subcontinent (39).
3
4 The potential role of water limitation to play a central role C₄ grass evolution has sparked interest
5 in grass hydraulics and the anatomical shifts in C₃ grasses that were prerequisites to C₄ evolution
6 (24, 22, 40). The modeling effort of Osborne and Sack (22) suggests a hydrological underpinning
7 to the evolution of C₄ grasses, but found a much smaller environmental window for C₄ evolution
8 than we did. At 400 ppm CO₂ and soil water potential of -1 MPa— a common occurrence in
9 grasslands (41)— they showed that C₄ hydraulic conductance must be twice that of C₃ grasses
10 for C₄ grasses to achieve slightly greater carbon uptake. In contrast, we find a clear C₄ advantage
11 under these, and even drier, conditions by allowing for optimal solutions of stomatal
12 conductance and leaf/fine-root ratio to maximize photosynthesis. Plant hydraulic conductance
13 was kept equal across C₃ and C₄ throughout simulations, and increasing hydraulic conductance
14 had little impact on our major results and conclusion (Fig. S4, S5), the implication being that the
15 C₄ pathway itself is enough to result in greater carbon gain under water stress without any
16 required increase in hydraulic conductance. These results do not necessarily contradict the idea
17 that larger bundle sheaths and smaller interveinal distance— which were clear prerequisites for
18 C₄ evolution (24, 42) — led to greater hydraulic conductance and drought tolerance among C₃
19 grass progenitors (24), but they do suggest that greater hydraulic conductance is not necessary to
20 give C₄ plants an advantage once the carbon-concentrating mechanism evolved. In fact, we
21 hypothesize that once C₄ evolves in a lineage, selection on increased hydraulic conductance
22 would not only lessen, but invert, leading to the development of even narrower xylem conduits
23 and greater drought resistance. There is some empirical support for such a prediction in eudicots
24 (43).
25
26 We assumed that during the early evolution of C₄, both C₃ and C₄ plants had a similar balance of
27 nitrogen across the light and dark reactions, and that the allocation of nitrogen could be treated

1 separately from the evolution of the C₄ CCM as a target of selection. We propose that different
2 environmental conditions can select for a shift in the degree of reallocation (assessed here by a
3 change in J_{max}/V_{cmax}) both through evolutionary time and across species in extant plants. In
4 general, CCMs allow for less investment in nitrogen-rich Rubisco (44), and the nitrogen not used
5 for Rubisco could be either reinvested in light harvesting machinery, or simply not used at all;
6 thus reducing the plant nitrogen requirement. Modeling studies have long assumed a high
7 J_{max}/V_{cmax} for C₄ photosynthesis (22, 31, 45) and measurements show lower Rubisco content and
8 higher chlorophyll and thylakoid content, giving evidence of reallocation in extant C₄ species
9 (26, 27, 46). Empirical estimates of J_{max}/V_{cmax} in C₄ plants paint a more variable picture, ranging
10 from 2 to above 6, with a mean of around 4.5 (47, 48, 49, 50, 51, 52), which is higher than the
11 mean J_{max}/V_{cmax} estimates for C₃ plants of 2.1 (53). Increasing J_{max}/V_{cmax} almost always increases
12 the photosynthesis rate of C₄ grasses (Fig. 4, Fig. S6), and therefore could lead to a competitive
13 advantage over C₃ grasses as well as C₄ grasses that do not reallocate. Assuming there is little
14 cost or no genetic constraints for reallocation, the selection pressure to reallocate would have
15 been strongest when CO₂ was high, e.g. during the initial evolutionary events in the
16 Oligocene/Miocene, when the CCM alone does not give C₄ a large advantage (Fig. 2 c, e, Fig. 3
17 c). When CO₂ was low during the C₄ radiation 5-10 MYA, however, the CCM alone would give
18 C₄ an advantage and reallocation would not change the competitive balance between C₃ and C₄
19 (Fig. 2 a and Fig. 3a). As CO₂ remained low through to the Pleistocene, selection for nitrogen
20 reallocation to the light reactions would lessen further, especially during the CO₂ minima of the
21 Pleistocene glacial periods (~ 180 ppm). In this context, an interesting question is how the
22 evolutionary picture of J_{max} and V_{cmax} allocation was coordinated with the formation of C₄
23 pathway in response to the high CO₂ in Oligocene, to the CO₂ decrease through Pleistocene and,
24 further, to the increase of CO₂ in the last 150 years.

25

26 C₄ photosynthesis first evolved 25 – 32 MYA, and many subsequent and independent
27 evolutionary origins occurred through to the Pleistocene 2.8 MYA. Each evolutionary origin

1 represents both different selective pressures and taxonomic (genetic) constraints as climate and
2 CO₂ changed. Taking the Chloridoideae as an example, we can use our model to develop
3 hypotheses along the evolutionary trajectory of C₄ in this grass subfamily. The initial evolution
4 of C₄ photosynthesis 25 – 32 MYA while CO₂ was high was driven by aridity, acting to decrease
5 stomatal conductance that increased photorespiration in C₃ progenitors initially, and led to higher
6 water use efficiency upon the evolution of the CCM. There would have been enough of a
7 reduction in water use that selection for increased hydraulic conductance would relax, allowing
8 for the development of more resilient— and less conductive— xylem. Also at this point, there
9 would have been strong selection for reallocation of nitrogen from the dark reactions to the light
10 reactions. The large radiation of C₄ within the Chloridoideae that occurred 5 – 10 MYA was
11 likely driven by low CO₂ and high light, and the previously-evolved hydraulic resilience would
12 lead to this subfamily becoming dry-site specialists observed in current-day distributions (35).
13 There would have been much less selective pressure to reallocate N during the large radiation,
14 but such a reorganization was likely already in place within the clade. In contrast, for the
15 lineages that first evolved C₄ in the late Miocene (e.g. *Stipagrostis*, *Eriachne*, *Neurachne*), CO₂
16 would have been the primary impetus for C₄ evolution, but for these lineages there would have
17 been little selection to reallocate nitrogen until the dawn of the industrial revolution. We would
18 also expect these more recently evolved lineages to have greater hydraulic conductance than
19 those of the Chloridoideae. By optimizing carbon gain over water loss, we developed a plausible
20 physiological explanation for the early evolution of C₄ and further proposed hypotheses about
21 how the variety of traits that comprise the C₄ syndrome developed in concert with the climate
22 changes that occurred through the evolutionary trajectory (54). By selecting extant species within
23 select lineages, these hypotheses can be examined empirically, ultimately providing an
24 integrative view of the selection pressures that led to the current physiologies and distribution of
25 C₄ plants.

26

27 **Materials and Methods**

1 ***Overview of the model***

2 We used different modeling scenarios to examine the advantage of C₄ photosynthesis for the
3 initial origin, expansion and current distribution. Initially, we assume that the CCM is the only
4 difference between C₃ and C₄. This comparison corresponds to two closely related species whose
5 other traits have not had time to diverge in response to differential selection pressures. We then
6 examine shifts in N allocation between the light and dark reactions of C₄, which may have
7 happened in subsequent divergence of C₃ and C₄ after the CCM evolved.

8
9 The soil-plant-air water continuum was incorporated in C₃ photosynthesis models (55) and C₄
10 models (29) to examine interactions of CO₂, water availability, light and temperature. We used
11 the optimality approach of Givnish (1986) (56), where C₃ and C₄ plants optimize stomatal
12 resistance and leaf/fine-root allocation to balance carbon gain and water loss. A full model
13 description is in SI in supporting information with Table S1 for parameter abbreviation and
14 Table S2 for input parameters. The model derivation using Mathematica (Wolfram Research,
15 Inc.) and methods for numerical solutions are in SII.

16 17 ***Photosynthesis model***

18 We are using the traditional C₃ photosynthesis models (55) and C₄ models (29) for the
19 photosynthesis modeling (SI in supporting information).

20 21 ***Hydraulic system***

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

$$24 \quad \frac{EfN}{\rho} = k(1-f)N(\Psi_l - \Psi_s) \quad (1)$$

25 where Ψ_s is soil water potential, k is the effective root hydraulic conductivity, N is the total
26 biomass of fine root and leaves, ρ is the leaf mass density (gcm⁻²) and E is the transpiration rate
27 per leaf area. E could be written as δ/r_s , where δ is the water partial pressure deficit between

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

$$4 \quad \Psi_l = \Psi_s - \frac{f\delta}{\rho k r_s (1-f)} \quad (2)$$

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

7 Reduced leaf water potential inhibits photosynthesis (57, 58, 59). We model this cost of
 8 transpiration as Weibull-type vulnerability curves relating leaf Ψ_l and photosynthetic parameters
 9 (45):

$$10 \quad V_{cmax,\Psi_l} = V_{cmax} e^{-\left(\frac{\Psi_l}{d_v}\right)^{b_v}} \quad (3)$$

$$11 \quad J_{max,\Psi_l} = J_{max} e^{-\left(\frac{\Psi_l}{d_j}\right)^{b_j}} \quad (4)$$

$$12 \quad V_{pmax,\Psi_l} = V_{pmax} e^{-\left(\frac{\Psi_l}{d_p}\right)^{b_p}}, \quad (5)$$

13 where b and d are curve fitting parameters. Since Ψ_l is a function of r_s and f , all those parameters
 14 are functions of r_s and f .

16 ***Optimal stomatal resistance and optimal allocation of energy between leaves and fine roots***

17 We assume that the plant adjusts the r_s and f to optimize the total carbon gain by

$$18 \quad A_{total} = \frac{fNA_n}{\rho} \quad (6)$$

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

1
$$\frac{\partial(fA_n)}{\partial r_s} = f \frac{\partial A_n}{\partial r_s} = 0 \quad (7)$$

2
$$\frac{\partial(fA_n)}{\partial f} = A_n + f \frac{\partial A_n}{\partial f} = 0. \quad (8)$$

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

5

6 ***Allocation of nitrogen***

7 We examine how nitrogen allocation between RuBP carboxylation and RuBP regeneration in C₄
8 grasses affect competitive advantage over C₃ grasses. Despite great variation in V_{cmax} and J_{max}
9 based on the total leaf nitrogen content within C₃ plants, Wullschleger (1993) (53) found a mean
10 of $J_{max}/V_{cmax}=2.1$ across 109 C₃ species, which we use as a baseline for C₃ and C₄ pathways in
11 analyzing the initial evolution of C₄. Then, we used $J_{max}/V_{cmax}=4.5$ for C₄ (22, 45) to analyze the
12 role that nitrogen reallocation played in the evolutionary trajectory of C₄ plants. In determining
13 the values of J_{max} and V_{cmax} , we used a simplified stoichiometry: we consider the total of J_{max} and
14 V_{cmax} as a constant to hold nitrogen concentration constant (22, 45). Two assumptions are
15 underlying this simplified stoichiometry: (1) investing one molecule of nitrogen to the dark
16 reactions will increase of V_{cmax} equal to the increase of J_{max} by investing one molecule of
17 nitrogen to the light reactions; (2) nitrogen allocation to enzymes involved in photorespiration
18 and the CCM balanced each other.

19

20 ***Modeling scenarios***

21 We modeled the photosynthesis rates of C₃ and C₄ under temperature range from 10 °C to 40 °C
22 with an interval of 5 °C, under CO₂ mixing ratios ranging from 200 ppm to 600 ppm with an
23 interval of 50 ppm, under different water conditions (VPD=0.001, 1, 2, 3, 4kPa corresponding to
24 soil water potential (Ψ_s) =0, -0.5, -1, -1.5, -2 MPa) and under different light intensities (1400,
25 1000, 600, 200, 100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). We consider VPD=0.001 kPa and Ψ_s =0 MPa as
26 saturated water condition and light intensity of 1400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ as an average saturated
27 light intensity of a day.

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3

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- 12

1 Figure Legends

2 Fig. 1. Crossover temperatures of photosynthesis for C₃ and C₄ with the change of CO₂
3 concentration under different water conditions. Light intensity was 1400 μmol photons m⁻²s⁻¹ for
4 all model runs. $J_{\max}/V_{\text{cmax}}=2.1$ for C₃ and $J_{\max}/V_{\text{cmax}}=4.5$ for C₄. Solid black line: VPD=0.15kPa,
5 $\Psi_S=0$ MPa; dashed black line: VPD=1kPa, $\Psi_S=-0.5$ MPa; dot-dashed black line: VPD=2kPa,
6 $\Psi_S=-1$ MPa; dotted black line: VPD=3 kPa, $\Psi_S=-1.5$ MPa.

7
8 Fig. 2. The total difference in CO₂ assimilation between C₄ and C₃ ($A_n(C_4)-A_n(C_3)$) under various
9 CO₂ (200 ppm, 400 ppm and 600 ppm) and water conditions under saturated light intensity (1400
10 μmol photons m⁻²s⁻¹). $J_{\max}/V_{\text{cmax}}=2.1$ for C₃ and C₄ (a, c, e) and $J_{\max}/V_{\text{cmax}}=2.1$ for C₃ and
11 $J_{\max}/V_{\text{cmax}}=4.5$ for C₄ (b, d, f). Water limitation intensity is: 1: VPD =0.15 kPa, $\Psi_S=0$ MPa; 2: 1.5
12 kPa, -0.5MPa; 3: 2kPa, -1 MPa; 4: 3kPa, -1.5 MPa; 5: 4kPa, -2 MPa.

13
14 Fig. 3. The total difference in CO₂ assimilation between C₄ and C₃ ($A_n(C_4)-A_n(C_3)$) with
15 $J_{\max}/V_{\text{cmax}}=2.1$ for C₃ and C₄ under various CO₂ (200 ppm, 400 ppm) and different light
16 intensities (from 200 to 1400 μmol photons m⁻²s⁻¹) with saturated water condition
17 (VPD=0.15kPa, $\Psi_S=0$ MPa) (a, c) and with $J_{\max}/V_{\text{cmax}}=2.1$ for C₃ and $J_{\max}/V_{\text{cmax}}=4.5$ for C₄ (b,
18 d).

19
20 Fig. 4. Assimilation rates of C₃ with $J_{\max}/V_{\text{cmax}}=2.1$ (solid black line), C₄ with $J_{\max}/V_{\text{cmax}}=2.1$
21 (dashed black line) and C₄ with $J_{\max}/V_{\text{cmax}}=4.5$ (dotted black line) under light intensity of 1400
22 μmol photons m⁻²s⁻¹, CO₂ of 400 ppm and different water limitation conditions. (a) saturated
23 soils; (b) VPD=1kPa and $\Psi_S=-0.5$ MPa; (c) VPD=2kPa and $\Psi_S=-1$ MPa.

24
25 Fig. 5. Stomatal resistance (r_s) and leaf/fine-root allocation (f) as a function of temperature, with
26 $J_{\max}/V_{\text{cmax}}=2.1$ for both C₃ and C₄ with saturated light under CO₂ of 400 ppm and different water
27 conditions. Solid black line: C₃ with VPD=0.15kPa, $\Psi_S=0$ MPa; dashed black line: C₄ with

- 1 VPD=0.15kPa, $\Psi_S=0$; solid grey line: C₃ with VPD=4 kPa, $\Psi_S=-2$ MPa; dashed grey line: C₄
- 2 with VPD=4 kPa, $\Psi_S=-2$ MPa. Vertical lines indicate transition from RuBP carboxylation
- 3 limited condition to RuBP regeneration limited condition for C₃ and C₄.