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## **The legacy of C<sub>4</sub> evolution in the hydraulics of C<sub>3</sub> and C<sub>4</sub> grasses**

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## 17 **Abstract**

18 The anatomical reorganization required for optimal C<sub>4</sub> photosynthesis should also impact plant  
19 hydraulics. Most C<sub>4</sub> plants possess large bundle-sheath cells and high vein density, which should  
20 also lead to higher leaf hydraulic conductance ( $K_{\text{leaf}}$ ) and capacitance. Paradoxically, the C<sub>4</sub>  
21 pathway reduces water demand and increases water-use-efficiency, creating a potential mis-  
22 match between supply capacity and demand in C<sub>4</sub> plant water relations. We use phylogenetic  
23 analyses, physiological measurements, and models to examine the reorganization of hydraulics  
24 in closely-related C<sub>4</sub> and C<sub>3</sub> grasses. Evolutionarily young C<sub>4</sub> lineages have higher  $K_{\text{leaf}}$ ,  
25 capacitance, turgor-loss-point, and lower stomatal conductance than their C<sub>3</sub> relatives. In  
26 contrast, species from older C<sub>4</sub> lineages show decreased  $K_{\text{leaf}}$  and capacitance, indicating that  
27 over time, C<sub>4</sub> plants have evolved to optimize hydraulic investments while maintaining C<sub>4</sub>  
28 anatomical requirements. The initial “over-plumbing” of C<sub>4</sub> plants disrupts the positive  
29 correlation between maximal assimilation rate and  $K_{\text{leaf}}$ , decoupling a key relationship between  
30 hydraulics and photosynthesis generally observed in vascular plants.

31

## 32 **Introduction**

33 The evolution of C<sub>4</sub> photosynthesis in the grasses— and the attendant fine-tuning of both  
34 anatomical and biochemical components across changing selection landscapes<sup>[1,2,3]</sup>— likely  
35 impacted leaf hydraulics and hydraulics-photosynthesis relationships, both within the grass  
36 lineages in which C<sub>4</sub> evolved independently > 20 times<sup>[4]</sup>, and as compared to closely-related  
37 C<sub>3</sub><sup>[5,6]</sup>. C<sub>4</sub> plants typically exhibit lower stomatal conductance ( $g_s$ ) and consequently greater  
38 water-use efficiency than C<sub>3</sub>, because the concentration of CO<sub>2</sub> inside bundle sheath cells permits  
39 reduced intercellular CO<sub>2</sub> concentrations and conservative stomatal behavior<sup>[7,8,9]</sup>. At the same  
40 time, C<sub>4</sub> plants require high bundle sheath to mesophyll ratios (BS:M), which are accomplished  
41 with increased vein density and bundle sheath size as compared to C<sub>3</sub> plants. In C<sub>3</sub> species, leaf  
42 hydraulic conductance ( $K_{\text{leaf}}$ ) has a positive relationship with vein density<sup>[10,11,12,13]</sup>. The  
43 decreased inter-veinal distance and consequently higher vein density in C<sub>4</sub> species has been  
44 predicted to lead to a higher  $K_{\text{leaf}}$  than closely-related C<sub>3</sub> species<sup>[14,15]</sup>. Further, increased bundle  
45 sheath size was proposed to lead to a higher leaf capacitance in C<sub>4</sub> species<sup>[15,16]</sup>. This would lead  
46 to a potential physiological “mis-match”, where the evolution of the C<sub>4</sub> pathway simultaneously  
47 increases a plant’s hydraulic capacity while reducing its transpirational demand.

48

49 The significance of such a potential physiological mismatch depends on the potential costs and  
50 tradeoffs associated with the building of an ‘over-plumbed’ leaf. If the costs are high<sup>[12,17]</sup>, then  
51 one would expect to see a reduction of  $K_{\text{leaf}}$  over evolutionary time, as continued selection works  
52 to optimize the  $C_4$  metabolism<sup>[5,18]</sup>. Alternatively, a maintenance of high  $K_{\text{leaf}}$  over time could  
53 result from either a lack of strong selection to reduce  $K_{\text{leaf}}$ , or a strong evolutionary constraint  
54 imposed by the anatomical requirements of  $C_4$  photosynthesis. In other words, the high BS:M  
55 ratio required for an efficient  $C_4$  system may directly limit the ability of  $C_4$  plants to optimize  
56 their hydraulic architecture.

57

58 The evolution of a new photosynthetic pathway that results in multiple potential changes to the  
59 plant hydraulic system represents the ideal platform to expand our understanding of the  
60 relationship between photosynthesis and water transport. It is generally thought that maximum  
61 photosynthetic rate ( $A_{\text{max}}$ ) and hydraulic capacity ( $K_{\text{leaf}}$ ) are tightly linked, because the ability to  
62 transport water through leaves to the sites of evaporation at a high rate allows for the  
63 maximization of carbon gain. Studies have documented a positive correlation between  $A_{\text{max}}$  and  
64  $K_{\text{leaf}}$  across many scales, from a broad phylogenetic spectrum of species spanning vascular  
65 plants<sup>[11]</sup>, to smaller clades of closely related species<sup>[13]</sup>. Grasses are largely absent from previous  
66 efforts to examine this relationship, which is unfortunate because of the parallel venation found  
67 in grasses and other monocots. With over 20 origins of  $C_4$  photosynthesis with ages that span ~  
68 30 million years, grasses also present a unique opportunity to examine the influence of  $C_4$   
69 evolution on  $A_{\text{max}}$ - $K_{\text{leaf}}$  relationships. Using a broad sampling of grasses (Fig. 1), we determined  
70 whether anatomical differences associated with  $C_4$  evolution result in greater  $K_{\text{leaf}}$  and leaf  
71 capacitance compared to their  $C_3$  relatives. We then compared these properties between closely  
72 related  $C_3$  and  $C_4$  clades to determine how  $C_4$  evolution alters the predicted  $A_{\text{max}}$ - $K_{\text{leaf}}$   
73 relationships. Finally, we then quantified evolutionary trends in  $K_{\text{leaf}}$ , capacitance and turgor loss  
74 point after the evolution of  $C_4$  within a lineage by asking whether more recent origins of  $C_4$  are  
75 represented by higher  $K_{\text{leaf}}$  and a greater  $K_{\text{leaf}}$ - $A_{\text{max}}$  mismatch.

76

77 **Results**

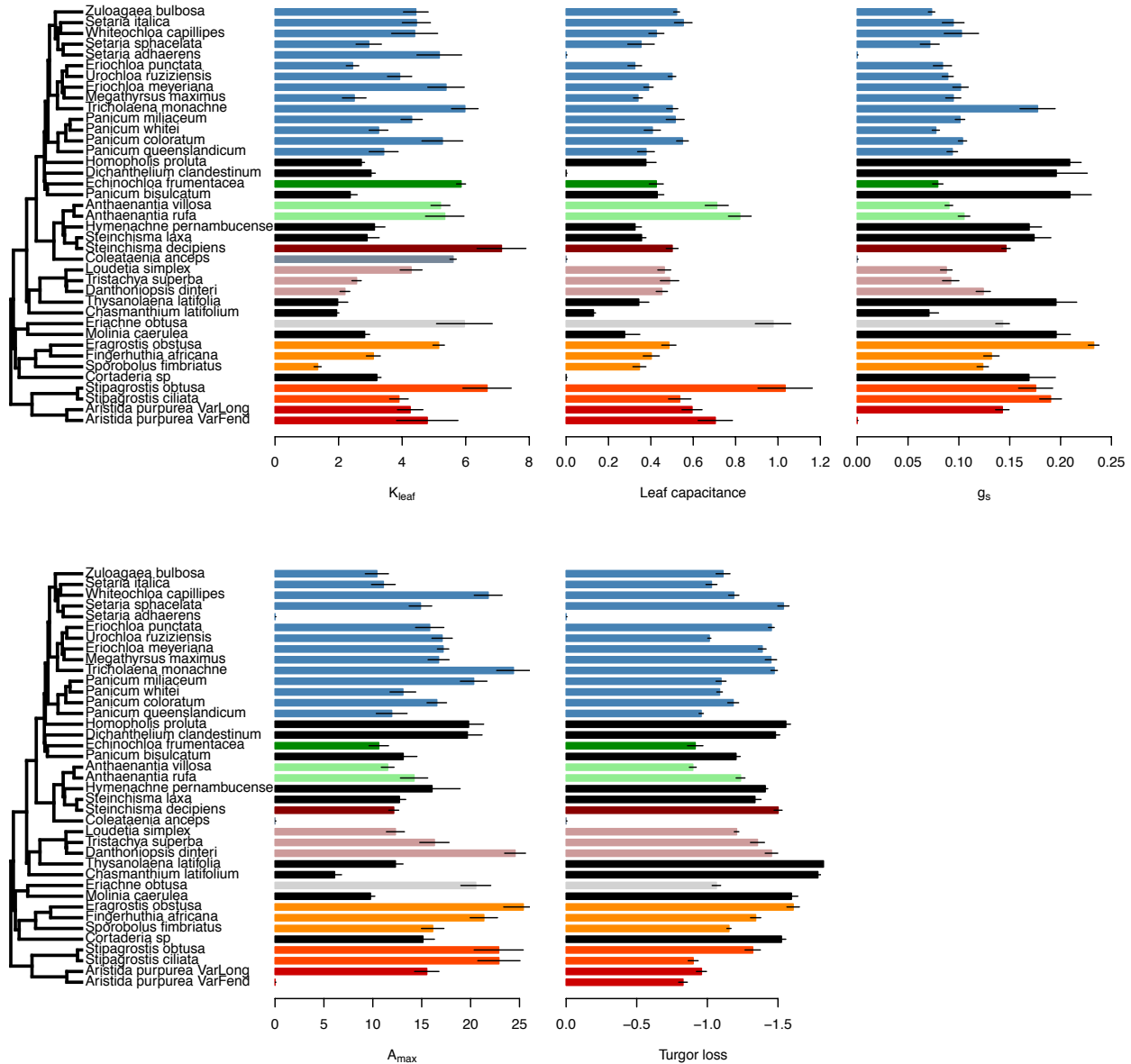
78 Within each phylogenetic cluster, there were no clear patterns between C<sub>3</sub> and C<sub>4</sub> hydraulic traits  
79 by conducting ANOVA tests only. C<sub>4</sub> grasses had higher or equivalent K<sub>leaf</sub>, leaf capacitance  
80 leaf turgor loss point, A<sub>max</sub> and lower or equivalent g<sub>s</sub> than their closest C<sub>3</sub> relatives (Fig. 2). The  
81 one C<sub>3</sub>-C<sub>4</sub> intermediate species, *Steinchisma decipiens*, in our analysis had K<sub>leaf</sub> similar or  
82 equivalent to C<sub>4</sub>, but leaf capacitance, leaf turgor loss point, g<sub>s</sub> and A<sub>max</sub> equivalent to C<sub>3</sub> (Fig. 2).  
83 By analyzing our data in the context of the evolutionary models (Supplementary Table S1),  
84 however, we found clear C<sub>3</sub>-C<sub>4</sub> differences in most measured traits. We first fitted evolutionary  
85 models of Brownian motion and Ornstein-Uhlenbeck processes to the hydraulic traits based on a  
86 reliable dated phylogenetic tree<sup>[19]</sup>. The best fitting evolutionary model to the data for K<sub>leaf</sub>, leaf  
87 turgor loss point, A<sub>max</sub> and g<sub>s</sub> was Ornstein-Uhlenbeck model, while the Brownian model is the  
88 best-fitting model for leaf capacitance, as determined by the AICc and Akaike weights and LRT  
89 test (Table 1, Supplementary Tables S2-S6). Higher K<sub>leaf</sub>, higher A<sub>max</sub>, lower leaf turgor loss  
90 point, and lower g<sub>s</sub> are detected C<sub>4</sub> species compared to C<sub>3</sub> (LRT test, all  $p < 0.01$ ; all  $\Delta AICc < -3$ ).  
91 For leaf capacitance, there is no significant difference for C<sub>3</sub> and C<sub>4</sub> species.

92  
93 We also looked for evolutionary trends in hydraulic traits after the evolution of a C<sub>4</sub> system to  
94 probe for an extended ‘optimization’ phase of C<sub>4</sub> evolution<sup>[3, 20]</sup>. Identifying directional trends in  
95 continuous character evolution is difficult without fossil taxa, and it is impossible to directly  
96 measure hydraulic traits for fossils; however, we can test for trends indirectly using extant  
97 species. For example, if reduction in K<sub>leaf</sub> is selected for subsequent to C<sub>4</sub> evolution we expect  
98 older C<sub>4</sub> lineages to have lower K<sub>leaf</sub> values than younger C<sub>4</sub> lineages. We extracted the  
99 evolutionary age of C<sub>4</sub> origin for each of our lineages from the dated phylogeny<sup>[19]</sup>. Regressions  
100 of evolutionary age versus hydraulic traits provide strong evidence for a long-term directional  
101 trend in hydraulic evolution following the origin of C<sub>4</sub> photosynthesis (Fig. 3). K<sub>leaf</sub>, leaf turgor  
102 loss point and capacitance showed significant negative correlations with evolutionary age, while  
103 A<sub>max</sub> had a significant positive correlation. In contrast, there was no significant relationship  
104 between g<sub>s</sub> and evolutionary age. No evolutionary relationships were detected in C<sub>3</sub> species,  
105 which indicated the correlations between evolutionary age and hydraulic traits were unique to C<sub>4</sub>  
106 species. We also tested for an evolutionary trend by modelling hydraulic trait evolution using a  
107 phylogeny with branch lengths scaled to molecular substitutions/site, which provides an estimate  
108 of differences in evolutionary rates between lineages<sup>[4]</sup>. While the second approach requires

109 many assumptions that are likely violated, the results also provide additional support to a  
 110 directional trend in  $K_{leaf}$  and capacitance in  $C_4$  lineages: comparing 12 different types of models  
 111 with or without evolutionary trends (supplementary Table S7), we found  $K_{leaf}$  and leaf  
 112 capacitance were best fitted by the Brownian motion model with a significant negative trend for  
 113  $C_4$  (Supplementary Table S8, Table S9-13).  
 114  
 115



116  
 117 Fig. 1 Phylogenetic sampling of the species for measuring physiological traits and the  
 118 independent evolutionary lineages corresponding to grass lineages. The figure on the left was a  
 119 grass phylogeny adapted from GPWGII (2012), on which the tags represent the recommended  
 120 independent evolution of  $C_4$  for comparative studies in grasses (numbers represent there are  
 121 multiple origins within a lineage). The figure on the right is the phylogeny for our species,  
 122 extracted from a dated phylogeny <sup>[19]</sup> for species sampled in our experiments. We sampled nine  
 123 independent evolution of  $C_4$  in total.  
 124



125

126

127 Fig. 2 Hydraulic conductance ( $K_{leaf}$ , mmol m<sup>-2</sup> s<sup>-1</sup> MPa<sup>-1</sup>), leaf capacitance (mmol m<sup>-2</sup> MPa<sup>-1</sup>),  
 128 maximal stomatal conductance ( $g_s$ , mmol m<sup>-2</sup> s<sup>-1</sup>), maximal assimilation rate ( $A_{max}$ ,  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>),  
 129 and leaf turgor loss points (Turgor loss, -MPa) of closely related C<sub>3</sub> and C<sub>4</sub> species. Different  
 130 colored clusters of bars show nine different origins of closely-related C<sub>3</sub> and C<sub>4</sub> species. C<sub>3</sub>  
 131 species are colored black. Error bars indicated standard errors.

132

133 Table 1 Phylogenetic results of the best-fitted models and their parameters for hydraulic  
134 conductance ( $K_{\text{leaf}}$ ), leaf capacitance (Capacitance), stomatal conductance ( $g_s$ ), and leaf turgor  
135 loss point (Turgor loss) (summarizing Table S2-S6; model description: Table S1).

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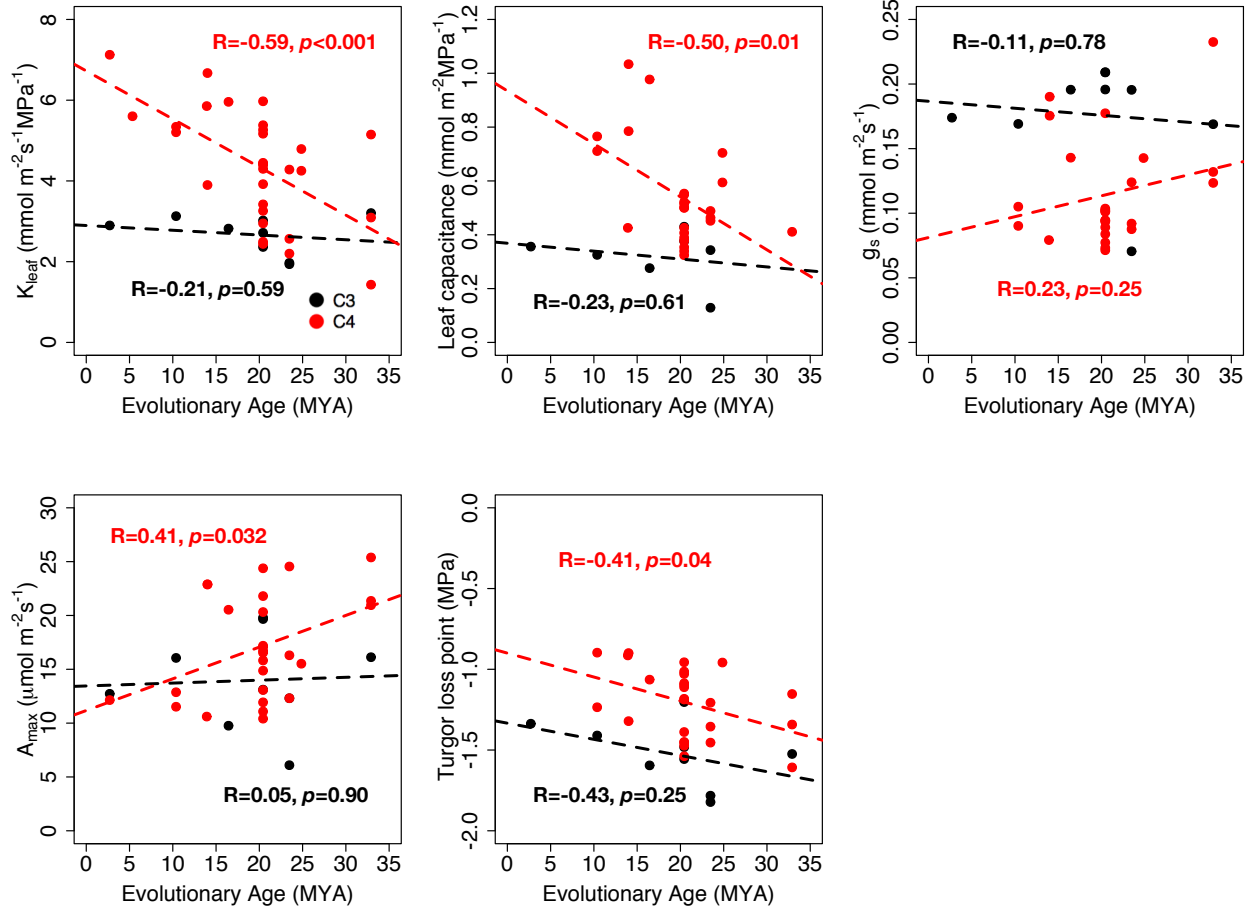
Property	Model	Model type	AICw	Root/Theta	
				C <sub>3</sub>	C <sub>4</sub>
$K_{\text{leaf}}$	Model 6*	OU2	0.984	2.682	4.295
Capacitance	Model 2	BM1	0.323	0.523	
$g_s$	Model 6*	OU2	0.980	0.183	0.102
Turgor loss	Model 6*	OU2	0.996	-1.522	-1.192
$A_{\text{max}}$	Model 6*	OU2	0.5292	13.66	17.34

137 \* indicates the model fit significantly better than all the other models. Different root or theta values for C<sub>3</sub>  
138 and C<sub>4</sub> indicates that the evolutionary model with two different values of the root or theta for C<sub>3</sub> and C<sub>4</sub>  
139 species is a significantly better fit than the evolutionary model with the same root or theta.

140

141 We next explored how  $A_{\text{max}}$  and hydraulic traits are correlated across the phylogeny, and whether  
142 this relationship is different for C<sub>3</sub> and C<sub>4</sub> lineages. The correlations between  $A_{\text{max}}$  and  $K_{\text{leaf}}$  were  
143 different between C<sub>3</sub> and C<sub>4</sub> (Fig. 4, Table 2, Table S13).  $A_{\text{max}}$  was significantly positively  
144 correlated with  $K_{\text{leaf}}$  for C<sub>3</sub>, but not for C<sub>4</sub> (Fig. 4, Table 2, Table S13).  $A_{\text{max}}$  was weakly  
145 positively correlated with leaf capacitance and  $g_s$  and the correlations were not significantly  
146 different for C<sub>3</sub> and C<sub>4</sub> (Fig. 4, Table 2, Supplementary Table S21, S22).  $A_{\text{max}}$  was negatively,  
147 but not significantly related with leaf turgor loss point in C<sub>3</sub> and C<sub>4</sub> species (Supplementary  
148 Table S23).

149



150

151

152 Fig. 3 The regression for hydraulic conductance ( $K_{leaf}$ ), leaf capacitance, leaf turgor loss point,  
 153 stomatal conductance ( $g_s$ ) and maximal assimilation rate ( $A_{max}$ ) vs. the evolutionary age for the  
 154 nine origins of C<sub>4</sub> to show the evolutionary trend within C<sub>4</sub> and within their closely-related C<sub>3</sub>  
 155 species. The evolutionary age for each sampled origin is derived from the dated phylogeny<sup>[19]</sup>.

156

157

158 Table 2 Phylogenetic Correlations between maximal assimilation rates ( $A_{max}$ ) and hydraulic traits  
 159 for C<sub>3</sub> and C<sub>4</sub> species (summarizing Table S20-S23; model description: Table S19).

Parameter 1	Parameter 2	Best Model	r for C <sub>3</sub>	r for C <sub>4</sub>	p value
$A_{max}$	$K_{leaf}$	CorModel 3	0.695	0.129	0.012/0.51
$A_{max}$	Capacitance	CorModel 2	0.259		0.027
$A_{max}$	$g_s$	CorModel 1	0.533		0.003

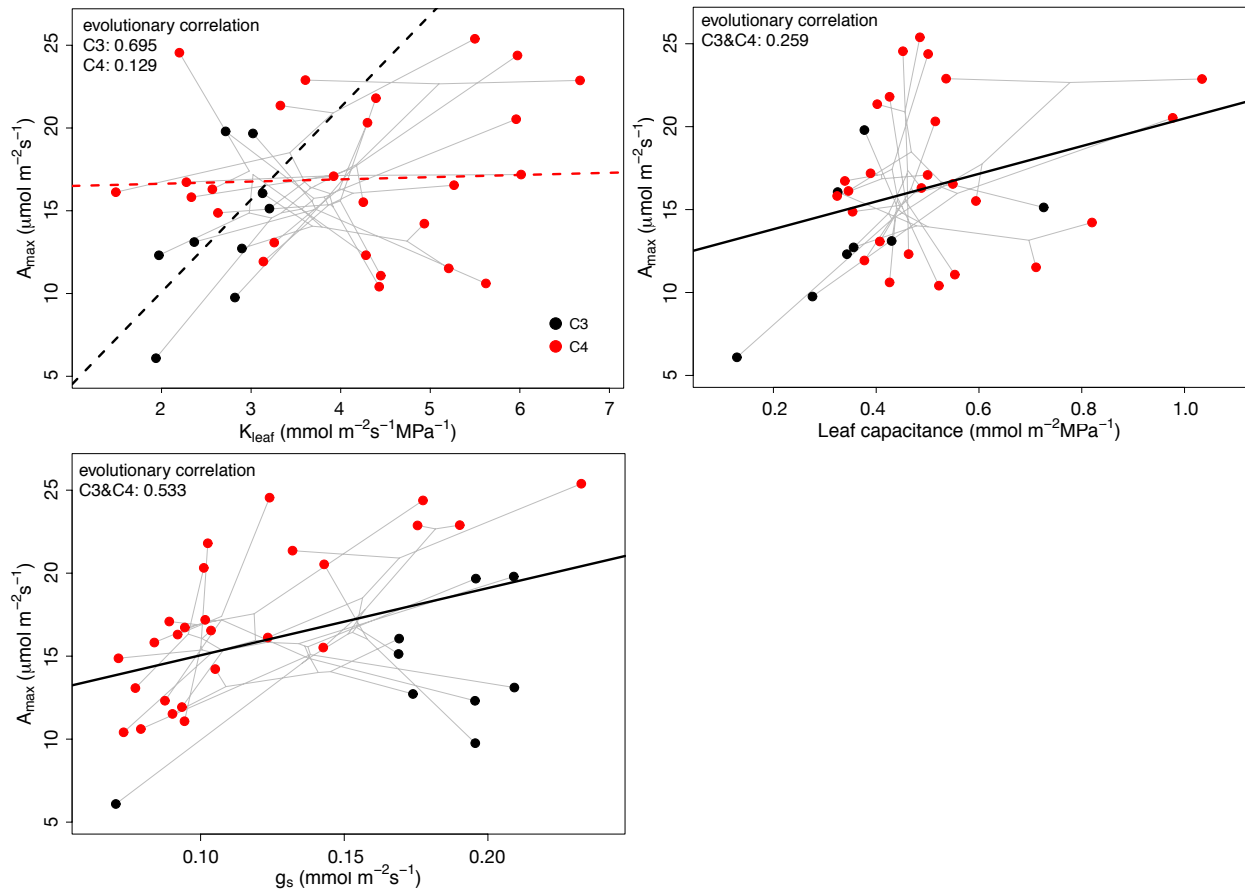


$A_{\max}$	leaf turgor loss	CorModel 1	-0.223	0.256
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160 Different  $r$  means the best fitted model assuming different correlations for  $C_3$  and  $C_4$ . One  $r$  means the  
161 best fitted model assuming similar correlations for  $C_3$  and  $C_4$ .  $p$  values indicated whether the correlation  
162 coefficients are significant.

163  
164 We used our mechanism-based physiological model<sup>[32]</sup> to consider how the evolution of higher  
165  $K_{\text{leaf}}$  would affect the optimal  $g_s$  and photosynthesis in  $C_3$  and  $C_4$  plants. An increase in  $K_{\text{leaf}}$  in  
166 the  $C_3$  ancestor selects for higher  $g_s$  and increases the steady-state leaf water potential to a  
167 limited extent (Fig. 5, S1). Changing  $K_{\text{leaf}}$  has a smaller effect on the photosynthesis rate of  $C_4$   
168 than that of  $C_3$  (Fig. 6, Table S25). Decreasing  $K_{\text{leaf}}$  by half or doubling it changes the  
169 photosynthesis rate of a  $C_4$  plant by an average of -4.27% and 3.48%, respectively. In contrast,  
170 the same shifts in  $K_{\text{leaf}}$  has average effects of -10.07% and 9.14% on the assimilation rate of a  $C_3$   
171 plant. The sensitivity of the assimilation rate to changes in  $K_{\text{leaf}}$  decreases with increasing  $\text{CO}_2$   
172 concentration and increasing water-limitation for both  $C_3$  and  $C_4$  plants (Table S25). These  
173 differences in sensitivity to  $K_{\text{leaf}}$  were robust to differences in physiological properties between  
174  $C_3$  and  $C_4$  (specifically, the temperature response properties and  $J_{\max}/V_{\text{cmax}}$  ratio; Table S25). The  
175 assimilation rate of  $C_4$  plants was still less sensitive to  $K_{\text{leaf}}$  than that of  $C_3$  species under  
176 different  $\text{CO}_2$  concentration and water-limited conditions (Table S25). The physiological  
177 modeling results indicates that  $C_4$  species maintain lower  $g_s$  and higher leaf water potential  
178 compared to closely related  $C_3$  species because the CCM reduces transpirational demand. The  
179 modeling effects of varying  $K_{\text{leaf}}$  on photosynthesis confirmed the diminished returns for high-  
180 efficiency water transport in  $C_4$  species mentioned above.

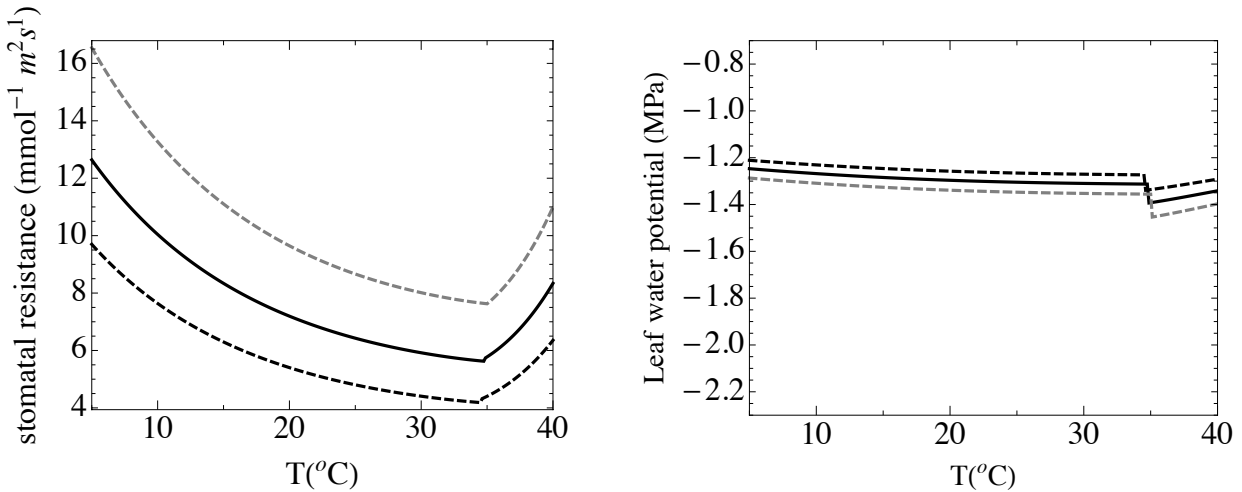
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183 Fig. 4 Phylogenetic correlation for C<sub>3</sub> and C<sub>4</sub> between  $A_{max}$  and other hydraulic traits ( $K_{leaf}$ , leaf  
184 capacitance and  $g_s$ ). Different/same correlation values on the figure mean C<sub>3</sub> and C<sub>4</sub> have  
185 significantly different/same correlations. Detailed phylogenetic correlation models and analysis  
186 results are shown in Table 2. Dashed black line: C<sub>3</sub>; dashed red line: C<sub>4</sub>; solid black line: C<sub>3</sub> and  
187 C<sub>4</sub> have the same correlation; grey lines indicate the phylogeny.

188



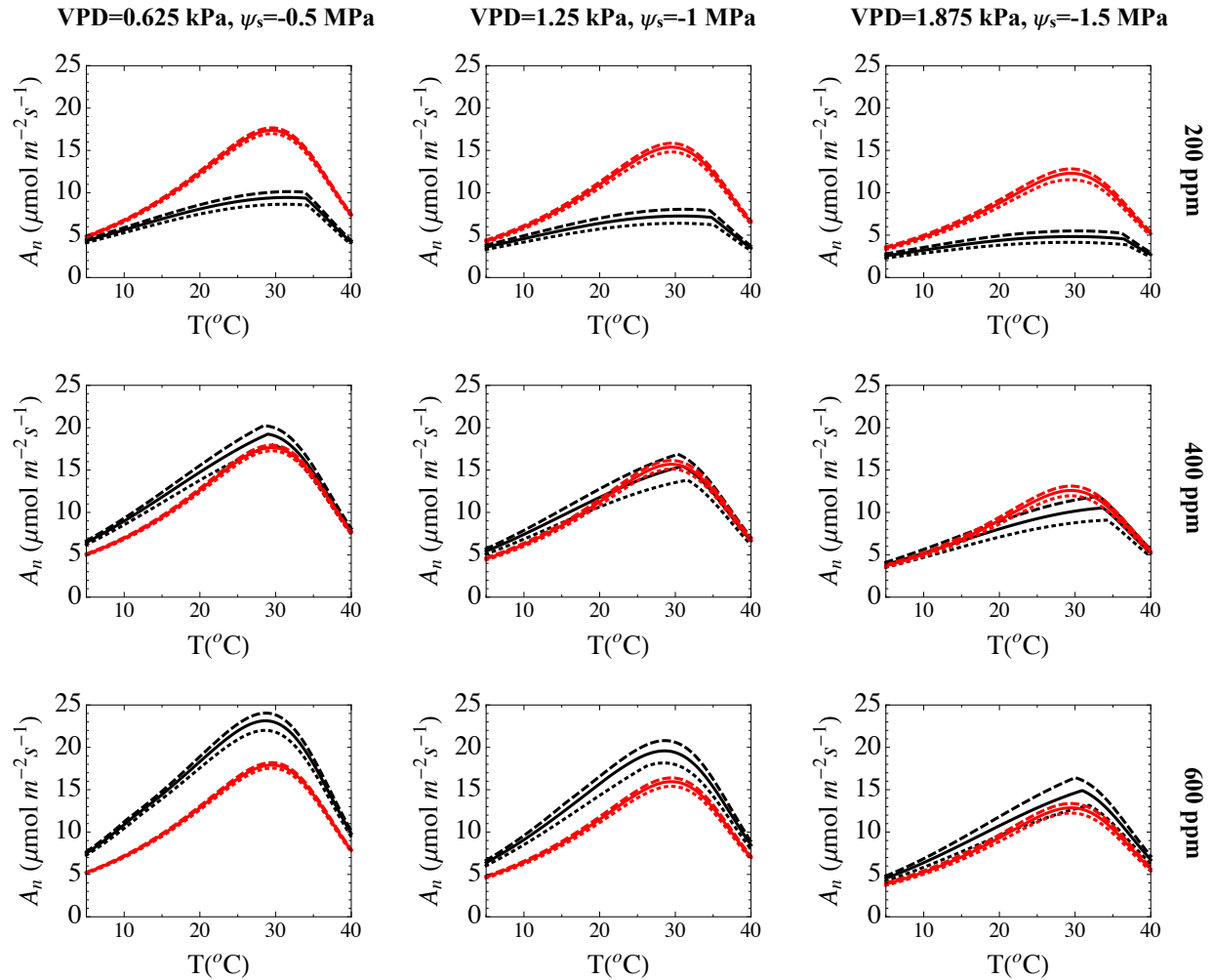
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190 Fig. 5 The effect of changing  $K_{\text{leaf}}$  on stomatal resistance (the inverse of  $g_s$ ) and leaf water  
191 potential under  $\text{VPD}=1.25$  kPa,  $\psi_s = -1$  MPa and  $\text{CO}_2$  concentration of 200 ppm for the  $\text{C}_3$  model.  
192 Solid black line: measured  $K_{\text{leaf}}$ , dashed black line:  $K_{\text{leaf}}$  doubled; dashed grey line:  $K_{\text{leaf}}$  reduced  
193 by 50%.

194

195 To see if  $\text{C}_4$  subtypes varied in hydraulic traits and their evolutionary rates or variance, we also  
196 considered evolutionary models where we allowed each variable to have a subtype-specific value  
197 (Supplementary Table S1). We found no significant differences in  $K_{\text{leaf}}$ , leaf capacitance,  $g_s$ ,  
198 leaf turgor loss point and  $A_{\text{max}}$  among  $\text{C}_4$  subtypes (all  $\Delta\text{AICc} > 0$ ,  $\Delta\text{AICc}$  obtained by  $\text{AICc}$  of  
199 subtype models minus  $\text{AICc}$  model not considering subtypes; Supplementary Tables S14-18).  
200 Although different decarboxylation enzymes are utilized by the three major subtypes (NADP-  
201 ME, NAD-ME and PCK), there does not seem to be an evolutionary effect on hydraulic traits.  
202 However, a previous study documenting PCK species from the Chloridoideae and Panicoideae  
203 lineages with lower leaf turgor loss point<sup>[23]</sup>. Such differences were not apparent when we  
204 compared  $\text{C}_4$  subtypes with multiple lineages. Our current representation of different subtypes is,  
205 however, somewhat limited. It would be advantageous to increase both lineage and species  
206 diversity and to balance subtypes within lineages to more deeply examine  $\text{C}_4$  subtypes.

207



208

209 Fig. 6 Modeling results of photosynthesis rates along with different CO<sub>2</sub> concentration, different  
 210 temperatures and different water limited conditions for C<sub>3</sub> (black lines) and for C<sub>4</sub> (red lines).

211 Solid lines: modeling results for C<sub>3</sub> and C<sub>4</sub> with measured leaf hydraulic conductance; dashed  
 212 lines: modeling results for C<sub>3</sub> and C<sub>4</sub> with twice of the regular leaf hydraulic conductance; dotted  
 213 lines: modeling results for C<sub>3</sub> and C<sub>4</sub> with half of the regular leaf hydraulic conductance. C<sub>3</sub> and  
 214 C<sub>4</sub> parameters are kept the same except for C<sub>4</sub> has the carbon concentration mechanism.

215

216

## 217 Discussion

218 The evolution of the C<sub>4</sub> pathway in the grasses caused a series of shifts in hydraulic properties as  
 219 compared to closely-related C<sub>3</sub> grasses. The anatomical requirements of C<sub>4</sub> initially increased  
 220 K<sub>leaf</sub> and leaf capacitance, as predicted by previous studies<sup>[14,15,16]</sup>; however, K<sub>leaf</sub> and leaf

221 capacitance appear to decline over evolutionary time, suggesting a long period of physiological  
222 optimization after the initial assembly of a new photosynthetic system. Previous examination of  
223 leaf hydraulic traits in grasses focused on investigating single species or were not developed  
224 within a phylogenetic framework when comparing multiple species<sup>[21,22]</sup>, and phylogenetic  
225 studies have assumed trait evolution as simple Brownian motion<sup>[23,24]</sup>. Hydraulic traits, however,  
226 may have evolved along different trajectories before and after the evolution of the C<sub>4</sub> pathway  
227 and associated anatomical reorganization, resulting in more complicated evolutionary dynamics.  
228 Our evolutionary models indicated C<sub>4</sub> grasses initially had higher K<sub>leaf</sub>, leaf capacitance, turgor  
229 loss point than corresponding C<sub>3</sub>, and a lower stomatal conductance (g<sub>s</sub>) than grasses consistent  
230 with previous studies<sup>[25,26]</sup>. Decreased vein distance and increased bundle sheath size are thought  
231 to be anatomical precursors to the evolution of C<sub>4</sub><sup>[27,28]</sup>, and both are thought to increase K<sub>leaf</sub>  
232 and/or leaf capacitance<sup>[14,15]</sup>. Therefore, the shifts of K<sub>leaf</sub> and leaf capacitance likely occurred  
233 before, or at the initial formation of, the C<sub>4</sub> CCM. After the full formation of C<sub>4</sub>, K<sub>leaf</sub> and/or leaf  
234 capacitance started to decrease, which led to higher or equivalent K<sub>leaf</sub> and leaf capacitance in the  
235 current C<sub>3</sub> and C<sub>4</sub> species (Fig. 2). Liu et al. (2019) found that K<sub>leaf</sub> in C<sub>4</sub> grasses overlapped with  
236 C<sub>3</sub> values<sup>[24]</sup>. The positive correlation between A<sub>max</sub> and the evolutionary age also supports an  
237 extended optimization phase for C<sub>4</sub>. Previous studies have indicated that species from the oldest  
238 C<sub>4</sub> lineages (*Chloridoideae* and *Andropogoneae* for example) contain the most productive crops  
239 (Sage, 2016), while some recent C<sub>4</sub> lineages are not more productive than C<sub>3</sub> (Ripley et al., 2008;  
240 Lundgren et al., 2016). In contrast, the significant decrease of g<sub>s</sub> and the increase of leaf turgor  
241 loss point occurred with the evolution of a fully operational C<sub>4</sub> CCM, as suggested by our  
242 physiological models discussed below. Consistent with this prediction, in clades that possess a  
243 range of C<sub>3</sub>, C<sub>3</sub>-C<sub>4</sub> intermediate and C<sub>4</sub> physiologies, the increased water use efficiency,  
244 decreased g<sub>s</sub>, and a broadened ecological niche are observed only in plants with a full C<sub>4</sub>  
245 CCM<sup>[29,30]</sup>.

246  
247 The evolution of C<sub>4</sub> significantly alters the widely-accepted A<sub>max</sub>-K<sub>leaf</sub> relationships existing in  
248 vascular plants. A<sub>max</sub> is limited by the efficient transport of water through leaves to replace water  
249 loss through open stomata, which is the likely cause of a positive correlation between K<sub>leaf</sub> and  
250 A<sub>max</sub> across and within plant taxa<sup>[11,13,31]</sup>. We found that A<sub>max</sub> and K<sub>leaf</sub> are positively correlated  
251 in our C<sub>3</sub> species but not in C<sub>4</sub> (Fig 4). Ocheltree et al. (2016)<sup>[22]</sup> similarly found no relationship

252 between  $K_{\text{leaf}}$  and  $A_{\text{max}}$  in a set of nine  $C_4$  species. We see possible explanations that are not  
253 necessarily mutually exclusive. First, the positive relationship of  $A_{\text{max}}$  and  $K_{\text{leaf}}$  is weakened  
254 under high  $K_{\text{leaf}}$ , possibly due to diminished returns of further increasing the efficiency of water  
255 transport<sup>[11,31]</sup>, a conclusion supported by our physiological modeling results below. As  $K_{\text{leaf}}$   
256 tends to be lower in grasses than in other species, it is possible that the diminishing returns from  
257 increasing  $K_{\text{leaf}}$  manifest at lower values in grasses, and the initial high  $K_{\text{leaf}}$  resulting from  $C_4$   
258 anatomy could be in the  $A_{\text{max}}$  “saturation” zone. Lastly, we see evidence here that the time-since-  
259  $C_4$ -evolution affects several hydraulic traits across and within lineages, and it could be that a  
260 walk towards  $A_{\text{max}}-K_{\text{leaf}}$  optimality is slowly occurring within  $C_4$  grass lineages in relatively  
261 newfound ecological niches. However, the similar correlations of  $g_s$  vs.  $A_{\text{max}}$  in  $C_3$  and  $C_4$  and  
262 lack of evolutionary trend in  $g_s$  indicated the evolutionary processes of  $g_s$  might be already near  
263 the optimal condition or stabilized quickly. Other hydraulic traits of leaf capacitance and leaf  
264 turgor loss point do not seem to contribute to the  $A_{\text{max}}$  directly because of weak correlations.

265  
266 We identified the mode and direction of evolution for hydraulic traits in  $C_3$  and  $C_4$  lineages and  
267 found evidence that different traits followed different evolutionary processes. Hydraulic  
268 conductance and leaf capacitance could therefore evolve with directions in a step-wise fashion  
269 due to anatomical constraints, but  $g_s$  and leaf turgor loss point might have a more quick process  
270 of readjustments, which allows them to stabilize soon. This suggests that there could be greater  
271 diversification of  $K_{\text{leaf}}$  and leaf capacitance in the existing  $C_4$  species and maybe in the future.  
272 Also, these rearrangements of hydraulic properties interacted with each other throughout the  
273 evolutionary trajectory. For example, increased  $K_{\text{leaf}}$  and leaf capacitance would lead to an  
274 increased water transport efficiency, which enabled greater  $g_s$  of the  $C_4$  ancestor (either a  $C_3$   
275 grass or a  $C_3$ - $C_4$  intermediate), but the formation of the full  $C_4$  CCM enables a decrease of  $g_s$ .  
276 Therefore, observed  $g_s$  in  $C_4$  grasses reflects a balance of these two contrasting physiologies  
277 playing out in a given ecological and phenological background, which may explain why although  
278  $C_4$   $g_s$  was lower than the  $C_3$ , the difference was not large. This line of reasoning might also  
279 explain the inconsistent observations of  $g_s$  comparisons between  $C_3$  and  $C_4$ . Most previous  
280 studies found that  $C_4$  grasses had lower  $g_s$  than  $C_3$  grasses in both closely related and unrelated  
281 species<sup>[25,33]</sup>, yet Taylor et al. (2014) found that  $C_4$  grasses maintained a higher or equivalent  $g_s$   
282 to closely-related  $C_3$  grasses<sup>[34]</sup>. Likewise, artificial selection or genetic engineering might have

283 more success in adjusting these hydraulic traits in advance. Consciously selecting or  
284 manipulating narrower xylem, decreasing the expression of aquaporins, or other mechanisms of  
285 decreasing leaf conductance while maintain high bundle sheath to mesophyll ratio, together with  
286 CCM may increase the water use efficiency of C<sub>4</sub> species further. Our phylogenetic analyses can  
287 thus inform both the evolutionary history of C<sub>4</sub> plants and future efforts to modify C<sub>4</sub> crops.

288

289 By capitalizing on the multiple origins of C<sub>4</sub> photosynthesis in grasses, we have shown that the  
290 vascular organization that is a hallmark of C<sub>4</sub> plants also impacts leaf hydraulics, and disrupts the  
291 established link between hydraulic and photosynthetic capacity demonstrated in C<sub>3</sub> plants. C<sub>4</sub>  
292 grasses are “overplumbed” relative to their C<sub>3</sub> counterparts, suggesting that the costs associated  
293 with the production of an extensive leaf vasculature require re-evaluation in plants with C<sub>4</sub>  
294 photosynthetic systems. The gradual decline in K<sub>leaf</sub> in C<sub>4</sub> lineages over millions of years also  
295 requires an explanation. The C<sub>4</sub>-K<sub>leaf</sub> conundrum provides an opportunity to examine what we  
296 mean by “evolutionary constraint” and highlights the very dynamic nature of evolutionary trade-  
297 offs and functional optimization. First, we assume that the costs of building and maintaining a  
298 high K<sub>leaf</sub> are still significant in C<sub>4</sub> plants<sup>[12,35,36,37,38]</sup>. The most efficient way to reduce K<sub>leaf</sub> costs  
299 would be to reduce venation density, as veins come with high construction costs<sup>[12,17]</sup>, and also  
300 reduce the leaf area that is available for carbon fixation. Yet the anatomical requirements of the  
301 C<sub>4</sub> system preclude this option: reducing vein density would result in a highly inefficient C<sub>4</sub>  
302 system<sup>[15]</sup>, which would negatively impact the plant’s carbon budget, presumably to a much  
303 greater extent than the cost of an overbuilt venation system. As vein construction is a primary  
304 contribution to the cost of a high K<sub>leaf</sub>, and high vein densities are now linked to a new function  
305 (C<sub>4</sub> carbon fixation), the cost-benefit calculations in optimizing K<sub>leaf</sub> have shifted, and the  
306 tradeoff is in favor of overplumbing in order to maintain a highly efficient new carbon fixation  
307 system. In evolutionary vocabulary, what emerges is a new constraint – and in this example, it is  
308 clear that the emergence of a new constraint to organismal evolution is simply due to a shift in  
309 the tradeoffs associated with characters that influence multiple aspects of organismal function. In  
310 other words, we assume a low vein density is a phenotype that is still developmentally  
311 achievable for C<sub>4</sub> grasses; what has prevented its emergence is the shift in functional costs  
312 associated with reduced vein densities.

313

314 And yet, we documented a gradual reduction in  $K_{\text{leaf}}$  over time, which we presume was  
315 accomplished via changes in other factors that influence leaf hydraulic capacity— perhaps by  
316 changing xylem conduit diameters, shifts in extra-xylary mesophyll conductance, decreased  
317 expression of aquaporins, and reorganization of internal air spaces<sup>[6,12,37,39,40]</sup>. It is possible that  
318 these changes resulted from a continued and direct selection pressure to reduce investment in an  
319 underutilized hydraulic system. An alternative explanation is that all of the traits that influence  
320  $K_{\text{leaf}}$  also play important roles in other aspects of leaf function – and the emergent of a new  
321 constraint (a high vein density to maintain  $C_4$  function) has *released* still other constraints on  
322 other traits so that they may be optimized for their other functions. A striking pattern in our data  
323 is that older  $C_4$  lineages have achieved both lower  $K_{\text{leaf}}$  and higher  $A_{\text{max}}$  – suggesting that they  
324 are continuing to optimize their photosynthetic capacity, long after the initial origin of  $C_4$ . We  
325 suspect that the slow evolutionary decline in  $K_{\text{leaf}}$  is due in large part to the optimization of traits  
326 to increase  $A_{\text{max}}$  at the expense of  $K_{\text{leaf}}$ , which is possible only because hydraulic capacity was  
327 already “buffered” by the vein density requirements of  $C_4$  – allowing for continued reductions of  
328  $K_{\text{leaf}}$  at no functional cost. Increased suberization of bundle sheath cells is one example of a  
329 potential release of constraint<sup>[22]</sup>: it allows  $C_4$  plants to gain higher  $A_{\text{max}}$  through reducing bundle  
330 sheath leakiness, but it likely simultaneously reduces water flow from veins out into the  
331 mesophyll. Since  $C_4$  plants are already operating in hydraulic excess, bundle sheath suberization  
332 may be optimized for  $C_4$  function without any negative repercussions for plant water relations.  
333 This hypothesis could also explain the opposing trends in  $A_{\text{max}}$  and  $K_{\text{leaf}}$  when viewed as a  
334 function of evolutionary age. The examination of  $C_4$  evolution in grasses provides an exciting  
335 system to study the evolutionary dynamics of constraints highlighted by the interplay between  
336 photosynthesis and plant hydraulics.

337

## 338 **Methods**

### 339 ***Plant material***

340 We collected seeds of 39 closely related  $C_3$  (9 species),  $C_4$  species (29 species), representing  
341 three  $C_4$  subtypes, nine  $C_4$  origins, and one  $C_3$ - $C_4$  intermediate species. The selected  $C_3$  and  $C_4$   
342 species fall into nine identified  $C_4$  lineages belong to the 11 recommended grass lineages for  $C_3$   
343 and  $C_4$  study (11 out of the total 24 grass lineages have clear  $C_3$  sister species and are  
344 recommended for comparative studies in GPWGII, 2012<sup>[4]</sup>): *Aristida*, *Stipagrostis*,



345 *Chloridoideae* (*Eragrostideae*), *Eriachne*, *Tristachyideae*, *Arthropogoninae*, *Otachyrinae*  
346 (*Anthaenantia*), *Panicinae*, *Melinidinae*, and *Cenchrinae* (Fig. 1). In 2015, seeds were surface  
347 sterilized before germination and the seedlings were transferred to 6 inch pots with the soil of  
348 Fafard #52 (Sungro, Ajawam, MA). Six replicates of each species were randomized in the  
349 greenhouse of the University of Pennsylvania supplemented with artificial lighting. The plants  
350 were watered twice daily. Daytime/night temperature was controlled at 23.9-29.4/18.3-23.8 °C;  
351 relative humidity was around 50-70%. Plants were fertilized once per week with 300 ppm  
352 Nitrogen solution (Jacks Fertilizer; JR Peters, Allentown, PA) and 0.5 tsp of 18-6-8 slow release  
353 Nutricote Total (Arysta LifeScience America Inc, NY) per pot was applied when plants were  
354 potted into 6 inch pots. To maintain optimal plant growth a 15-5-15 cal-mg fertilizer was used  
355 every third week. All measurements were performed on the most-recent fully expanded leaves.  
356

### 357 ***Hydraulic traits***

358 Leaf hydraulic conductance ( $K_{\text{leaf}}$ ) was measured using the evaporative flux method<sup>[41]</sup>, with  
359 some adjustments to maintain stability of the evaporative environment to which the leaf was  
360 exposed (Supplementary Methods). The evening before measurements, potted plants were  
361 brought to the laboratory, watered, and then covered by black plastic bags filled with wet paper  
362 towels to rehydrate overnight. For the leaf gasket, a 1 cm diameter, ~ 1 cm long solid silicone  
363 rubber cylinder was cut nearly in two, leaving a hinge on one end. The cylinder was placed  
364 around the leaf blade near the ligule and glued shut with superglue<sup>[42]</sup>. The leaf was cut from the  
365 plant with a razor blade while submerged in a 15 mmol L<sup>-1</sup> KCl solution; the rubber gasket was  
366 then attached to tubing filled with the same KCl solution. The other end of the tubing was inside  
367 a graduated cylinder that sat on a digital balance (Mettler-Toledo). The leaf was then placed  
368 inside a custom, environmentally controlled cuvette that allowed for the measurement of entire  
369 grass blades. Throughout measurements, cuvette temperature was controlled at 25 °C and the  
370 humidity was 55-65% (VPD range of 1.1-1.4 kPa) across measurements, but remained constant  
371 during a particular measurement. Photosynthetically active radiation in the system is 1000  $\mu\text{mol}$   
372  $\text{m}^{-2} \text{s}^{-1}$ . Flow from the balance was monitored for 45 m to 1h until the flow rates reach steady  
373 state. After the measurements, the leaf was detached and was put into a plastic bag to equilibrate  
374 for 20 minutes to measure the leaf water potential (Model 1000, PMS Instrument, USA).  $K_{\text{leaf}}$   
375 values were further standardized to 25 °C and leaf area to make the  $K_{\text{leaf}}$  comparable among

376 studies and across species. Data indicating a sudden change of flow and whose leaf water  
377 potential was an obvious outlier were deleted.

378

379 We measured pressure-volume (PV) curves for six leaves per species using the bench-drying  
380 method<sup>[43,44]</sup>. A leaf was cut directly from the same plants rehydrated in the lab (as described  
381 above) using a razor blade and leaf water potential was measured immediately. Then, the leaf  
382 weight was recorded. The leaf was initially allowed to dry on the bench for 2-minute intervals  
383 and put into a ziplock bag and under darkness for 10-minute equilibration before measuring the  
384 leaf water potential and leaf weight again. Then, the waiting intervals could be adjusted based on  
385 the decrease of the leaf water potential (from 2 minutes-1h). Ideally, a decreasing gradient of -  
386 0.2MPa for leaf water potential was obtained for the curves, until the leaf weight reached a  
387 steady state. At the end of the experiment, leaves were dried in the oven at 70°C for 48h to obtain  
388 the dry weight. The PV curves were used in curve fitting to obtain leaf capacitance, and leaf  
389 turgor loss point using an excel program from Sack and Pasquet-Kok (2010)<sup>[44]</sup>.

390

391 Maximal assimilation rate ( $A_{\max}$ ) and stomatal conductance ( $g_s$ ) were measured under saturated  
392 light intensity.  $A_{\max}$  and  $g_s$  were obtained using a standard 2 x 3 cm<sup>2</sup> leaf chamber with a red/blue  
393 LED light source of LI-6400XT (LI-COR Inc., Lincoln, NE, USA). Light curves were measured  
394 with light intensities of 2000, 1500, 1200, 1000, 800, 500, 300, 200, 150, 100, 75, 50, 20, 0  $\mu\text{mol}$   
395  $\text{m}^{-2} \text{s}^{-1}$  under CO<sub>2</sub> of 400 ppm. Then,  $A_{\max}$  was estimated from the light curve<sup>[45,46]</sup>. All the  
396 measurements were made under the temperature of 25°C and the leaf temperature to air vapor  
397 pressure deficit was controlled around 2kPa.  $g_s$  at the saturated light intensity of 2000  $\mu\text{mol} \text{m}^{-2}$   
398  $\text{s}^{-1}$  was recorded for each plant. The cuvette opening was covered by Fun-Tak to avoid and  
399 correct for the leakiness.

400

#### 401 ***Phylogenetic analysis***

402 **Phylogenetic analysis for C<sub>3</sub> and C<sub>4</sub>.** We pruned the dated phylogeny from a published grass  
403 phylogeny to include only the species in our physiological experiments<sup>[19]</sup>(Fig. 1). Using the  
404 dated phylogeny, for each of the hydraulic traits, we fitted evolutionary models to test which  
405 evolutionary model best explains observed distribution of traits along the phylogeny and how  
406 these models differ between C<sub>3</sub> and C<sub>4</sub> (Table S1). We fitted evolutionary models belonging

407 Brownian Motion model and Ornstein-Uhlenbeck Model using the package “mvMORPH” in  
408 R<sup>[47]</sup>. To determine the best fitted evolutionary model, we compared two criteria, the small-  
409 sample-size corrected version of Akaike information criterion (AICc, the lower AICc, the better  
410 fit) and Akaike weights (AICw, the higher AICw, the better fit)<sup>[48,49,50]</sup>. The evolutionary models  
411 have nested variants (Models 1-4; Models 5-6), varying in whether C<sub>3</sub> and C<sub>4</sub> species had the  
412 same or different fluctuation rates, root states for Brownian motion model and optima for  
413 Ornstein-Uhlenbeck model. We used likelihood-ratio test (LRT) to verify whether a specific  
414 model variant performs significantly better. The AICc, AICw and LRT allowed us to test  
415 evolutionary hypotheses, for instance, if the model in which C<sub>3</sub> and C<sub>4</sub> have different root states  
416 fit significantly better than model in which C<sub>3</sub> and C<sub>4</sub> have the same root states, it means there is  
417 a shift of physiological trait along with the formation of C<sub>4</sub>. To examine the further evolution of  
418 hydraulic traits after a full C<sub>4</sub> evolved, we extracted the evolutionary ages for each represented  
419 C<sub>4</sub> origin from the dated phylogenetic trees. Then, we regressed the hydraulic traits with  
420 evolutionary age. A significant negative correlation between evolutionary age and hydraulic trait  
421 will indicate a further decreasing evolutionary direction after C<sub>4</sub> evolved. We also performed an  
422 additional analysis to test the original states and further direction together. We extracted  
423 molecular phylogeny for all the species from Edwards, GPWG II (2012)<sup>[4]</sup>. Except for the six  
424 evolutionary models mentioned above, the molecular phylogeny allows us to fit for additional six  
425 Brownian motion models with trend (Supplementary Table S7). Likewise, if Brownian motion  
426 model with trend fits the phylogenetic patterns better than Brownian motion model without trend  
427 it means there is an evolutionary trend, and a significant LRT test for a two-trend model suggests  
428 that C<sub>3</sub> and C<sub>4</sub> lineages differ in the speed or direction of hydraulic evolution. We also mapped  
429 the traits on the phylogeny for potential further references (Fig. S2-S5).

430 To further test whether there are significant differences among C<sub>4</sub> subtypes, evolutionary models  
431 with subtypes (Table S1) were used to fit the data. We again used AICc, AICw and LRT  
432 methods to find the best model variants: whether there are significant differences for hydraulic  
433 shifts and evolutionary trends among three different subtypes. For the leaf capacitance analysis,  
434 *Dichanthelium clandestinum* is deleted as it is an obvious outlier.

435 **Phylogenetic analysis for correlations among traits.** Multivariate analysis in “mvMORPH”  
436 was used to estimate the correlations between  $A_{\max}$  and each of the hydraulic traits and to test the

437 hypotheses that whether such correlations are different between  $C_3$  and  $C_4$ . The process of  
438 brownian motion with different root for  $C_3$  and  $C_4$  was used for  $K_{\text{leaf}}$ ,  $g_s$  and leaf turgor loss and  
439 brownian motion with the same root was used for leaf capacitance. Since the Ornstein-  
440 Uhlenbeck process is difficult to take the root state difference into consideration, here we used  
441 Brownian motion assumptions as approximation for leaf turgor loss. Seven different correlation  
442 models are fitted (Table S19). We used LRT for the seven correlation models to test whether the  
443 correlation of the two traits is significantly different from 0 and whether the correlation of two  
444 traits is significantly different between  $C_3$  and  $C_4$ . Such correlation analysis is similar to PGLS  
445 considering  $C_3$  and  $C_4$ , but with more varieties on the setting of variance and covariance matrix.  
446

#### 447 ***Physiological Modeling***

448  
449 Furthermore, we used physiological models that couples the photosynthesis systems and  
450 hydraulic systems to predict the effect of changing  $K_{\text{leaf}}$  on assimilation rate<sup>[32]</sup>. The change of  
451  $K_{\text{leaf}}$  was assumed to change the plant hydraulic conductance ( $K_{\text{plant}}$ ) proportionally in the  
452 modeling process. We double or reduce by half  $K_{\text{leaf}}$  relative to the original value to predict the  
453 effects on assimilation rates for  $C_3$  and  $C_4$  pathways. We assumed  $C_4$  had the same  
454 photosynthetic properties with  $C_3$  species (e.g., Rubisco affinity and specificity, Supplementary  
455 Table S24) other than the carbon concentration mechanism, which mimics the initial evolution of  
456  $C_4$  and the closely-related  $C_3$ - $C_4$  system. We also model the additional scenarios in which  $C_4$  had  
457 different photosynthetic properties to support the above condition further (Supplementary Table  
458 S25).

459

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466

#### 467 **Data availability**

468 The data that support the findings of this study are available from the corresponding author upon  
469 request.

470

#### 471 **Code availability**

472 All source code is available upon request.

473

474

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476

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