The legacy of C₄ evolution in the hydraulics of C₃ and C₄ grasses

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Abstract

The anatomical reorganization required for optimal C₄ photosynthesis should also impact plant hydraulics. Most C₄ plants possess large bundle-sheath cells and high vein density, which should also lead to higher leaf hydraulic conductance (K_{leaf}) and capacitance. Paradoxically, the C₄ pathway reduces water demand and increases water-use-efficiency, creating a potential mismatch between supply capacity and demand in C₄ plant water relations. We use phylogenetic analyses, physiological measurements, and models to examine the reorganization of hydraulics in closely-related C₄ and C₃ grasses. Evolutionarily young C₄ lineages have higher K_{leaf} , capacitance, turgor-loss-point, and lower stomatal conductance than their C₃ relatives. In contrast, species from older C₄ lineages show decreased K_{leaf} and capacitance, indicating that over time, C₄ plants have evolved to optimize hydraulic investments while maintaining C₄ anatomical requirements. The initial “over-plumbing” of C₄ plants disrupts the positive correlation between maximal assimilation rate and K_{leaf} , decoupling a key relationship between hydraulics and photosynthesis generally observed in vascular plants.

Introduction

The evolution of C₄ photosynthesis in the grasses— and the attendant fine-tuning of both anatomical and biochemical components across changing selection landscapes^[1,2,3]— likely impacted leaf hydraulics and hydraulics-photosynthesis relationships, both within the grass lineages in which C₄ evolved independently > 20 times^[4], and as compared to closely-related C₃^[5,6]. C₄ plants typically exhibit lower stomatal conductance (g_s) and consequently greater water-use efficiency than C₃, because the concentration of CO₂ inside bundle sheath cells permits reduced intercellular CO₂ concentrations and conservative stomatal behavior^[7,8,9]. At the same time, C₄ plants require high bundle sheath to mesophyll ratios (BS:M), which are accomplished with increased vein density and bundle sheath size as compared to C₃ plants. In C₃ species, leaf hydraulic conductance (K_{leaf}) has a positive relationship with vein density^[10,11,12,13]. The decreased inter-veinal distance and consequently higher vein density in C₄ species has been predicted to lead to a higher K_{leaf} than closely-related C₃ species^[14,15]. Further, increased bundle sheath size was proposed to lead to a higher leaf capacitance in C₄ species^[15,16]. This would lead to a potential physiological “mis-match”, where the evolution of the C₄ pathway simultaneously increases a plant’s hydraulic capacity while reducing its transpirational demand.

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

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

Results

Within each phylogenetic cluster, there were no clear patterns between C₃ and C₄ hydraulic traits by conducting ANOVA tests only. C₄ grasses had higher or equivalent K_{leaf}, leaf capacitance leaf turgor loss point, A_{max} and lower or equivalent g_s than their closest C₃ relatives (Fig. 2). The one C₃-C₄ intermediate species, *Steinchisma decipiens*, in our analysis had K_{leaf} similar or equivalent to C₄, but leaf capacitance, leaf turgor loss point, g_s and A_{max} equivalent to C₃ (Fig. 2). By analyzing our data in the context of the evolutionary models (Supplementary Table S1), however, we found clear C₃-C₄ differences in most measured traits. We first fitted evolutionary models of Brownian motion and Ornstein-Uhlenbeck processes to the hydraulic traits based on a reliable dated phylogenetic tree^[19]. The best fitting evolutionary model to the data for K_{leaf}, leaf turgor loss point, A_{max} and g_s was Ornstein-Uhlenbeck model, while the Brownian model is the best-fitting model for leaf capacitance, as determined by the AICc and Akaike weights and LRT test (Table 1, Supplementary Tables S2-S6). Higher K_{leaf}, higher A_{max}, lower leaf turgor loss point, and lower g_s are detected C₄ species compared to C₃ (LRT test, all $p < 0.01$; all $\Delta AICc < -3$). For leaf capacitance, there is no significant difference for C₃ and C₄ species.

We also looked for evolutionary trends in hydraulic traits after the evolution of a C₄ system to probe for an extended ‘optimization’ phase of C₄ evolution^[3, 20]. Identifying directional trends in continuous character evolution is difficult without fossil taxa, and it is impossible to directly measure hydraulic traits for fossils; however, we can test for trends indirectly using extant species. For example, if reduction in K_{leaf} is selected for subsequent to C₄ evolution we expect older C₄ lineages to have lower K_{leaf} values than younger C₄ lineages. We extracted the evolutionary age of C₄ origin for each of our lineages from the dated phylogeny^[19]. Regressions of evolutionary age versus hydraulic traits provide strong evidence for a long-term directional trend in hydraulic evolution following the origin of C₄ photosynthesis (Fig. 3). K_{leaf}, leaf turgor loss point and capacitance showed significant negative correlations with evolutionary age, while A_{max} had a significant positive correlation. In contrast, there was no significant relationship between g_s and evolutionary age. No evolutionary relationships were detected in C₃ species, which indicated the correlations between evolutionary age and hydraulic traits were unique to C₄ species. We also tested for an evolutionary trend by modelling hydraulic trait evolution using a phylogeny with branch lengths scaled to molecular substitutions/site, which provides an estimate of differences in evolutionary rates between lineages^[4]. While the second approach requires

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

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

Fig. 2 Hydraulic conductance (K_{leaf} , $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$), leaf capacitance ($\text{mmol m}^{-2} \text{MPa}^{-1}$), maximal stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$), maximal assimilation rate (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), and leaf turgor loss points (Turgor loss, -MPa) of closely related C₃ and C₄ species. Different colored clusters of bars show nine different origins of closely-related C₃ and C₄ species. C₃ species are colored black. Error bars indicated standard errors.

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

Property	Model	Model type	AICw	Root/Theta	
				C ₃	C ₄
K_{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_{max}	Model 6*	OU2	0.5292	13.66	17.34

* indicates the model fit significantly better than all the other models. Different root or theta values for C₃ and C₄ indicates that the evolutionary model with two different values of the root or theta for C₃ and C₄ species is a significantly better fit than the evolutionary model with the same root or theta.

We next explored how A_{max} and hydraulic traits are correlated across the phylogeny, and whether this relationship is different for C₃ and C₄ lineages. The correlations between A_{max} and K_{leaf} were different between C₃ and C₄ (Fig. 4, Table 2, Table S13). A_{max} was significantly positively correlated with K_{leaf} for C₃, but not for C₄ (Fig. 4, Table 2, Table S13). A_{max} was weakly positively correlated with leaf capacitance and g_s and the correlations were not significantly different for C₃ and C₄ (Fig. 4, Table 2, Supplementary Table S21, S22). A_{max} was negatively, but not significantly related with leaf turgor loss point in C₃ and C₄ species (Supplementary Table S23).

Fig. 3 The regression for hydraulic conductance (K_{leaf}), leaf capacitance, leaf turgor loss point, stomatal conductance (g_s) and maximal assimilation rate (A_{max}) vs. the evolutionary age for the nine origins of C₄ to show the evolutionary trend within C₄ and within their closely-related C₃ species. The evolutionary age for each sampled origin is derived from the dated phylogeny^[19].

Table 2 Phylogenetic Correlations between maximal assimilation rates (A_{max}) and hydraulic traits for C₃ and C₄ species (summarizing Table S20-S23; model description: Table S19).

Parameter 1	Parameter 2	Best Model	r for C ₃	r for C ₄	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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Different r means the best fitted model assuming different correlations for C_3 and C_4 . One r means the best fitted model assuming similar correlations for C_3 and C_4 . p values indicated whether the correlation coefficients are significant.

We used our mechanism-based physiological model^[32] to consider how the evolution of higher K_{leaf} would affect the optimal g_s and photosynthesis in C_3 and C_4 plants. An increase in K_{leaf} in the C_3 ancestor selects for higher g_s and increases the steady-state leaf water potential to a limited extent (Fig. 5, S1). Changing K_{leaf} has a smaller effect on the photosynthesis rate of C_4 than that of C_3 (Fig. 6, Table S25). Decreasing K_{leaf} by half or doubling it changes the photosynthesis rate of a C_4 plant by an average of -4.27% and 3.48%, respectively. In contrast, the same shifts in K_{leaf} has average effects of -10.07% and 9.14% on the assimilation rate of a C_3 plant. The sensitivity of the assimilation rate to changes in K_{leaf} decreases with increasing CO_2 concentration and increasing water-limitation for both C_3 and C_4 plants (Table S25). These differences in sensitivity to K_{leaf} were robust to differences in physiological properties between C_3 and C_4 (specifically, the temperature response properties and $J_{\max}/V_{\max}$ ratio; Table S25). The assimilation rate of C_4 plants was still less sensitive to K_{leaf} than that of C_3 species under different CO_2 concentration and water-limited conditions (Table S25). The physiological modeling results indicates that C_4 species maintain lower g_s and higher leaf water potential compared to closely related C_3 species because the CCM reduces transpirational demand. The modeling effects of varying K_{leaf} on photosynthesis confirmed the diminished returns for high-efficiency water transport in C_4 species mentioned above.

Fig. 4 Phylogenetic correlation for C₃ and C₄ between A_{max} and other hydraulic traits (K_{leaf} , leaf capacitance and g_s). Different/same correlation values on the figure mean C₃ and C₄ have significantly different/same correlations. Detailed phylogenetic correlation models and analysis results are shown in Table 2. Dashed black line: C₃; dashed red line: C₄; solid black line: C₃ and C₄ have the same correlation; grey lines indicate the phylogeny.

Fig. 5 The effect of changing K_{leaf} on stomatal resistance (the inverse of g_s) and leaf water potential under VPD=1.25 kPa, $\psi_s = -1$ MPa and CO₂ concentration of 200 ppm for the C₃ model. Solid black line: measured K_{leaf} ; dashed black line: K_{leaf} doubled; dashed grey line: K_{leaf} reduced by 50%.

To see if C₄ subtypes varied in hydraulic traits and their evolutionary rates or variance, we also considered evolutionary models where we allowed each variable to have a subtype-specific value (Supplementary Table S1). We found no significant differences in K_{leaf} , leaf capacitance, g_s , leaf turgor loss point and A_{max} among C₄ subtypes (all $\Delta\text{AICc} > 0$, ΔAICc obtained by AICc of subtype models minus AICc model not considering subtypes; Supplementary Tables S14-18). Although different decarboxylation enzymes are utilized by the three major subtypes (NADP-ME, NAD-ME and PCK), there does not seem to be an evolutionary effect on hydraulic traits. However, a previous study documenting PCK species from the Chloridoideae and Panicoideae lineages with lower leaf turgor loss point^[23]. Such differences were not apparent when we compared C₄ subtypes with multiple lineages. Our current representation of different subtypes is, however, somewhat limited. It would be advantageous to increase both lineage and species diversity and to balance subtypes within lineages to more deeply examine C₄ subtypes.

Fig. 6 Modeling results of photosynthesis rates along with different CO₂ concentration, different temperatures and different water limited conditions for C₃ (black lines) and for C₄ (red lines). Solid lines: modeling results for C₃ and C₄ with measured leaf hydraulic conductance; dashed lines: modeling results for C₃ and C₄ with twice of the regular leaf hydraulic conductance; dotted lines: modeling results for C₃ and C₄ with half of the regular leaf hydraulic conductance. C₃ and C₄ parameters are kept the same except for C₄ has the carbon concentration mechanism.

Discussion

The evolution of the C₄ pathway in the grasses caused a series of shifts in hydraulic properties as compared to closely-related C₃ grasses. The anatomical requirements of C₄ initially increased K_{leaf} and leaf capacitance, as predicted by previous studies^[14,15,16]; however, K_{leaf} and leaf

capacitance appear to decline over evolutionary time, suggesting a long period of physiological optimization after the initial assembly of a new photosynthetic system. Previous examination of leaf hydraulic traits in grasses focused on investigating single species or were not developed within a phylogenetic framework when comparing multiple species^[21,22], and phylogenetic studies have assumed trait evolution as simple Brownian motion^[23,24]. Hydraulic traits, however, may have evolved along different trajectories before and after the evolution of the C₄ pathway and associated anatomical reorganization, resulting in more complicated evolutionary dynamics. Our evolutionary models indicated C₄ grasses initially had higher K_{leaf}, leaf capacitance, turgor loss point than corresponding C₃, and a lower stomatal conductance (g_s) than grasses consistent with previous studies^[25,26]. Decreased vein distance and increased bundle sheath size are thought to be anatomical precursors to the evolution of C₄^[27,28], and both are thought to increase K_{leaf} and/or leaf capacitance^[14,15]. Therefore, the shifts of K_{leaf} and leaf capacitance likely occurred before, or at the initial formation of, the C₄ CCM. After the full formation of C₄, K_{leaf} and/or leaf capacitance started to decrease, which led to higher or equivalent K_{leaf} and leaf capacitance in the current C₃ and C₄ species (Fig. 2). Liu et al. (2019) found that K_{leaf} in C₄ grasses overlapped with C₃ values^[24]. The positive correlation between A_{max} and the evolutionary age also supports an extended optimization phase for C₄. Previous studies have indicated that species from the oldest C₄ lineages (*Chloridoideae* and *Andropogoneae* for example) contain the most productive crops (Sage, 2016), while some recent C₄ lineages are not more productive than C₃ (Ripley et al., 2008; Lundgren et al., 2016). In contrast, the significant decrease of g_s and the increase of leaf turgor loss point occurred with the evolution of a fully operational C₄ CCM, as suggested by our physiological models discussed below. Consistent with this prediction, in clades that possess a range of C₃, C₃-C₄ intermediate and C₄ physiologies, the increased water use efficiency, decreased g_s, and a broadened ecological niche are observed only in plants with a full C₄ CCM^[29,30].

The evolution of C₄ significantly alters the widely-accepted A_{max}-K_{leaf} relationships existing in vascular plants. A_{max} is limited by the efficient transport of water through leaves to replace water loss through open stomata, which is the likely cause of a positive correlation between K_{leaf} and A_{max} across and within plant taxa^[11,13,31]. We found that A_{max} and K_{leaf} are positively correlated in our C₃ species but not in C₄ (Fig 4). Ocheltree et al. (2016)^[22] similarly found no relationship

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

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

more success in adjusting these hydraulic traits in advance. Consciously selecting or manipulating narrower xylem, decreasing the expression of aquaporins, or other mechanisms of decreasing leaf conductance while maintain high bundle sheath to mesophyll ratio, together with CCM may increase the water use efficiency of C₄ species further. Our phylogenetic analyses can thus inform both the evolutionary history of C₄ plants and future efforts to modify C₄ crops.

By capitalizing on the multiple origins of C₄ photosynthesis in grasses, we have shown that the vascular organization that is a hallmark of C₄ plants also impacts leaf hydraulics, and disrupts the established link between hydraulic and photosynthetic capacity demonstrated in C₃ plants. C₄ grasses are “overplumbed” relative to their C₃ counterparts, suggesting that the costs associated with the production of an extensive leaf vasculature require re-evaluation in plants with C₄ photosynthetic systems. The gradual decline in K_{leaf} in C₄ lineages over millions of years also requires an explanation. The C₄- K_{leaf} conundrum provides an opportunity to examine what we mean by “evolutionary constraint” and highlights the very dynamic nature of evolutionary tradeoffs and functional optimization. First, we assume that the costs of building and maintaining a high K_{leaf} are still significant in C₄ plants^[12,35,36,37,38]. The most efficient way to reduce K_{leaf} costs would be to reduce venation density, as veins come with high construction costs^[12,17], and also reduce the leaf area that is available for carbon fixation. Yet the anatomical requirements of the C₄ system preclude this option: reducing vein density would result in a highly inefficient C₄ system^[15], which would negatively impact the plant’s carbon budget, presumably to a much greater extent than the cost of an overbuilt venation system. As vein construction is a primary contribution to the cost of a high K_{leaf} , and high vein densities are now linked to a new function (C₄ carbon fixation), the cost-benefit calculations in optimizing K_{leaf} have shifted, and the tradeoff is in favor of overplumbing in order to maintain a highly efficient new carbon fixation system. In evolutionary vocabulary, what emerges is a new constraint – and in this example, it is clear that the emergence of a new constraint to organismal evolution is simply due to a shift in the tradeoffs associated with characters that influence multiple aspects of organismal function. In other words, we assume a low vein density is a phenotype that is still developmentally achievable for C₄ grasses; what has prevented its emergence is the shift in functional costs associated with reduced vein densities.

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

Methods

Plant material

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

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

Hydraulic traits

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

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

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

Maximal assimilation rate ($A_{\max}$) and stomatal conductance (g_s) were measured under saturated light intensity. $A_{\max}$ and g_s were obtained using a standard 2 x 3 cm² leaf chamber with a red/blue LED light source of LI-6400XT (LI-COR Inc., Lincoln, NE, USA). Light curves were measured with light intensities of 2000, 1500, 1200, 1000, 800, 500, 300, 200, 150, 100, 75, 50, 20, 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under CO₂ of 400 ppm. Then, $A_{\max}$ was estimated from the light curve^[45,46]. All the measurements were made under the temperature of 25°C and the leaf temperature to air vapor pressure deficit was controlled around 2kPa. g_s at the saturated light intensity of 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was recorded for each plant. The cuvette opening was covered by Fun-Tak to avoid and correct for the leakiness.

Phylogenetic analysis

Phylogenetic analysis for C₃ and C₄. We pruned the dated phylogeny from a published grass phylogeny to include only the species in our physiological experiments^[19](Fig. 1). Using the dated phylogeny, for each of the hydraulic traits, we fitted evolutionary models to test which evolutionary model best explains observed distribution of traits along the phylogeny and how these models differ between C₃ and C₄ (Table S1). We fitted evolutionary models belonging

Brownian Motion model and Ornstein-Uhlenbeck Model using the package “mvMORPH” in R^[47]. To determine the best fitted evolutionary model, we compared two criteria, the small-sample-size corrected version of Akaike information criterion (AICc, the lower AICc, the better fit) and Akaike weights (AICw, the higher AICw, the better fit)^[48,49,50]. The evolutionary models have nested variants (Models 1-4; Models 5-6), varying in whether C₃ and C₄ species had the same or different fluctuation rates, root states for Brownian motion model and optima for Ornstein-Uhlenbeck model. We used likelihood-ratio test (LRT) to verify whether a specific model variant performs significantly better. The AICc, AICw and LRT allowed us to test evolutionary hypotheses, for instance, if the model in which C₃ and C₄ have different root states fit significantly better than model in which C₃ and C₄ have the same root states, it means there is a shift of physiological trait along with the formation of C₄. To examine the further evolution of hydraulic traits after a full C₄ evolved, we extracted the evolutionary ages for each represented C₄ origin from the dated phylogenetic trees. Then, we regressed the hydraulic traits with evolutionary age. A significant negative correlation between evolutionary age and hydraulic trait will indicate a further decreasing evolutionary direction after C₄ evolved. We also performed an additional analysis to test the original states and further direction together. We extracted molecular phylogeny for all the species from Edwards, GPWG II (2012)^[4]. Except for the six evolutionary models mentioned above, the molecular phylogeny allows us to fit for additional six Brownian motion models with trend (Supplementary Table S7). Likewise, if Brownian motion model with trend fits the phylogenetic patterns better than Brownian motion model without trend it means there is an evolutionary trend, and a significant LRT test for a two-trend model suggests that C₃ and C₄ lineages differ in the speed or direction of hydraulic evolution. We also mapped the traits on the phylogeny for potential further references (Fig. S2-S5).

To further test whether there are significant differences among C₄ subtypes, evolutionary models with subtypes (Table S1) were used to fit the data. We again used AICc, AICw and LRT methods to find the best model variants: whether there are significant differences for hydraulic shifts and evolutionary trends among three different subtypes. For the leaf capacitance analysis, *Dichanthelium clandestinum* is deleted as it is an obvious outlier.

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

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

Physiological Modeling

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

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Data availability

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

Code availability

All source code is available upon request.

References

1. Sage, R.F., 2017. A portrait of the C4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. *Journal of experimental botany*, 68(2), pp.e11-e28.
2. Ehleringer JR, Monson RK (1993). Evolutionary and ecological aspects of photosynthesis pathway variation. *Annu Rev Ecol Syst* 24: 411-439.
3. Edwards, E. J. (2019). Evolutionary trajectories, accessibility and other metaphors: the case of C4 and CAM photosynthesis. *New Phytol.* 223(4), 1742-1755.
4. Grass Phylogeny Working Group II. (2012). New grass phylogeny resolves deep evolutionary relationships and discovers C4 origins. *New Phytol* 193: 304-312.
5. Christin PA, Osborne CP (2014) Tansley Review. The evolutionary ecology of C4 photosynthesis. *New Phytol* 204: 765-781.
6. Kocacinar F, Sage RF (2003) Photosynthetic pathway alters xylem structure and hydraulic function in herbaceous plants. *Plant, Cell & Environment* 26(12): 2015-2026.
7. Pearcy RW, Ehleringer J (1984) Comparative ecophysiology of C3 and C4 plants. *Plant, Cell & Environment* 7(1): 1-13.
8. Huxman TE, Monson RK (2003) Stomatal responses of C3, C3-C4 and C4 *Flaveria* species to light and intercellular CO2 concentration: implications for the evolution of stomatal behaviour. *Plant, Cell & Environment* 26(2): 313-322.
9. Way DA, Katul GG, Manzoni S, Vico G (2014) Increasing water use efficiency along the C3 to C4 evolutionary pathway: a stomatal optimization perspective. *Journal of Experimental Botany* 65(13): 3683-3693.
10. Sack L, Frole K (2006) Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology* 87: 483-491.
11. Brodribb TJ, Feild TS, Jordan GJ (2007) Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiol* 144: 1890-1898.
12. McKown AD, Cochard H, Sack L (2010) Decoding leaf hydraulics with a spatially explicit model: principles of venation architecture and implications for its evolution. *Am Nat* 175: 447-460.
13. Scoffoni C, Chatelet DS, Pasquet-kok J, Rawls M, Donoghue MJ, Edwards EJ, Sack L (2016) Hydraulic basis for the evolution of photosynthetic productivity. *Nature plants* 2:16072.
14. Osborne CP, Sack L (2012). Evolution of C4 plants: a new hypothesis for an interaction of CO2 and water relations mediated by plant hydraulics. *Philos Trans R Soc Lond B Biol Sci* 367: 583-600.

15. Griffiths H, Weller G, Toy L, Dennis RJ (2012) You're so vein: bundle sheath physiology, phylogeny and evolution in C₃ and C₄ plants. *Plant Cell Environ* 36: 249–261.
16. Sage RF (2001) Environmental and evolutionary preconditions for the origin and diversification of the C₄ photosynthetic syndrome. *Plant Biology* 3: 202–213.
17. McCulloh, KA, Sperry, JS, Adler, FR (2003) Water transport in plants obeys Murray's law. *Nature* 421: 939–942.
18. Sage RF, Sage TL, Kocacinar F (2012) Photorespiration and the evolution of C₄ photosynthesis. *Annual review of plant biology* 63: 19–47.
19. Spriggs EL, Christin PA and Edwards EJ (2014) C₄ photosynthesis promoted species diversification during the Miocene grassland expansion. *Plos one* 9(5): p.e97722.
20. Heyduk ., Moreno-Villena JJ, Gilman IS, Christin PA, and Edwards EJ (2019). The genetics of convergent evolution: insights from plant photosynthesis. *Nature Review Genetics*, 20, 485–493.
21. Martre P, Cochard H, Durand JL (2001) Hydraulic architecture and water flow in growing grass tillers (*Festuca arundinacea* Schreb.). *Plant, Cell and Environment* 24: 65–76.
22. Ocheltree TW, Nippert JB, Prasad PV (2016) A safety vs efficiency trade-off identified in the hydraulic pathway of grass leaves is decoupled from photosynthesis, stomatal conductance and precipitation. *New Phytologist* 210(1): 97–107.
23. Liu H, Osborne CP (2015) Water relations traits of C₄ grasses depend on phylogenetic lineage, photosynthetic pathway, and habitat water availability. *Journal of Experimental Botany* 66, 761–773.
24. Liu H, Taylor SH, Xu Q, Lin Y, Hou H, Wu G, Ye, Q (2019) Life history is a key factor explaining functional trait diversity among subtropical grasses, and its influence differs between C₃ and C₄ species. *Journal of experimental botany* 70(5): 1567–1580.
25. Taylor SH, Hulme SP, Rees M, Ripley BS, Woodward FI, Osborne CP (2010). Ecophysiological traits in C₃ and C₄ grasses: a phylogenetically controlled screening experiment *New Phytol* 185: 780–791
26. Taylor SH, Franks PJ, Hulme SP, Spriggs E, Christin PA, Edwards EJ, Woodward FI, Osborne CP (2012) Photosynthetic pathway and ecological adaptation explain stomatal trait diversity amongst grasses. *New Phytol* 193: 387–396.
27. Sage RF (2004) The evolution of C₄ photosynthesis. *New Phytol* 161(2):341–370.
28. Christin PA, Osborne CP, Chatelet DS, Columbus JT, Besnard G, Hodkinson TR, Garrison LM, Vorontsova MS, Edwards EJ (2013) Anatomical enablers and the evolution of C₄ photosynthesis in grasses. *Proc Natl Acad Sci USA* 110: 1381–1386.
29. Lundgren MR, Besnard G, Ripley BS, Lehmann CE, Chatelet DS, Kynast RG, Namaganda M, Vorontsova MS, Hall RC, Elia J, Osborne CP (2015) Photosynthetic innovation broadens the niche within a single species. *Ecology Letters* 18(10): 1021–1029.
30. Sage RF, Monson RK, Ehleringer JR, Adachi S, Pearcy RW (2018) Some like it hot: the physiological ecology of C₄ plant evolution. *Oecologia* 187(4):941–966.
31. Brodribb TJ, Holbrook NM, Zwieniecki MA, Palma B (2005) Leaf hydraulic capacity in ferns, conifers and angiosperms: impacts on photosynthetic maxima. *New Phytol* 165: 839–846
32. Zhou H, Helliker BR, Huber M, Dicks A, Akçay E (2018) C₄ photosynthesis and climate through the lens of optimality. *Proceedings of the National Academy of Sciences* 115:

- 12057-12062.
33. Ripley BS, Cunniff J, Osborne CP (2013) Photosynthetic acclimation and resource use by the C₃ and C₄ subspecies of *Alloteropsis semialata* in low CO₂ atmospheres. *Glob Change Biol* 19: 900–910.
34. Taylor SH, Ripley BS, Martin T, De-Wet L-A, Woodward FI, Osborne CP (2014). Physiological advantages of C₄ grasses in the field: a comparative experiment demonstrating the importance of drought. *Glob Change Biol* 20: 1922-2003.
35. Tyree MT, Sperry JS (1989) Vulnerability of xylem to cavitation and embolism. *Annu Rev Plant Physiol Plant Mol Biol* 40(1):19–36.
36. Niinemets Ü, Portsmouth A, Tobias M (2007) Leaf shape and venation pattern alter the support investments within leaf lamina in temperate species: a neglected source of leaf physiological differentiation? *Functional Ecology* 21: 28-40.
37. Scoffoni C, Rawls M, McKown A, Cochard H, Sack L (2011) Decline of leaf hydraulic conductance with dehydration: relationship to leaf size and venation architecture. *Plant Physiology* 156(2): 832-843.
38. Wolf A, Anderegg WR, Pacala SW (2016) Optimal stomatal behavior with competition for water and risk of hydraulic impairment. *Proceedings of the National Academy of Sciences* 113(46): E7222-E7230.
39. Scoffoni C, Albuquerque C, Brodersen CR, Townes SV, John GP, Bartlett MK, Buckley TN, McElrone AJ, Sack L (2017) Outside-xylem vulnerability, not xylem embolism, controls leaf hydraulic decline during dehydration. *Plant Physiology* 173: 1197-1210.
40. Pathare VS, Sonawane BV, Koteyeva N, Cousins AB (2020) C₄ grasses adapted to low precipitation habitats show traits related to greater mesophyll conductance and lower leaf hydraulic conductance. *Plant Cell & Environment* doi: 10.1111/pce.13807.
41. Sack L, Scoffoni C (2012) Measurement of Leaf Hydraulic Conductance and Stomatal Conductance and Their Responses to Irradiance and Dehydration Using the Evaporative Flux Method (EFM). *J Vis Exp* (70), e4179, doi:10.3791/4179.
42. Ocheltree T, Gleason S, Cao KF, Jiang GF (2020) Loss and recovery of leaf hydraulic conductance: Root pressure, embolism, and extra-xylary resistance. *Journal of Plant Hydraulics* 7
43. Tyree MT, Hammel HT (1972) The measurement of the turgor pressure and the water relations of plants by the pressure-bomb technique. *Journal of Experimental Botany* 23(1): 267-282.
44. Sack L, Pasquet-Kok J and PrometheusWiki contributors. Leaf pressure-volume curve parameters. PrometheusWiki. May 20, 2010, 17:08 UTC. Available at: /wiki/pagehistory.php?page=Leaf pressure-volume curve parameters&preview=16.
45. Marshall B, Biscoe PV (1980) A model for C₃ leaves describing the dependence of net photosynthesis on irradiance. *Journal of Experimental Botany* 31:29-39.
46. Thornley JHM (1998) Dynamic model of leaf photosynthesis with acclimation to light and nitrogen. *Annals of Botany* 81(3): 421-430.
47. Clavel J, Escarguel G, Merceron G (2015) mvMORPH: an R package for fitting multivariate evolutionary models to morphometric data. *Methods in Ecology and Evolution*, 6(11):1311-1319.<doi:10.1111/2041-210X.12420>
48. Akaike H (1974) A new look at the statistical model identification. In *Selected Papers of Hirotugu Akaike* (pp. 215-222). Springer, New York, NY.
49. Cavanaugh JE (1997) Unifying the derivations for the Akaike and corrected Akaike

603 information criteria. Statistics & Probability Letters 33(2): 201-208.
604 50. Burnham KP and Anderson DR (1998) Practical use of the information-theoretic
605 approach. In Model Selection and Inference (pp. 75-117). Springer, New York, NY.
606