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$_{\text{leaf}}$) 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$_{\text{leaf}}$, capacitance, turgor-loss-point, and lower stomatal conductance than their C3 relatives. In contrast, species from older C4 lineages show decreased K$_{\text{leaf}}$ 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$_{\text{leaf}}$, 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 CO$_2$ inside bundle sheath cells permits reduced intercellular CO$_2$ 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$_{\text{leaf}}$) 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$_{\text{leaf}}$ 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\cite{12,17}, then one would expect to see a reduction of $K_{\text{leaf}}$ over evolutionary time, as continued selection works to optimize the C$_4$ metabolism\cite{5,18}. Alternatively, a maintenance of high $K_{\text{leaf}}$ over time could result from either a lack of strong selection to reduce $K_{\text{leaf}}$, 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 ($A_{\text{max}}$) and hydraulic capacity ($K_{\text{leaf}}$) 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 $A_{\text{max}}$ and $K_{\text{leaf}}$ across many scales, from a broad phylogenetic spectrum of species spanning vascular plants\cite{11}, to smaller clades of closely related species\cite{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 $A_{\text{max}}$-$K_{\text{leaf}}$ relationships. Using a broad sampling of grasses (Fig. 1), we determined whether anatomical differences associated with C$_4$ evolution result in greater $K_{\text{leaf}}$ 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 $A_{\text{max}}$-$K_{\text{leaf}}$ relationships. Finally, we then quantified evolutionary trends in $K_{\text{leaf}}$, 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 $K_{\text{leaf}}$ and a greater $K_{\text{leaf}}$-$A_{\text{max}}$ 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 Kₗₑᵃ𝑓, leaf capacitance leaf turgor loss point, Aₘ₃ₓ and lower or equivalent gₛ than their closest C₃ relatives (Fig. 2). The one C₃-C₄ intermediate species, *Steinchisma decipiens*, in our analysis had Kₗₑᵃ𝑓 similar or equivalent to C₄, but leaf capacitance, leaf turgor loss point, gₛ and Aₘ₃ₓ 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 Kₗₑᵃᶠ, leaf turgor loss point, Aₘ₃ₓ and gₛ 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 Kₗₑᵃᶠ, higher Aₘ₃ₓ, lower leaf turgor loss point, and lower gₛ are detected C₄ species compared to C₃ (LRT test, all \( p<0.01 \); all ΔAICc<-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 Kₗₑᵃᶠ is selected for subsequent to C₄ evolution we expect older C₄ lineages to have lower Kₗₑᵃᶠ 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). Kₗₑᵃᶠ, leaf turgor loss point and capacitance showed significant negative correlations with evolutionary age, while Aₘ₃ₓ had a significant positive correlation. In contrast, there was no significant relationship between gₛ 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[44]. While the second approach requires
many assumptions that are likely violated, the results also provide additional support to a
directional trend in $K_{\text{leaf}}$ and capacitance in $C_4$ lineages: comparing 12 different types of models
with or without evolutionary trends (supplementary Table S7), we found $K_{\text{leaf}}$ 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 ($K_{\text{leaf}}$, mmol m$^{-2}$ s$^{-1}$ MPa$^{-1}$), leaf capacitance (mmol m$^{-2}$ MPa$^{-1}$), maximal stomatal conductance ($g_s$, mmol m$^{-2}$ s$^{-1}$), maximal assimilation rate ($A_{\text{max}}$, µmol m$^{-2}$ s$^{-1}$), and leaf turgor loss points (Turgor loss, -MPa) of closely related C$_3$ and C$_4$ species. Different colored clusters of bars show nine different origins of closely-related C$_3$ and C$_4$ species. C$_3$ 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  |
|---------------|---------|------------|------|------------|
| $K_{leaf}$    | Model 6* | OU2         | 0.984 | 2.682     |
| 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 $C_3$ and $C_4$ indicates that the evolutionary model with two different values of the root or theta for $C_3$ and $C_4$ 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 $C_3$ and $C_4$ lineages. The correlations between $A_{max}$ and $K_{leaf}$ were different between $C_3$ and $C_4$ (Fig. 4, Table 2, Table S13). $A_{max}$ was significantly positively correlated with $K_{leaf}$ for $C_3$, but not for $C_4$ (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 $C_3$ and $C_4$ (Fig. 4, Table 2, Supplementary Table S21, S22). $A_{max}$ was negatively, but not significantly related with leaf turgor loss point in $C_3$ and $C_4$ species (Supplementary Table S23).
Fig. 3 The regression for hydraulic conductance ($K_{\text{leaf}}$), leaf capacitance, leaf turgor loss point, stomatal conductance ($g_s$) and maximal assimilation rate ($A_{\text{max}}$) vs. the evolutionary age for the nine origins of $C_4$ to show the evolutionary trend within $C_4$ and within their closely-related $C_3$ species. The evolutionary age for each sampled origin is derived from the dated phylogeny[^19].

Table 2 Phylogenetic Correlations between maximal assimilation rates ($A_{\text{max}}$) and hydraulic traits for $C_3$ and $C_4$ 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 |
|-------------|-------------|------------|---------------|---------------|-----------|
| $A_{\text{max}}$ | $K_{\text{leaf}}$ | CorModel 3 | 0.695 | 0.129 | 0.012/0.51 |
| $A_{\text{max}}$ | Capacitance | CorModel 2 | 0.259 | 0.027 | 0.003 |
| $A_{\text{max}}$ | $g_s$ | CorModel 1 | 0.533 | 0.003 | 0.003 |
A\text{max} & leaf\ turgor\ loss & CorModel\ 1 & -0.223 & 0.256 \\

Different r means the best fitted model assuming different correlations for C\text{3} and C\text{4}. One r means the best fitted model assuming similar correlations for C\text{3} and C\text{4}. p values indicated whether the correlation coefficients are significant.

We used our mechanism-based physiological model\textsuperscript{[32]} to consider how the evolution of higher $K_{\text{leaf}}$ would affect the optimal $g_s$ and photosynthesis in C\text{3} and C\text{4} plants. An increase in $K_{\text{leaf}}$ in the C\text{3} ancestor selects for higher $g_s$ and increases the steady-state leaf water potential to a limited extent (Fig. 5, S1). Changing $K_{\text{leaf}}$ has a smaller effect on the photosynthesis rate of C\text{4} than that of C\text{3} (Fig. 6, Table S25). Decreasing $K_{\text{leaf}}$ by half or doubling it changes the photosynthesis rate of a C\text{4} plant by an average of -4.27\% and 3.48\%, respectively. In contrast, the same shifts in $K_{\text{leaf}}$ has average effects of -10.07\% and 9.14\% on the assimilation rate of a C\text{3} plant. The sensitivity of the assimilation rate to changes in $K_{\text{leaf}}$ decreases with increasing CO\text{2} concentration and increasing water-limitation for both C\text{3} and C\text{4} plants (Table S25). These differences in sensitivity to $K_{\text{leaf}}$ were robust to differences in physiological properties between C\text{3} and C\text{4} (specifically, the temperature response properties and $J_{\text{max}}/V_{\text{c,max}}$ ratio; Table S25). The assimilation rate of C\text{4} plants was still less sensitive to $K_{\text{leaf}}$ than that of C\text{3} species under different CO\text{2} concentration and water-limited conditions (Table S25). The physiological modeling results indicates that C\text{4} species maintain lower $g_s$ and higher leaf water potential compared to closely related C\text{3} species because the CCM reduces transpirational demand. The modeling effects of varying $K_{\text{leaf}}$ on photosynthesis confirmed the diminished returns for high-efficiency water transport in C\text{4} species mentioned above.
Fig. 4 Phylogenetic correlation for C$_3$ and C$_4$ between $A_{\text{max}}$ and other hydraulic traits ($K_{\text{leaf}}$, leaf capacitance and $g_s$). Different/same correlation values on the figure mean C$_3$ and C$_4$ have significantly different/same correlations. Detailed phylogenetic correlation models and analysis results are shown in Table 2. Dashed black line: C$_3$; dashed red line: C$_4$; solid black line: C$_3$ and C$_4$ have the same correlation; grey lines indicate the phylogeny.
Fig. 5 The effect of changing $K_{\text{leaf}}$ on stomatal resistance (the inverse of $g_s$) and leaf water potential under VPD=1.25 kPa, $\psi_s = -1$ MPa and CO$_2$ concentration of 200 ppm for the C$_3$ model. Solid black line: measured $K_{\text{leaf}}$; dashed black line: $K_{\text{leaf}}$ doubled; dashed grey line: $K_{\text{leaf}}$ reduced by 50%.

To see if C$_4$ 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 $K_{\text{leaf}}$, leaf capacitance, $g_s$, leaf turgor loss point and $A_{\text{max}}$ among C$_4$ subtypes (all $\Delta$AICc>0, $\Delta$AICc 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 C$_4$ 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 C$_4$ subtypes.
Fig. 6 Modeling results of photosynthesis rates along with different CO$_2$ concentration, different temperatures and different water limited conditions for C$_3$ (black lines) and for C$_4$ (red lines). Solid lines: modeling results for C$_3$ and C$_4$ with measured leaf hydraulic conductance; dashed lines: modeling results for C$_3$ and C$_4$ with twice of the regular leaf hydraulic conductance; dotted lines: modeling results for C$_3$ and C$_4$ with half of the regular leaf hydraulic conductance. C$_3$ and C$_4$ parameters are kept the same except for C$_4$ has the carbon concentration mechanism.

Discussion
The evolution of the C$_4$ pathway in the grasses caused a series of shifts in hydraulic properties as compared to closely-related C$_3$ grasses. The anatomical requirements of C$_4$ initially increased $K_{\text{leaf}}$ and leaf capacitance, as predicted by previous studies$^{[14,15,16]}$; however, $K_{\text{leaf}}$ 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\cite{21,22}, and phylogenetic studies have assumed trait evolution as simple Brownian motion\cite{23,24}. Hydraulic traits, however, may have evolved along different trajectories before and after the evolution of the C\textsubscript{4} pathway and associated anatomical reorganization, resulting in more complicated evolutionary dynamics. Our evolutionary models indicated C\textsubscript{4} grasses initially had higher K\textsubscript{leaf}, leaf capacitance, turgor loss point than corresponding C\textsubscript{3}, and a lower stomatal conductance (g\textsubscript{s}) than grasses consistent with previous studies\cite{25,26}. Decreased vein distance and increased bundle sheath size are thought to be anatomical precursors to the evolution of C\textsubscript{4}\cite{27,28}, and both are thought to increase K\textsubscript{leaf} and/or leaf capacitance\cite{14,15}. Therefore, the shifts of K\textsubscript{leaf} and leaf capacitance likely occurred before, or at the initial formation of, the C\textsubscript{4} CCM. After the full formation of C\textsubscript{4}, K\textsubscript{leaf} and/or leaf capacitance started to decrease, which led to higher or equivalent K\textsubscript{leaf} and leaf capacitance in the current C\textsubscript{3} and C\textsubscript{4} species (Fig. 2). Liu et al. (2019) found that K\textsubscript{leaf} in C\textsubscript{4} grasses overlapped with C\textsubscript{3} values\cite{24}. The positive correlation between A\textsubscript{max} and the evolutionary age also supports an extended optimization phase for C\textsubscript{4}. Previous studies have indicated that species from the oldest C\textsubscript{4} lineages (Chloridoideae and Andropogoneae for example) contain the most productive crops (Sage, 2016), while some recent C\textsubscript{4} lineages are not more productive than C\textsubscript{3} (Ripley et al., 2008; Lundgren et al., 2016). In contrast, the significant decrease of g\textsubscript{s} and the increase of leaf turgor loss point occurred with the evolution of a fully operational C\textsubscript{4} CCM, as suggested by our physiological models discussed below. Consistent with this prediction, in clades that possess a range of C\textsubscript{3}, C\textsubscript{3}-C\textsubscript{4} intermediate and C\textsubscript{4} physiologies, the increased water use efficiency, decreased g\textsubscript{s}, and a broadened ecological niche are observed only in plants with a full C\textsubscript{4} CCM\cite{29,30}. The evolution of C\textsubscript{4} significantly alters the widely-accepted A\textsubscript{max}-K\textsubscript{leaf} relationships existing in vascular plants. A\textsubscript{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\textsubscript{leaf} and A\textsubscript{max} across and within plant taxa\cite{11,13,31}. We found that A\textsubscript{max} and K\textsubscript{leaf} are positively correlated in our C\textsubscript{3} species but not in C\textsubscript{4} (Fig 4). Ocheltree et al. (2016)\cite{22} similarly found no relationship
between $K_{\text{leaf}}$ and $A_{\text{max}}$ in a set of nine C$_4$ species. We see possible explanations that are not necessarily mutually exclusive. First, the positive relationship of $A_{\text{max}}$ and $K_{\text{leaf}}$ is weakened under high $K_{\text{leaf}}$, 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 $K_{\text{leaf}}$ tends to be lower in grasses than in other species, it is possible that the diminishing returns from increasing $K_{\text{leaf}}$ manifest at lower values in grasses, and the initial high $K_{\text{leaf}}$ resulting from C$_4$ anatomy could be in the $A_{\text{max}}$ “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 $A_{\text{max}}$–$K_{\text{leaf}}$ optimality is slowly occurring within C$_4$ grass lineages in relatively newfound ecological niches. However, the similar correlations of g$_s$ vs. $A_{\text{max}}$ 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 $A_{\text{max}}$ 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 $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ 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. Consiously 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 trade-
offs 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 $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ and higher $A_{\text{max}}$—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 $K_{\text{leaf}}$ is due in large part to the optimization of traits to increase $A_{\text{max}}$ at the expense of $K_{\text{leaf}}$, which is possible only because hydraulic capacity was already “buffered” by the vein density requirements of C$_4$—allowing for continued reductions of $K_{\text{leaf}}$ 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 $A_{\text{max}}$ 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 $A_{\text{max}}$ and $K_{\text{leaf}}$ 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 (Anthaenantia), 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 ($K_{\text{leaf}}$) was measured using the evaporative flux method\cite{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\cite{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 µmol m$^{-2}$ 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). $K_{\text{leaf}}$ values were further standardized to 25 °C and leaf area to make the $K_{\text{leaf}}$ 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 (\(A_{\text{max}}\)) and stomatal conductance (\(g_s\)) were measured under saturated
light intensity. \(A_{\text{max}}\) and \(g_s\) were obtained using a standard 2 x 3 cm\(^2\) 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 µmol
m\(^{-2}\) s\(^{-1}\) under CO\(_2\) of 400 ppm. Then, \(A_{\text{max}}\) 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 µmol m\(^{-2}\)
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 C\(_3\) and C\(_4\).** 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 C\(_3\) and C\(_4\) (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 C\(_3\) and C\(_4\) 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 C\(_3\) and C\(_4\) have different root states fit significantly better than model in which C\(_3\) and C\(_4\) have the same root states, it means there is a shift of physiological trait along with the formation of C\(_4\). To examine the further evolution of hydraulic traits after a full C\(_4\) evolved, we extracted the evolutionary ages for each represented C\(_4\) 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 C\(_4\) 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 C\(_3\) and C\(_4\) 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 C\(_4\) 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 \(A_{\text{max}}\) and each of the hydraulic traits and to test the
hypotheses that whether such correlations are different between C3 and C4. The process of brownian motion with different root for C3 and C4 was used for $K_{\text{leaf}}$, $g_s$ 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 C3 and C4. Such correlation analysis is similar to PGLS considering C3 and C4, 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 $K_{\text{leaf}}$ on assimilation rate$^{32}$. The change of $K_{\text{leaf}}$ was assumed to change the plant hydraulic conductance ($K_{\text{plant}}$) proportionally in the modeling process. We double or reduce by half $K_{\text{leaf}}$ relative to the original value to predict the effects on assimilation rates for C3 and C4 pathways. We assumed C4 had the same photosynthetic properties with C3 species (e.g., Rubisco affinity and specificity, Supplementary Table S24) other than the carbon concentration mechanism, which mimics the initial evolution of C4 and the closely-related C3-C4 system. We also model the additional scenarios in which C4 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.

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