The legacy of C_4 evolution in the hydraulics of C_3 and C_4 grasses

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Abstract
Most studies concerning the functional implications of C_4 photosynthesis have focused on enhanced carbon fixation under high temperature, low atmospheric CO_2, and/or water limitation, yet the biochemical and anatomical reorganization required for optimal C_4 function should also impact plant hydraulics and water use. C_4 grasses have increased bundle-sheath size and vein density, and these are thought to have been anatomical precursors for the evolution of C_4 from C_3 ancestors. Paradoxically, these traits should also lead to higher leaf capacitance and higher leaf hydraulic conductance, yet C_4 photosynthesis lowers water demand and increases plant water use efficiency. Here, we use phylogenetic analyses, physiological measurements and photosynthetic modeling to examine the reorganization of hydraulic traits in C_4 grass lineages and in closely-related C_3 grasses. Evolutionarily young C_4 lineages have higher leaf hydraulic conductance, capacitance, turgor loss point, and lower stomatal conductance than their C_3 relatives. In contrast, species from older C_4 lineages show decreased leaf hydraulic conductance and capacitance, indicating that over time, C_4 plants have optimized hydraulic investments while maintaining their C_4 anatomical requirements. The "overplumbing" of young C_4 lineages lead to a reduced positive correlation between maximal assimilation rate and leaf hydraulic conductance, decoupling a key relationship between hydraulic traits and photosynthesis generally observed in vascular plants.

Key words C_4 evolution, leaf hydraulic conductance, capacitance, stomatal conductance, turgor loss point, maximal assimilation, grass

Introduction

C_4 photosynthesis is a carbon-concentrating mechanism that has evolved > 65 times in vascular plants over the ~30 million-years (Sage et al. 2011; 2016). The C_4 pathway increases the efficiency of photosynthesis by concentrating CO_2 around the enzyme Rubisco, hence reducing the oxygenase activity of Rubisco and photorespiration, and likely evolved in response to multiple climatic variables which increased photorespiration in C_3 plants (Edwards et al., 2010). While high temperature was a persistent selective pressure, water availability likely drove the evolution of the earliest C_4 lineages (Edwards and Smith, 2010, Zhou et al., 2018), and low atmospheric CO_2 drove the C_4 evolution and distribution within the last 15 MY (Ehleringer and Monson 1993; Ehleringer et al. 1997). Regardless of the primary selective driver, C_4 photosynthesis evolved independently more than 20 times in the grasses (Grass Phylogeny

Working Group II, 2012), and each separate event required significant anatomical and biochemical reorganization within the leaf blade (Sage et al., 2012; Edwards, 2019).

The evolution of C_4 photosynthesis— and subsequent fine-tuning of both anatomical and biochemical components across changing selection landscapes— likely impacted leaf hydraulics and hydraulics-photosynthesis relationships, both within C_4 grass lineages and as compared to closely-related C_3 grasses. C_4 plants typically exhibit lower stomatal conductance (g_s) and consequently greater water-use efficiency than C_3 plants, because the concentration of CO2 inside bundle sheath cells permits reduced intercellular CO2 concentrations and conservative stomatal behavior (Pearcy and Ehleringer, 1984; Huxman and Monson, 2003; Way et al., 2014). At the same time, C_4 plants have increased vein density and bundle sheath size as compared to C_3 plants. In C_3 species, leaf hydraulic conductance (K_{leaf}) has a positive relationship with vein density (Sack and Frole, 2006; Brodribb et al., 2007; McKown et al., 2010; Scoffoni et al., 2016). The decreased inter-veinal distance and consequently higher vein density in C_4 species has been proposed to lead to a higher K_{leaf} than closely related C_3 species (Osborne and Sack, 2012; Griffiths et al. 2012). Further, increased bundle sheath size was proposed to lead to a higher leaf capacitance in C_4 species (Sage, 2001; Griffiths et al. 2012). 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, then one would expect to see a reduction of Kleaf over evolutionary time, as continued selection works to optimize the C4 metabolism. Alternatively, a maintenance of high Kleaf over time could result from either a lack of strong selection to reduce Kleaf, or a strong evolutionary constraint imposed by the anatomical requirements of C4 photosynthesis. In other words, the high BS:M ratio required for an efficient C4 system may directly limit the ability of C4 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 (Amax) and hydraulic capacity (Kleaf) 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 Amax and K_{leaf} across many scales, from a broad phylogenetic spectrum of species spanning vascular plants (Brodribb et al. 2007), to smaller clades of closely related species (Scoffoni et al. 2016). 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 C4 photosynthesis with ages that span ~ 30 million years, grasses also present a unique opportunity to examine the influence of C4 evolution on Amax-Kleaf relationships. Here, we determined whether anatomical differences in C_4 grasses result in greater K_{leaf} and leaf capacitance compared to their corresponding C_3 relatives. We then compared these properties between closely related C3 and C4 clades to determine how C4 evolution alters the predicted Amax-Kleaf 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 C4 are represented by higher Kleaf and a greater Kleaf-Amax mismatch.

Results

Within each phylogenetic cluster, there were no clear patterns within C_3 and C_4 hydraulic traits by conducting ANOVA tests only. C_4 grasses had higher or equivalent K_{leaf}, leaf capacitance and leaf turgor loss point and lower or equivalent gs than their closest C_3 relatives (Fig. 1). The one C_3-C_4 intermediate species, *Steinchisma decipiens*, in our analysis had K_{leaf} similar or higher to C_4, but leaf capacitance, leaf turgor loss point and g_s equivalent to C_3 (Fig. 1).

By analyzing our data in the context of the evolutionary models (Table 1) and regression of hydraulic traits vs. lineage age (Fig. 2), however, we found clear C_3-C_4 differences that imply an interplay of anatomical precursors that initially increased K_{leaf} and leaf capacitance, and further evolutionary trends of decreasing K_{leaf} and leaf capacitance after the full C_4 CCM evolved. We first fitted evolutionary models of Brownian motion and Ornstein-Uhlenbeck processes to the hydraulic traits based on a reliable dated phylogenetic tree(Spriggs et al., 2014). The best fitting evolutionary model to the data for K_{leaf}, leaf turgor loss point 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 2, Supplementary Fig. S2, Supplementary Tables S3-S5). Higher K_{leaf}, lower leaf turgor loss point, and lower g_s are detected C_4 species compared to C_3 (LRT test, all $p<0.01$; all $\Delta AICc<-3$). For leaf capacitance, there is no significant difference for C_3 and C_4 species. Also, we extracted the evolutionary age for each C_4 lineages from the dated phylogeny. Regressions of evolutionary age versus hydraulic traits provide interesting evidence for their further evolutionary trends after C_4 evolved (Fig. 2). K_{leaf}, leaf turgor loss point and capacitance showed significant negative correlation with evolutionary age. There was no significant relationship between g_s, and the evolutionary age. The negative correlation between evolutionary age and K_{leaf} and leaf capacitance indicates that younger lineages have higher K_{leaf} and higher leaf capacitance and the older lineages have lower K_{leaf} and higher leaf capacitance. Such results indicated that at the initial origin of C_4, anatomical precursors lead to the higher K_{leaf} and higher leaf capacitance. After the full C_4 evolved, K_{leaf} and leaf capacitance started to decrease, the longer the lineages evolved as a C_4, the lower the K_{leaf} and leaf capacitance were.

In order to provide another level of support for the evolutionary trends and stable states mentioned above, we performed a secondary analysis. We extracted a phylogenetic tree with molecular branch length (Grass Phylogeny Working Group II, 2012). Except for the above six evolutionary models, we were able to fit additional six Brownian motion with trend models to the hydraulic traits to the phylogenetic tree with molecular branch length. Compared all the 12 different types of models, we found K_{leaf} and leaf capacitance could be best fitted by the Brownian motion model with trend (Table 2, Table S19-22). For K_{leaf}, there were significantly higher root states in C_4, which is consistent with Table 1, and a significantly negative evolutionary trend in C_4, which is consistent with Fig. 2a. For leaf capacitance, a decreasing evolutionary trend was detected in C_4, which support the above finding (Fig. 2b); however, a higher root state was also detected in C_4. The reason that such a higher root state was not detected in the first analysis could be that leaf capacitance increase at the initial formation of C_4 and, then, decreased rapidly to the similar values of C_3 compartment. Lower g_s and higher leaf turgor loss point in C_4 also confirmed the finding of the dated phylogeny analysis.

Since the hydraulic traits of evolutionary intermediate stage between C_3 and C_4 can never be obtained from fossil, we cannot estimate the evolutionary trend model with the dated tree. Thus, we attempted to use a molecular tree to fit the evolutionary model with a trend to provide a

secondary support to the evolutionary dynamics, although the molecular tree is less reliable. The molecular branch length represents a combination of the evolutionary age and the evolutionary rate (or mutation rate) for species. Thus, the potential meaning of detecting a negative evolutionary trends using a molecular tree in K_{leaf} and leaf capacitance could be that the quicker the evolutionary process and the longer the evolutionary time in C_4 species, the lower the K_{leaf} and leaf capacitance to reach an optimal operation in C_4. Although the molecular branch length was less reliable, together with the analysis using dated phylogenetic tree, we could obtain more robust results.

To see if C_4 subtypes varied in their root states and evolutionary rates or variance, we also considered evolutionary models where we allowed each variable to have a subtype-specific value (Table S1). Our analysis showed that stable states were not significantly different among subtypes. The best-fitted models for K_{leaf}, leaf capacitance, g_s, leaf turgor loss point and leaf elasticity with different parameters for C_4 subtypes did not fit significantly better than the model only considered C_3 and C_4 (all ΔAICc>0; Supplementary Tables S7-11). Although the C_4 subtypes evolved different enzymatic machinery to support the CCM, this does not seem to have affected the evolutionary direction of hydraulic traits. These results contrast what Liu and Osborne (2014) found in Chloridoideae, where NAD-ME grasses had more elastic leaves and PCK grasses had lower leaf turgor loss point. However, such differences were not apparent when we sampled C_4 subtypes from multiple lineages. Our sample size could still be limited, and in the future, it would be advantageous to increase both lineage and species diversity to more deeply examine C_4 subtypes.

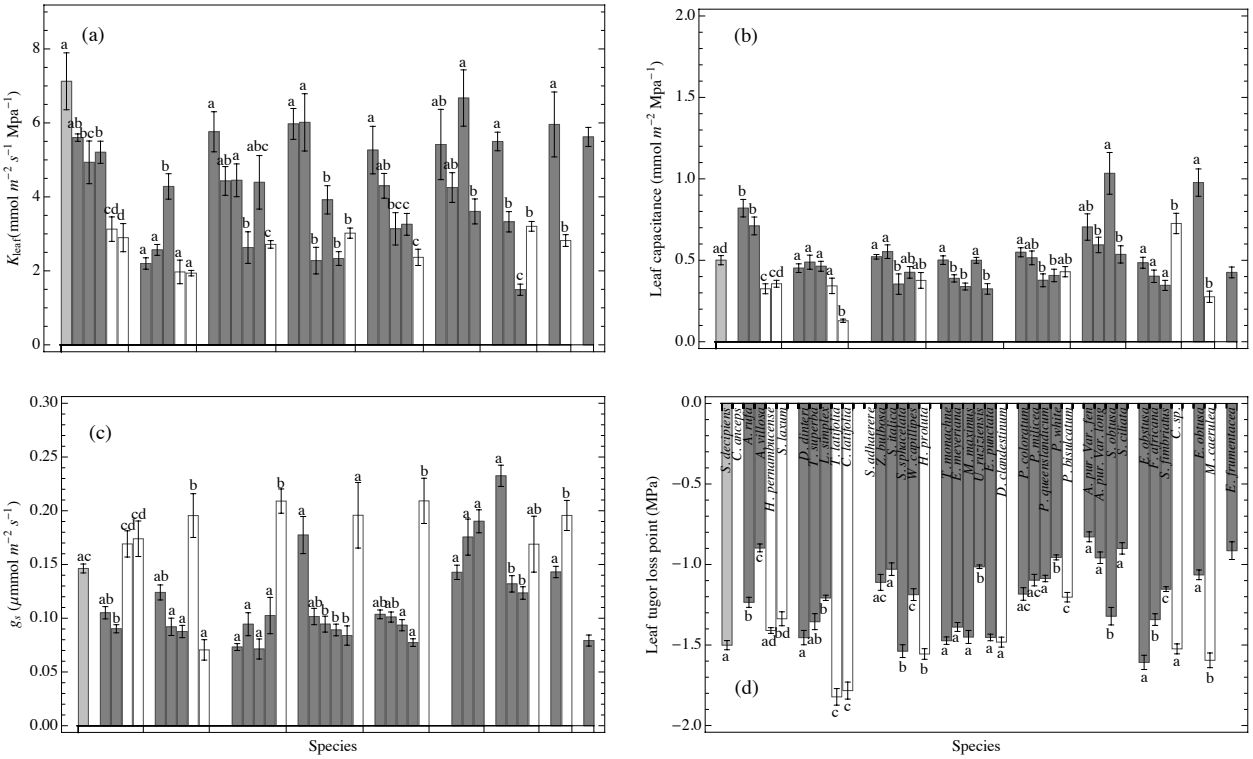

Fig. 1 Hydraulic conductance (K_{leaf}), leaf capacitance (CFT), maximal stomatal conductance (g_s) and leaf turgor loss points of closely related C_3 and C_4 species. Different clusters of bars show ten different origins of closely-related C_3 and C_4 species (Fig. S1). Small case letters indicate significant differences for the species within each cluster.

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-S5; model description: Table S1).

Property	Model	Model type	AICw	Root/Theta	
				C_3	C_4
K_{leaf}	Model 6*	OU	0.984	2.682	4.295
Capacitance	Model 2	BM	0.323	0.523	
g_s	Model 6*	OU	0.980	0.183	0.102
Turgor loss	Model 6*	OU	0.996	-1.522	-1.192

* indicates the model fit significantly better than all the other models. If the values for C_3 and C_4 were different, it meant there were significant different values for C_3 and C_4 species (the evolutionary model with two different values of the root or theta fits significantly better than the evolutionary model with the similar root or theta).

Fig. 2 The regression for hydraulic conductance (K_{leaf}), leaf capacitance, leaf turgor loss point, and stomatal conductance (g_s) vs. the evolutionary age for the ten origins of C_4 to show the evolutionary trend within C_4 further. The evolutionary age for each C_4 clusters is derived from the branch length of the C_3 ancestor to C_4 from the dated phylogeny of Spriggs et al. (2014). The evolutionary age indicates how long the species have evolved since the formation of C_4 photosynthesis pathway.

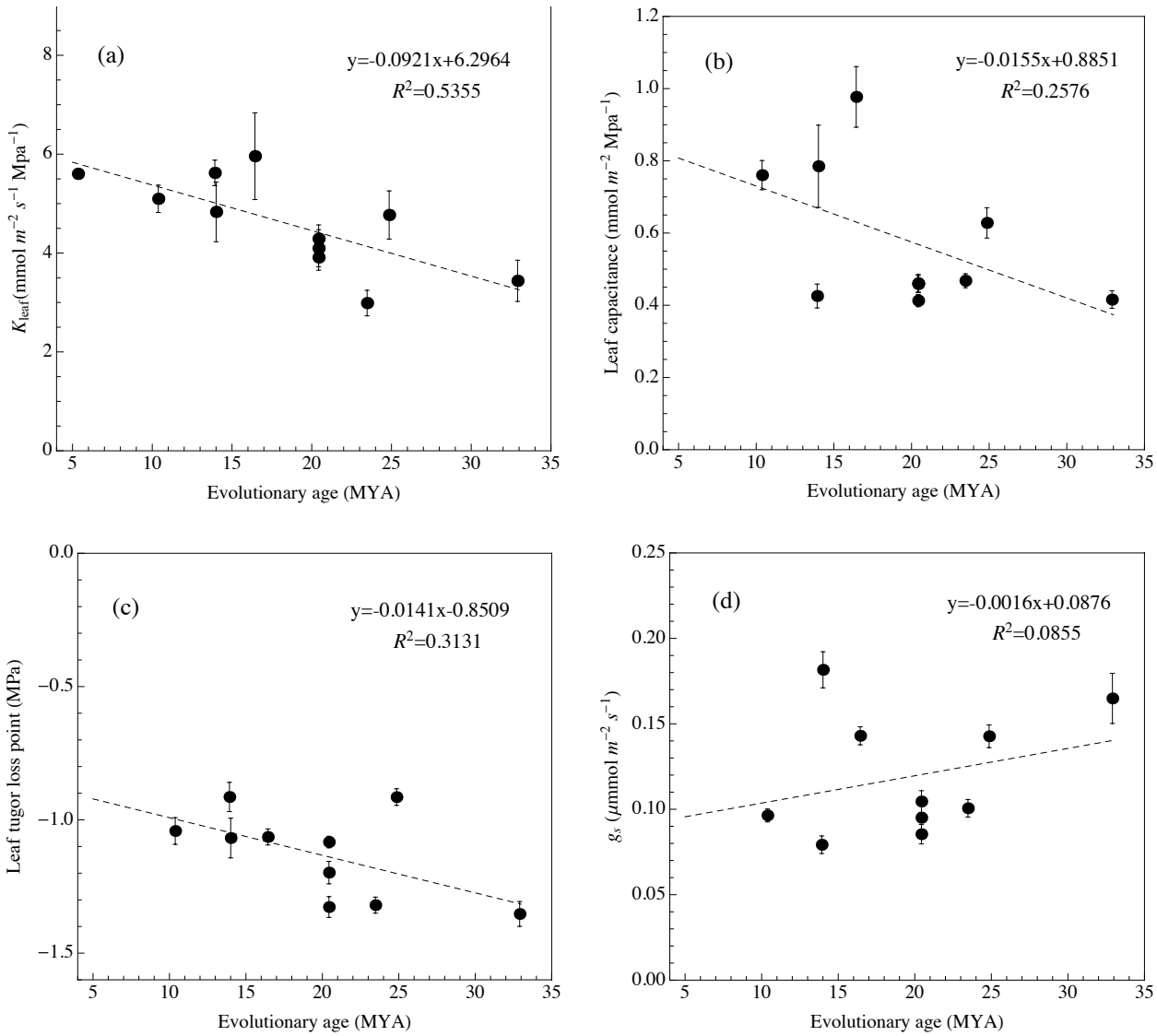

Table 2 Phylogenetic results of the best-fitted models and their parameters for hydraulic conductance (K_{leaf}), leaf capacitance (Capacitance), stomatal conductance (g_s), and leaf turgor loss point (Turgor loss) (summarizing Table S19-S22; model description: Table S18).

Property	Model	Model type	AICw	Trend		Root/Theta	
				C_3	C_4	C_3	C_4
K_{leaf}	Model 12*	BMT	0.849	1.53	-10.51	1.85	12.34
Capacitance	Model 11*	BMT	0.589	0.53	-0.81	0.097	1.18
g_s	Model 3*	BM	0.337	NA		0.19	0.12
Turgor loss	Model 6*	OU	0.975	NA		-1.52	-1.20

* indicates the model fit significantly better than all the other models. If the values for C_3 and C_4 were different, it meant there were significant different values for C_3 and C_4 species (the

evolutionary model with two different values of the root or theta or trend fits significantly better than the evolutionary model with the similar/no root or theta or trend).

We next explored how the maximum assimilation rate (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. 3, Table 3, Table S13). A_{max} was significantly positively correlated with K_{leaf} for both C_3 and C_4, but with a stronger correlation in C_3 species (Fig. 3, Table 3, Table S13). A_{max} was positively correlated with leaf capacitance and g_s and the correlations were similar for C_3 and C_4 (Fig. 3, Table 3, Table S14, S15). A_{max} was negatively, but not significantly related with leaf turgor loss point and leaf elasticity in C_3 and C_4 species (Table S16, S17).

Table 3 Phylogenetic Correlations between maximal assimilation rates (A_{max}) and other hydraulic traits for C_3 and C_4 species (summarizing Table S13-S16; model description: Table S12).

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/<0.001
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

p value indicated the comparison among models. A_{max} vs. K_{leaf}: compare best model with both model with one similar correlation model/no correlation if the best model estimate different correlation for C_3 and C_4 (if there are different correlations values for C_3 and C_4 and $p<0.05$, it meant there were significant different correlations for C_3 and C_4 species); A_{max} vs. Capacitance, g_s and leaf turgor loss: compare best model with no correlation (if there is one similar correlations values for C_3 and C_4 and $p<0.05$, it meant there were similar correlations for C_3 and C_4 species, the correlation is significant than 0).

We used our mechanism-based physiological model from Zhou et al. (2018) 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, S3). Changing K_{leaf} has a smaller effect on the photosynthesis rate of C_4 than that of C_3 (Fig. 4, 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_{cmax} 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 the reason why the higher g_s and leaf water potential reached in C_4 compared to closely related C_3 species that the CCM reduce the water demand for transpiration. Furthermore, because such a decrease in the water demand, a high efficient water supply system may not need to maintain the productivity, which results in decreasing K_{leaf}. The modeling

effects of varying K_{leaf} on photosynthesis confirmed a diminished returns of high efficiency of water transport in C_4 species mentioned above.

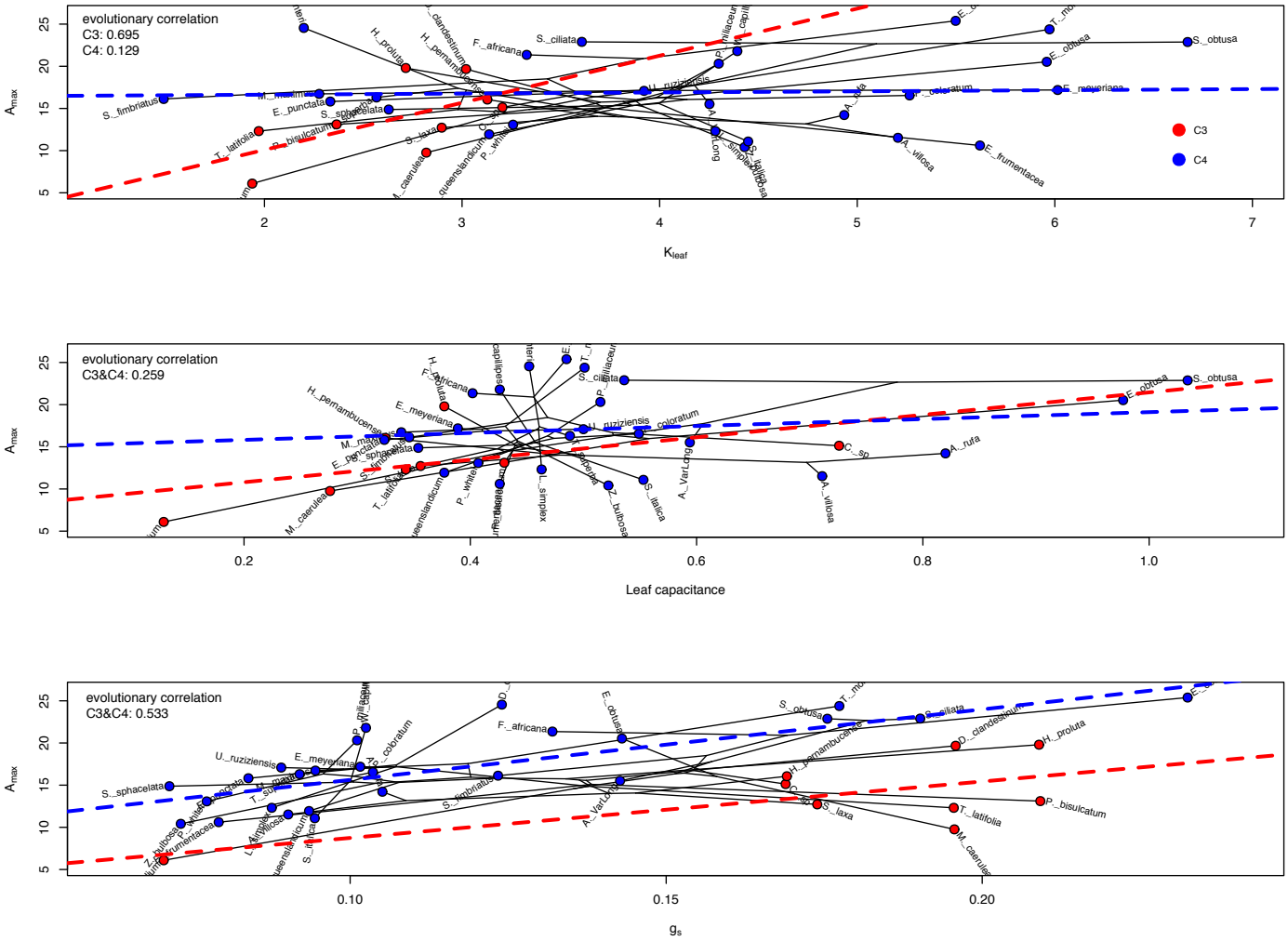

Fig. 3 Phylogenetic correlation for C_3 and C_4 between A_{max} and other hydraulic traits (K_{leaf}, leaf capacitance and g_s). Different/same correlation values on the figure mean C_3 and C_4 have significantly different/same correlations. Detailed phylogenetic correlation models and analysis results are shown in Table 3.

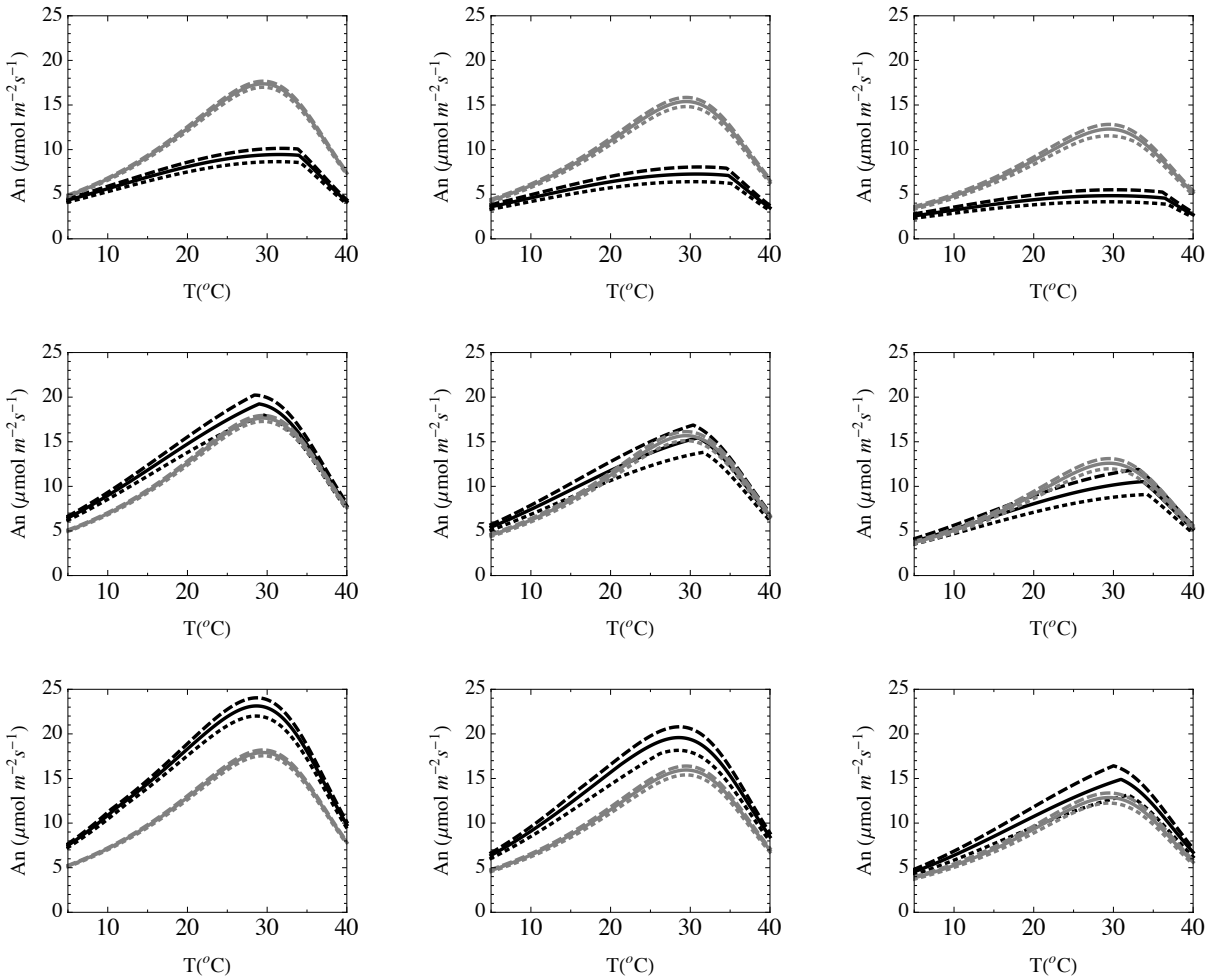

Fig. 4 Modeling results of photosynthesis rates along with different CO_2 concentration, different temperatures and different water limited conditions for C_3 (black lines) and for C_4 (grey lines). Solid lines: modeling results for C_3 and C_4 with the regular leaf hydraulic conductance; dashed lines: 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 the same except for C_4 has the carbon concentration mechanism. Upper row: CO2 of 200 ppm; middle row: CO2 of 400 ppm; lower row: CO2 of 600 ppm. Left column: water condition of VPD=0.625 kPa, ψ_s=-0.5 MPa; middle column: VPD=1.25 kPa, ψ_s =-1 MPa; right column water condition of VPD=1.875 kPa, ψ_s=-1.5 MPa.

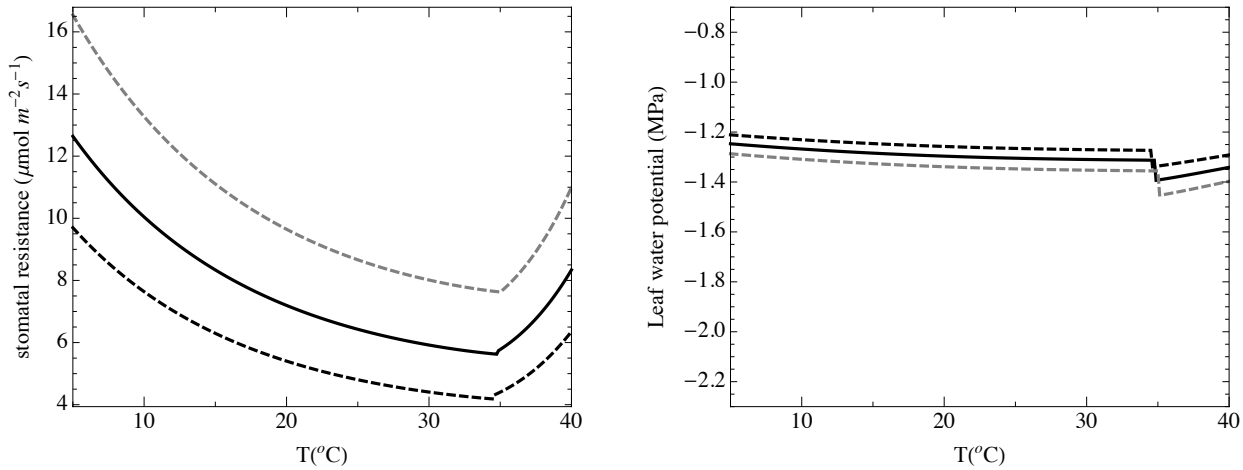

Fig. 5 The effect of changing K_{leaf} on stomatal resistance and leaf water potential under VPD=1.25 kPa, ψ_s =-1 MPa and CO_2 concentration of 200 ppm for the C_3 model. Solid black line: regular K_{leaf}; dashed black line: half regular K_{leaf}; dashed grey line: twice regular K_{leaf}.

Discussion

Our results demonstrate that evolution of the C_4 pathway in the grasses caused a variety shifts in hydraulic properties as compared to closely-related C_3 grasses, and allow us to speculate as to how these shifts occurred at different points along the evolutionary trajectory of C_4. 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 (Martre *et al.*, 2001; Ocheltree et al., 2016). Studies that have taken advantage of the newly-constructed grass phylogeny (Liu and Osborne, 2014; Liu et al., 2019) have assumed trait evolution as simple Brownian motion. Hydraulic traits, however, may have evolved along different trajectories before and after the evolution of the C_4 pathway and associated anatomical reorganization, resulting in non-monotonic evolutionary dynamics. Thus, the evolutionary dynamics of K_{leaf}, for example, could be concealed by the assumption of a simple Brownian motion process. We found that C_4 grasses had higher K_{leaf}, leaf capacitance, turgor loss point, and, as has been shown previously (Taylor et al., 2010; Taylor et al., 2012), a lower stomatal conductance (g_s) than corresponding C_3 grasses. Decreased vein distance and increased bundle sheath size are thought to be anatomical precursors to the evolution of C_4 (Sage, 2004; Christin et al. 2013), and both are thought to increase K_{leaf} and/or leaf capacitance (Osborne and Sack, 2012; Griffiths et al. 2012). Therefore, the shifts of K_{leaf} and leaf capacitance likely occurred before, or at the initial formation of, the C_4 CCM. After the full formation of C_4, K_{leaf} and/or leaf capacitance started to decrease, which led to higher or equivalent K_{leaf} and leaf capacitance in the current C_3 and C_4 species confirmed by our ANOVA analysis. Liu et al. (2019) found that K_{leaf} in C_4 grasses overlapped with the C_3. In contrast, the significant decrease of g_s and the increase of leaf turgor loss point occurred after the evolution of a fully operational C_4 CCM, as suggested by our model which shows increased K_{leaf} in C_3 plants selects for increased g_s. Consistent with this prediction, in genera that possess a range of C_3, C_3-C_4 intermediate and C_4 physiologies, the increased water use efficiency, decreased g_s, and a broadened ecological niche are observed only in plants with a full C_4 CCM (Lundgren et al., 2015; Sage et al. 2018).

Higher vein density is required for C_4 biochemical compartmentation (Griffiths et al. 2012), but we propose that the higher K_{leaf} that comes with higher vein density is an unsupportable cost. If anatomically-induced increases in K_{leaf} and leaf capacitance did indeed occur prior to the evolution of C_4 (Sage, 2001), and the subsequent evolution of C_4 led to lower g_s and higher turgor loss point, then we would predict that the selection on maintaining a high K_{leaf} and leaf capacitance would relax. Our analyses provides support to this prediction. K_{leaf} and leaf capacitance decreased in the C_4 species that either evolved C_4 first, based on the regression of lineage age with hydraulic traits, or evolved more rapidly after CCM evolution based on the evolutionary model for the molecular phylogeny. Our physiological model provides an explanation for this observation, in contrast to C_3 photosynthesis, changes in K_{leaf} had a small effect on photosynthesis in C_4. If one takes the potential cost of maintaining high K_{leaf} into consideration (Tyree and Sperry, 1989; Niinemets et al., 2007; McKown et al., 2010; Scoffoni et al., 2011; Wolf et al., 2016), then there would be little or no gain in maintaining high K_{leaf} in C_4. Additionally, each evolutionary origin of C_4 potentially represents both different selective pressures and taxonomic constraints as climate and CO_2 changed. C_4 photosynthesis first evolved in the grasses around 30 MYA, and many subsequent and independent evolutionary origins occurred well into the Pleistocene (Sage, 2004). CO_2 generally decreased through this period. Water limitation drove the earliest origin of C_4 30 MYA (Christin et al. 2008, Edwards and Smith 2010, Zhou et al. 2018) and there was a more intense selective pressure for water transport safety, which led to higher decrease of K_{leaf} in older lineages; however, the role played by water limitation was weakened and replaced by low CO_2 and high light as the CO_2 decreased with the multiple C_4 origins around 5-8 MYA (Zhou et al. 2018).

The results indicates the evolution of C_4 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 lost through open stomata, which is the likely cause of a positive correlation between K_{leaf} and A_{max} across and within plant taxa (Brodribb et al., 2005; Brodribb et al., 2007; Scoffoni et al., 2016). We found that A_{max} and K_{leaf} are positively correlated in both C_3 and C_4, but the strength of correlation is lower in C_4 species. We see three possible explanations for the weaker correlation in C_4 grasses that are not necessarily mutually exclusive. First, the positive relationship of A_{max} and K_{leaf} is weakened under high K_{leaf} (>10 or 15 mmol m^{-2} s^{-1} MPa^{-1}), possibly due to diminished returns of further increasing the efficiency of water transport (Brodribb et al., 2005; Brodribb et al., 2007). Such diminished returns was also supported by our physiological modeling results. K_{leaf} tends to be lower in grasses than in other species, and the evolution of C_4 provides such a significant benefit for water conservation that the diminishing returns from increasing K_{leaf} manifest at lower values. Second, Ocheltree et al. (2016), found no relationship between K_{leaf} and A_{max} in a set of nine C_4 species, and they propose that this decoupling of K_{leaf} and assimilation rate might be due to the evolution of tighter bundle sheath, which would increase photosynthesis capacity while decreasing water transport efficiency. 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 leaf capacitance vs. A_{max} and g_s vs. A_{max} in C_3 and C_4 indicated the evolutionary processes of leaf capacitance and g_s might be already near the optimal condition or stabilized.

By capitalizing on the many independent evolutionary events of C_4 photosynthesis in the grasses, we have shown that biochemical and vascular reorganization that are hallmarks of C_4 photosynthesis also impacted leaf hydraulics and hydraulics-photosynthesis relationships. We used alternative evolutionary models to identify the mode and direction of evolution of hydraulic traits in C_3 and C_4 lineages. Our analyses detected the effects of both anatomical precursors (evolutionary root states) and the subsequent selection pressures (evolutionary trends) in grass hydraulic systems. We found evidence that different hydraulic traits followed different evolutionary processes: leaf conductance and capacitance are more likely to evolve with decreasing trends supported by evidence of two analysis results, while leaf turgor loss point and stomatal conductance seemed to be stabilized soon after the CCM formation. Hydraulic conductance and leaf capacitance could therefore evolve 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. 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. 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 the root state of C_4 g_s was higher than the C_3 root state, 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 (Taylor et al., 2010; Ripley et al., 2013), yet Taylor et al. (2014) found that C_4 grasses maintained a higher or equivalent g_s to closely-related C_3 grasses. Likewise, artificial selection or genetic engineering might have more success in adjusting these hydraulic traits compared to leaf turgor loss point and elasticity. Our phylogenetic analyses can thus inform both the evolutionary history of C_4 plants and future efforts to modify C_4 crops.

Materials and 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 out of the 24 lineages (11 out of which have clear C_3 sister species) recommended for comparative studies in Grass Phylogeny Working Group II, 2012: *Aristida*, *Stipagrostis*, *Chloridoideae* (*Eragrostideae*), *Eriachne*, *Tristachyideae*, Arthropogoninae, *Otachyrinae (Anthaenantia)*, *Panicinae*, *Melinidinae*, and *Cenchrinae* (Fig. S1). 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 ºC; 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 (Sack and Scoffoni, 2012), 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 (gasket method from Troy Ocheltree, *personal communication*). 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 ^{o} 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_{leaf} values were further standardized to 25 ^{o}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 (Tyree and Hammel, 1972; Sack and Pasquet-Kok, 2010). 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).

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 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_{max} was estimated from the light curve (Marshall and Biscoe, 1980; Thornley, 1998). 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 (Spriggs et al., 2014). 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 (Clavel et al., 2015). 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) (Akaike, 1974; Cavanaugh, 1997; Burnham and Anderson, 1998). 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 and root states. 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 made 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, Grass Phylogeny Working Group II (2012). Except for the six evolutionary models mentioned above, the molecular phylogeny allows us to fit for Brownian motion models with trend (Table S17). 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. S5-S9).

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. In addition, ANOVA analysis is used within each cluster to test whether there are significant differences among C_4 species and their most closely-related C_3 species. 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 (Julien Clavel, *personal*

communication). Seven different correlation models are fitted (Table S2). 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 (Zhou et al., 2018). 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 S23) 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 S23, S24).

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