Mesophyll conductance response to short-term changes in CO₂ is related to leaf anatomy and biochemistry in diverse C₄ grasses

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Summary

- Mesophyll CO₂ conductance (g_m) in C₃ species responds to short-term (minutes) changes in environment potentially due to changes in some leaf anatomical and biochemical properties and due to measurement artifacts. Compared to C₃ species, there is less information about g_m responses to short-term changes in environment conditions like pCO_2 across diverse C₄ species and the potential determinants of these responses.
- Using 16 diverse C₄ grasses we investigated the response of g_m to short-term changes in CO₂ and how this response related to the leaf anatomical and biochemical traits.
- For all the measured C₄-grasses g_m increased as CO₂ decreased; however, the percent change in g_m varied (+13% to +250%) and significantly related to percent changes in leaf transpiration efficiency (TE_i). The percent increase in g_m was highest in grasses with thinner mesophyll cell walls and greater leaf nitrogen, activities of phosphoenolpyruvate carboxylase (PEPC), Rubisco and carbonic anhydrase, and a higher affinity of PEPC for bicarbonate.
- Our study demonstrates that CO₂ response of g_m varies greatly across diverse C₄ grasses and identifies the key leaf anatomical and biochemical traits related to this variation. These findings have implications for improving C₄ photosynthetic models, and in attempts to improve TE_i through manipulation of g_m .

Key words: C₄ photosynthesis, carbonic anhydrase (CA), CO₂ response of mesophyll conductance, leaf anatomy, mesophyll cell wall thickness, phosphoenolpyruvate carboxylase (PEPC), PEPC's affinity for bicarbonate (K_m), Rubisco activity, water-use efficiency

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48 **Introduction**

49 Mesophyll CO₂ conductance (g_m) describes the movement of CO₂ from substomatal cavities
 50 across the intercellular air space, cell walls and membranes to the site of first carboxylation. This
 51 carboxylation occurs in the mesophyll chloroplast in species with C₃ photosynthetic pathway and
 52 mesophyll cytosol in species with C₄ photosynthetic pathway (Evans & von Caemmerer, 1996).
 53 g_m varies across plant groups due to variation in leaf anatomy and biochemistry, changes
 54 dynamically in response to environmental stimuli and has a significant impact on the plant and
 55 ecosystem-level photosynthetic CO₂ uptake and water-use efficiency (Evans *et al.*, 2009; Flexas
 56 *et al.*, 2014; Knauer *et al.*, 2019b; Pathare *et al.*, 2020a). Despite its importance for
 57 photosynthetic CO₂ uptake and water-use at both plant and ecosystem levels and its variation
 58 across diverse plants groups, g_m is only beginning to be explicitly implemented into global
 59 models which upscale leaf-scale photosynthetic processes to canopy and global scales (Rogers *et*
 60 *al.*, 2017; Knauer *et al.*, 2019a; Knauer *et al.*, 2019b; von Caemmerer, 2021). The
 61 implementation of g_m is complicated because there is a lack of detailed information about
 62 responses of g_m to short and long-term changes in environmental conditions (e.g., light,
 63 precipitation, temperature, and CO₂ concentration) across diverse plant groups (Rogers *et al.*,
 64 2017; Knauer *et al.*, 2019a; Knauer *et al.*, 2019b). Most investigations on the responses of g_m to
 65 changes in environmental conditions focus on C₃ species (von Caemmerer & Evans, 2015; Xiong
 66 *et al.*, 2015; Carriqui *et al.*, 2018; Shrestha *et al.*, 2018), but there is less information about the
 67 response of g_m to changing environmental conditions in diverse C₄ species (Ubierna *et al.*, 2017;
 68 Kolbe & Cousins, 2018; Ubierna *et al.*, 2018; Sonawane *et al.*, 2021). A better understanding of
 69 how C₄- g_m responds to changing environmental conditions is essential for improving the models
 70 of C₄ photosynthetic at both the leaf and global scales (Rogers *et al.*, 2017; Knauer *et al.*, 2019a;
 71 Knauer *et al.*, 2019b; von Caemmerer, 2021) as well as for potentially increasing water-use
 72 efficiency of C₄ crops through manipulation of g_m (Cousins *et al.*, 2020; Pathare *et al.*, 2020a).

73 The influence of g_m on C₃ photosynthesis has been well studied for the past several years and
 74 there has been a significant understanding about C₃- g_m and its responses to changes in long-term
 75 growth conditions and short-term measurements conditions. In general, g_m varies greatly among
 76 diverse C₃ species and limits C₃ photosynthesis as much as g_{sw} (Flexas *et al.*, 2014; Muir *et al.*,
 77 2014; Barbour & Kaiser, 2016; Veromann-Jürgenson *et al.*, 2017). Variation in C₃- g_m is
 78 influenced by leaf ontogenic and anatomical traits like leaf development and senescence (Grassi

& Magnani, 2005; Barbour *et al.*, 2016), surface area of chloroplasts appressed to intercellular air space (S_c) (Tosens *et al.*, 2012; Peguero-Pina *et al.*, 2016), mesophyll cell wall thickness (M_{CW}) (Veromann-Jürgenson *et al.*, 2017; Ellsworth *et al.*, 2018; Evans, 2021) and leaf thickness (Flexas *et al.*, 2008; Muir *et al.*, 2014). In terms of responses to long-term growth conditions, C_3 - g_m is influenced by water stress, elevated CO_2 , salinity, nutrient supplement, and growth latitude (Flexas *et al.*, 2008; Momayyezi & Guy, 2017; Mizokami *et al.*, 2018; Shrestha *et al.*, 2018). Whereas rapid responses of g_m (within minutes) have been observed in response to short-term changes in leaf temperature, quantity and quality of light, relative humidity, and CO_2 concentrations (Hassiotou *et al.*, 2009; von Caemmerer & Evans, 2015; Xiong *et al.*, 2015), though not always (Loreto *et al.*, 1992; Tazoe *et al.*, 2009). However, there is no consensus on the exact cause of rapid responses of g_m to changes in environmental conditions like CO_2 .

Some studies suggest that rapid changes in g_m in C_3 plants can be explained by changes in chloroplast position and movement that could lead to short-term change in S_c (Oguchi *et al.*, 2005; Tholen *et al.*, 2008; Terashima *et al.*, 2011). Whereas mesophyll cell wall thickness (T_{CW}) and composition are considered invariable in short-term (minutes) and cannot explain the rapid responses of g_m (Evans *et al.*, 2009; Terashima *et al.*, 2011; Carriqui *et al.*, 2018). Alternatively, the rapid responses of C_3 - g_m have also been attributed to changes in activities of key photosynthetic enzymes like carbonic anhydrase (CA)- which catalyzes the conversion of CO_2 to HCO_3^- (Evans *et al.*, 2009; Momayyezi & Guy, 2017) and the facilitation effect of CO_2 -permeable aquaporins (Uehlein *et al.*, 2008; Flexas *et al.*, 2012; Kaldenhoff, 2012; Groszmann *et al.*, 2017), but see (Kromdijk *et al.*, 2019). Still, others suggest that rapid responses of C_3 - g_m could be the result of systematic methodological errors or the use of oversimplified models. These include mathematical dependency of g_m on other variables (like A_{net} and C_i) used to calculate it in the fluorescence method, neglecting the contribution of respiratory and photorespiratory CO_2 release to the total CO_2 pool in the leaf and inaccurate measurement of day respiration or estimates of the Rubisco fractionation factor in the $\Delta^{13}C$ method (Tholen & Zhu, 2011; Gu & Sun, 2014; Carriqui *et al.*, 2018; Ubierna *et al.*, 2019). The exact mechanism underlying the CO_2 responses of C_3 - g_m thus remains controversial. However, considerable evidence based on diverse species and methods of estimating g_m suggest that C_3 - g_m increases at low pCO_2 .

There has been a recent increase in research on the short- and long-term variability of g_m in diverse C_4 species and the leaf traits that could explain this variability (Barbour *et al.*, 2016;

Cano *et al.*, 2019; Pathare *et al.*, 2020a; Pathare *et al.*, 2020b). Our recent study demonstrated that, like C₃ species, g_m varies significantly among diverse C₄ grasses and has significant effects on photosynthetic rates and leaf water-use efficiencies (Pathare *et al.*, 2020a). This variation in C₄-g_m was correlated with leaf-level traits like leaf thickness, stomatal ratio, adaxial stomatal densities and S_{mes}. We also demonstrated that C₄ grasses adapted to low precipitation habitats exhibit traits related to greater g_m but lower leaf hydraulic conductance compared to grasses from habitats with relatively high precipitation (Pathare *et al.*, 2020b). These studies have advanced our understanding of the variability of g_m in C₄ grasses as well as how g_m is influenced by leaf-level traits and is affected by long-term growth conditions like precipitation. However, there is still a limited understanding of how g_m in diverse C₄ grasses responds to short-term changes in environmental conditions like CO₂. The few previous studies have largely focused on a few species like sorghum, maize and *Setaria* (Osborn *et al.*, 2017; Kolbe & Cousins, 2018; Ubierna *et al.*, 2018; Sonawane & Cousins, 2020). To the best of our knowledge, no studies to date have explored if g_m responses to short-term changes in pCO₂ vary among diverse C₄ grasses and which potential anatomical and biochemical traits could explain this variation in CO₂ response of C₄-g_m.

The overall objectives of the current study were (1) to investigate the response of g_m to short-term changes in pCO₂ in diverse C₄ species, (2) identify the anatomical and biochemical traits that may explain the variable CO₂ response of C₄-g_m, and (3) evaluate the impact of varying CO₂ responses of g_m on changes in photosynthesis and leaf water-use efficiency. To address above objectives, we estimated g_m under changing pCO₂ in 16 diverse C₄ grasses (see Pathare *et al.*, 2020a for details). Several methods have been used to estimate C₄-g_m. Pfeffer & Peisker (1998) calculated g_m from the initial slope of a photosynthetic CO₂ response curve and assumed no CO₂ dependence of g_m. However, C₄-g_m is sensitive to pCO₂ (Kolbe & Cousins, 2018; Ubierna *et al.*, 2018) and hence the initial slope method may be problematic. Using anatomical traits like S_{mes} for estimating C₄-g_m could also be subjected to errors due to assumptions made for values of T_{CW}, porosity, and membrane permeability (Pengelly *et al.*, 2010). The Δ¹³C *in vitro* V_{pmax} method (Ubierna *et al.*, 2017) estimates C₄-g_m by retrofitting models of C₄ photosynthesis model (von Caemmerer, 2000) and the Δ¹³C (Farquhar & Cernusak, 2012) with gas exchange, kinetic constants and *in vitro* PEPC activities. The Δ¹³C *in vitro* V_{pmax} method may also result in inaccurate estimates of C₄-g_m due to errors associated with estimation of *in vitro* PEPC and CA activities and enzyme kinetic parameters. The Δ¹⁸O method (Gillon & Yakir, 2000; Barbour *et*

al., 2016) utilizes simultaneous measurements of the oxygen isotope composition ($\delta^{18}\text{O}$) of transpired H_2O and oxygen isotope discrimination ($\Delta^{18}\text{O}$) in CO_2 to calculate the CO_2 concentration at the site of isotope exchange by CA. The $\Delta^{18}\text{O}$ method assumes full isotopic equilibrium between CO_2 and H_2O at the site of CA ($\theta = 1$), which may not always be true and hence g_m could be underestimated (Barbour *et al.*, 2016). In the current study we used the method described by (Ogee *et al.*, 2018) that builds upon the $\Delta^{18}\text{O}$ method by accounting for the possibility for an incomplete CO_2 - H_2O equilibrium inside leaves, the physical separation between mesophyll and bundle sheath cells in C_4 leaves and the contribution of respiratory fluxes.

We also investigated the relationship of leaf anatomical traits, previously known to influence C_4 - g_m (Pathare *et al.*, 2020a), with variable CO_2 response of g_m in the 16 C_4 grasses. Furthermore, we investigated the impacts of photosynthetic capacity on the CO_2 response of g_m in these C_4 grasses, through measurements of maximum photosynthetic rates ($A_{\max}$), leaf nitrogen content (N_{area}), activities of key enzymes of C_4 photosynthetic pathway like CA, phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and PEPC's affinity for its substrate (HCO_3^-). N_{area} is integral to the proteins of photosynthetic machinery (like PEPC, Rubisco and CA) which, along with the leaf structure, are responsible for drawdown of CO_2 inside the leaf (Parkhurst, 1994; Wright *et al.*, 2004; Evans *et al.*, 2009; DiMario *et al.*, 2018).

Material and methods

Plant growth

Sixteen C_4 grasses (Table 1) were selected for this study. In the graphs, each species is identified by four letter word which combines the first letter of genera and first three letters of species name. CO_2 response of g_m was not measured for two C_4 grasses, *Sporobolus airoides* Torr. and *Echinochloa esculenta* (A.Braun) H. Scholz that have been included in our previous studies (Pathare *et al.*, 2020a; Pathare *et al.*, 2020b). Details about growth conditions have been described in our previous study (Pathare *et al.*, 2020a). In short, plants were raised from seeds and grown in 3-L free drainage pots in a controlled environment growth chamber (model GC-16; Enconair Ecological Chambers Inc., Winnipeg, MB, Canada). The photoperiod was 14 h including a 2 h ramp at the beginning and end of the light period. Light and dark temperatures

were maintained at 26 and 22 °C, respectively. Light was provided by 400-W metal halide and high-pressure sodium lamps with maximum photosynthetic photon flux density (PPFD) of ca. 1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at plant height. One individual per species was grown per pot in a Sunshine mix LC-1 soil (Sun Gro Horticulture, Agawam, MA, USA) with 5-6 replicate pots per species. The plants were irrigated daily to pot saturation and fertilized twice a week with Peters 20-20-20 (2.5 g L⁻¹). Pots were randomized daily within the growth chamber.

Gas exchange measurements and estimation of g_m

The measurements of net photosynthetic rates (A_{net} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance to water vapor (g_{sw} , $\mu\text{mol water m}^{-2} \text{ s}^{-1}$), intercellular CO₂ concentrations (C_i , Pa), and mesophyll conductance to CO₂ (g_m , $\mu\text{mol m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$) were performed (Pathare *et al.*, 2020a) at four different CO₂ levels inside chamber (CO₂S) -34, 27, 20 and 14 Pa. Intrinsic water-use efficiency (TE_i , $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ water}$) was calculated as $A_{\text{net}}/g_{\text{sw}}$ for each CO₂ level. Maximum photosynthetic rates (A_{max} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) were measured at saturating light of ~1200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and $p\text{CO}_2$ of ~1500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$. For estimating g_m , isotopologs of CO₂ and H₂O were measured using the LI-6400XT infrared gas analyzer (LiCor, Lincoln, NE, USA) coupled to a tunable diode laser absorption spectroscopy (TDLAS, model TGA 200A, Campbell Scientific, Logan, UT, USA) and a cavity-ring down absorption spectroscopy (Picarro, Sunnyvale, CA, USA) as described previously (Ubierna *et al.*, 2017; Kolbe & Cousins, 2018; Pathare *et al.*, 2020a). The entire LI6400XT, the 2 cm x 6 cm leaf chamber (6400-11, Li-Cor), and LI-6400-18-RGB light source were placed in a growth cabinet (model EF7, Conviron; Controlled Environments Inc., MN, USA) with fluorescent lamps (F48T12/CW/ VHO; Sylvania, Wilmington, MA, USA) set at a PPFD of ~250 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and air temperature was maintained at 25 °C. g_m was estimated using the most recent method described by Ogee *et al.*, (2018) as discussed in Pathare *et al.*, (2020a). This method utilizes a newly developed model of C₄ photosynthetic discrimination that provides an estimate of the isotopic equilibration between CO₂ and H₂O inside the leaf and g_m . The CO₂ responses of g_m , g_{sw} , A_{net} and TE_i were measured by maintaining a mole fraction of CO₂ in the leaf chamber. For each species, four-point CO₂ response curve was performed. During measurements CO₂ sample (CO₂S) was set to about 34, 27, 20 and 14 Pa. During measurement at each CO₂ level, the leaves were allowed to adjust for at least 30 minutes or until stable values of A_{net} and g_{sw} were achieved. Data were subsequently collected and averaged over the next 20-30 min and the Li6400XT was set to log data only when

the TDLAS analyzed the sample line. Three to six biological replicates were measured per species.

Percent change in key physiological traits like A_{net} , g_{sw} , g_m and TE_i in response to decrease in CO_2S (from 34 to 14 Pa) was calculated as :

(mean trait value at 14 Pa- mean trait value at 34 Pa)*100/ (mean trait value at 34 Pa)

Measurement of anatomical traits and habitat mean annual precipitation

Light and electron microscopy techniques were used to measure important structural and anatomical traits like adaxial stomatal density (SD_{ada} , number mm^{-2}), abaxial stomatal density (SD_{aba} , number mm^{-2}), stomatal ratio (SR, unitless) expressed as ratio of $SD_{\text{ada}} : SD_{\text{aba}}$, leaf thickness (μm), mesophyll surface area exposed to intercellular air spaces (S_{mes} , $\mu\text{m}^2 \mu\text{m}^{-2}$) and mesophyll cell wall thickness (T_{CW} , μm). The details of sample preparation for light and electron microscopy and measurements are presented in Pathare *et al.*, (2020a). Light microscopy images of leaf cross sections were used to measure length of mesophyll cell walls exposed to intercellular air spaces (IAS) using 10-15 different fields of view for each leaf ($n = 3$ per species) taken at x 50 and x 100 magnifications. The S_{mes} was calculated from measurements of total length of mesophyll cell walls exposed to IAS and width of section analyzed using equation from Evans *et al.*, (1994) with curvature correction factor (F) of 1.34. T_{CW} was measured from TEM micrographs using at least 15 images for each leaf. Values for all the leaf anatomical traits used in current study have been published in Pathare *et al.*, 2020a. Values of mean annual precipitation (MAP) for habitats where the C_4 grasses commonly occur, were obtained as indicated previously in Pathare *et al.*, 2020b.

Enzyme assays and measurement of leaf nitrogen content

Immediately following gas exchange measurements, leaf samples were taken from the same leaf and frozen in liquid nitrogen for enzyme assays. Measurements of carbonic anhydrase (CA), phosphoenolpyruvate carboxylase (PEPC, $\mu\text{mol m}^{-2} \text{s}^{-1}$), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, $\mu\text{mol m}^{-2} \text{s}^{-1}$) activities were performed at 25°C as described previously (Sharwood *et al.*, 2016; Sonawane & Cousins, 2019; Pathare *et al.*, 2020a). CA activities were expressed as first order rate constant (k_{CA} , $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$). In addition to enzyme activities, PEPC's affinity for HCO_3^- (K_m , $\mu\text{M HCO}_3^-$) values were derived using the membrane-inlet mass spectrometer. K_m values have been published in Dimario *et al.*, 2021. For measuring leaf nitrogen content, leaf samples were taken from the same leaf on which gas

exchange measurements were performed. Samples were dried in hot air oven at 60 °C for 72 hours. Leaf nitrogen content was measured using a Eurovector elemental analyzer and expressed on leaf area basis (N_{area} , gm^{-2}).

Statistical analyses

Statistical analyses were performed using R software (v 4.1.0, R Foundation for Statistical Computing, Vienna, Austria). Normality and equal variances were tested and when necessary square root or log transformations were used to improve the data homoscedasticity (Zar, 2007). One-way ANOVA with post-hoc Tukey's test was used to examine differences in leaf-level physiological, anatomical, and biochemical traits among the 16 diverse C_4 grasses. Results for post-hoc Tukey's test are given in Table 1 and S1 to S4. For the one-way ANOVA, P -values ≤ 0.05 were considered as statistically significant. Results of one-way ANOVA for traits used in the current study are given in Table S1. In addition to the one-way ANOVA, Pearson correlation coefficients were calculated for relationships of percent change in physiological traits (A_{net} , g_{sw} , g_{m} and TE_i) with important anatomical and biochemical traits like T_{CW} , S_{mes} , SR, SD_{ada} , Leaf thickness, PEPC activity, Rubisco activity, k_{CA} , K_{m} , A_{max} and N_{area} . For the trait-to-trait correlations, P -values ≤ 0.05 were considered as statistically significant, whereas P -values ≤ 0.1 were considered as marginally significant.

To complement the trait-to-trait correlations, we also performed a principal component analysis (PCA), to identify the major axes of variation based on the important anatomical and biochemical traits and the percent change in physiological traits (Fig. S5, Table S2). The R package FactoMineR (Le *et al.*, 2008) was used to perform PCA. All traits were scaled during the analysis. The first three principal components (PC) had eigenvalues greater than one (Table S2) and were retained according to Kaiser's rule (Kaiser, 1960). For each trait, factor loadings greater than 0.5 in absolute value were considered important.

Results:

CO₂ response of physiological traits

The 16 C_4 grasses (listed in Table 1) with previously demonstrated variation in leaf-level anatomical traits and mesophyll conductance (Pathare *et al.*, 2020a; Pathare *et al.*, 2020b) were chosen to explore potential variation in CO_2 response of g_{m} and its relation to leaf anatomical and

biochemical traits as well as TE_i . Species differed significantly in CO_2 response of g_m (Fig.1 and Fig. S1) and g_{sw} (Fig. S2). In general, across the 16 C_4 grasses, g_m increased as CO_2S and C_i decreased (Fig.1 and S1) with percent increase at lowest C_i ranging from +13% to +250%. Like g_m , g_{sw} increased with decreasing CO_2S (Fig. S2). However, unlike g_m , the magnitude of increase in g_{sw} was lower. Specifically, the percent increase in g_{sw} ranged from about 40-80% when CO_2S decreased from 34 to 14 Pa. Alternatively, both A_{net} and TE_i decreased with decreases in CO_2S (Fig. S3 and S4). Particularly, A_{net} decreased by about 23-40% and TE_i decreased by about 53-64% when CO_2S decreased from 34 to 14 Pa.

Variation in leaf anatomical and biochemical traits

The results of one-way ANOVA suggest that the 16 C_4 grasses vary significantly ($p < 0.001$, Table 1 and S1, Pathare *et al.*, 2020a, DiMario *et al.*, 2021) in all the leaf-level anatomical and biochemical traits listed in Table 1. Mesophyll cell wall thickness (T_{CW}) showed a significant 2.6-fold variation with values ranging from about 0.08 to 0.21 μm . S_{mes} showed a 2.4-fold variation with values ranging from about 8.5 to 19 $\mu m^2 \mu m^{-2}$. The C_4 grasses measured here also showed a highly significant variation in stomatal traits like SR and SD_{ada} ($p < 0.001$; Table 1 and S1, Pathare *et al.*, 2020a). SR varied by 4.7-fold, with values ranging from 0.5 to 2.4, whereas SD_{ada} showed a 13-fold variation with values ranging from 12 to 160 number mm^{-2} . Leaf thickness showed a significant 2-fold variation with values ranging from 120 to 240 μm ($p < 0.001$, Table 1 and S1). Similar to the variation in anatomical traits mentioned above, the biochemical traits (that is leaf-level activities of PEPC, Rubisco and k_{CA}) also varied significantly among the 16 C_4 grasses included in the current study ($p < 0.001$, Table 1 and S1). PEPC activities showed a 7.5-fold variation with values from 96 to 721 $\mu mol m^{-2} s^{-1}$. Rubisco activities showed a 2.6-fold variation with values from 30 to 80 $\mu mol m^{-2} s^{-1}$. k_{CA} showed an 18-fold variation with values from 9 to 165 $\mu mol m^{-2} s^{-1} Pa^{-1}$. PEPC's affinity for HCO_3^- (K_m) showed a 1.6-fold variation across the 16 C_4 grasses with values ranging from around 29 to 46 $\mu M HCO_3^-$ ($p < 0.001$, Table 1 and S1, DiMario *et al.*, 2021). There was a 1.7-fold variation in maximum photosynthetic rates (A_{max}) with values ranging from ~25 to 42.5 $\mu mol CO_2 m^{-2} s^{-1}$ ($p < 0.001$, Table 1). N_{area} also varied significantly among the grasses ($p < 0.001$, Table 1) with values ranging from 1.24 to 2.26 gm^{-2} (Pathare *et al.*, 2020a).

Relationship of CO_2 response of g_m with CO_2 response of TE_i , g_{sw} and A_{net}

We investigated the impacts of changes in g_m with pCO_2 on corresponding changes in TE_i , g_{sw} and A_{net} (Fig. 2). For this we calculated percent change in the trait's value when CO_2S decreases from 34 to 14 Pa. Since g_m and g_{sw} increase with decreases in CO_2S , percent change for both conductances is reported as percent increase in the manuscript (Figs. 2-5). Alternatively, A_{net} and TE_i decrease with decreases in CO_2S , thus the percent change for these two traits have been reported as percent decrease in the manuscript (Fig. 2 and S6-S8).

Percent change in TE_i showed a strong negative correlation with percent change in g_m ($r = -0.74$, $p < 0.01$, Fig. 2a). Particularly, C_4 grasses which were able to achieve a greater increase in g_m at lower CO_2S (for example, udic and eari) showed a lesser decrease in TE_i (indicated by a less negative value for TE_i in Fig. 2a). Whereas, percent change in g_{sw} showed a strong negative relationship with percent change in g_m ($r = -0.57$, $p = 0.019$, Fig. 2b), that is, species which showed a greater increase in g_m at lower CO_2S also showed a lesser increase in g_{sw} . For example, species eari showed the greatest increase in g_m (250 %) but the lowest increase in g_{sw} (40%). A significant relationship was not observed between percent change in g_m and A_{net} (Fig. 2c).

Relationships of CO_2 response of physiological traits with anatomical and biochemical traits

Relationship of percent change in g_m with anatomical and biochemical traits are shown in Figs. 3 and 4. Percent change in g_m was negatively related to T_{CW} ($r = -0.60$, $p = 0.01$, Fig. 3a), that is, species with thinner cell walls showed greater increases in g_m at lower CO_2S . Whereas, percent change in g_m showed a statistically significant positive relationship with SR ($r = 0.54$, $p = 0.03$, Fig. 3c) and leaf thickness ($r = 0.52$, $p < 0.05$, Fig. 3e) and a non-significant but positive relationship with S_{mes} ($r = 0.25$, $p > 0.1$, Fig. 3b) and SD_{ada} ($r = 0.34$, $p > 0.1$, Fig. 3d). Percent change in g_m also showed a non-significant but positive relationship with biochemical traits like PEPC activity ($r = 0.29$, $p > 0.1$, Fig. 4a) and K_m ($r = 0.26$, $p > 0.1$, Fig. 4d) and a significant positive relationship with Rubisco activity ($r = 0.46$, $p = 0.07$, Fig. 4b) and k_{CA} ($r = 0.42$, $p = 0.1$, Fig. 4c).

To account for effects of anatomical traits, particularly T_{CW} which was strongly related to CO_2 response of C_4 g_m , and biochemical traits on CO_2 response of g_m , we derived the ratio of T_{CW} with PEPC activity, Rubisco activity, k_{CA} , K_m , A_{max} and N_{area} and investigated the relationship of percent change in g_m with these ratios (Fig. 5). Percent change in g_m showed significant negative relationship with $T_{CW}/PEPC$ ($r = -0.52$, $p = 0.05$, Fig. 5a), $T_{CW}/Rubisco$ ($r = -0.59$, $p < 0.05$, Fig. 5b), T_{CW}/K_m ($r = -0.55$, $p < 0.05$, Fig. 5c) and T_{CW}/k_{CA} ($r = -0.47$, $p = 0.06$, Fig. 5d). In

summary, C_4 grasses with thinner cell walls and greater enzyme activities or K_m were able to achieve greater increase in g_m at lower CO_2S . Also, in contrast to percent change in g_m , percent change in g_{sw} was positively related to T_{CW} and negatively related to enzyme activities (as indicated by PCA Fig. S5 and Table S1). Thus, grasses with lower T_{CW} but greater A_{max} , N_{area} , enzyme activities and greater values for K_m showed a lesser increase in g_{sw} at lower CO_2S .

Like percent change in g_m , we also investigated the relationship of percent change in TE_i with above mentioned anatomical (Fig. S6), biochemical traits (Fig. S7) and ratios of T_{CW} with biochemical traits (Fig. S8). Percent change in TE_i showed a statistically significant positive relationship with T_{CW} ($r = 0.68$, $p < 0.01$, Fig. S6a). Whereas percent change in TE_i showed a statistically significant negative relationship with SR ($r = -0.41$, $p = 0.1$, Fig. S6c) and a non-significant negative relationship with SD_{ada} ($r = -0.40$, $p > 0.1$, Fig. S6d). In terms of its relationship with biochemical traits, percent change in TE_i showed a non-significant negative relationship with PEPC activity ($r = -0.16$, $p > 0.1$, Fig. S7a), Rubisco activity ($r = -0.21$, $p > 0.1$, Fig. S7b), k_{CA} ($r = -0.27$, $p > 0.1$, Fig. S7c) and significant negative relationship with K_m ($r = -0.48$, $p = 0.05$, Fig. S7d). Furthermore, percent change in TE_i showed a significant positive relationship with $T_{CW}/PEPC$ ($r = 0.46$, $p = 0.07$, Fig. S8a), $T_{CW}/Rubisco$ ($r = 0.62$, $p = 0.01$, Fig. S8b), T_{CW}/K_m ($r = 0.67$, $p < 0.01$, Fig. S8c) and T_{CW}/k_{CA} ($r = 0.60$, $p = 0.013$, Fig. S8d). In summary, C_4 grasses with thinner cell walls but greater SR , leaf thickness, S_{mes} , SD_{ada} , enzyme activities and K_m showed a lesser decrease in TE_i at lower CO_2S .

Principal Component Analysis

To complement the trait-to-trait correlations, we also performed a PCA using the important leaf-level anatomical and biochemical traits and percent changes in g_m , A_{net} , g_{sw} and TE_i and mean annual precipitation. The PCA was performed on leaf traits from 16 C_4 grasses, where the first four axes with eigenvalues ≥ 1 were retained for analysis (Table S2). The first two major axes (PC1 and PC2) of leaf trait variation along with the average position of C_4 grasses in PC1-PC2 space are presented in Figure S5. PC1 explains about 44 % of total variation in the C_4 grasses and has a positive association with traits like SD_{ada} , S_{mes} , SR , leaf thickness, PEPC activity, Rubisco activity, k_{CA} , A_{max} , N_{area} and percent change in g_m and TE_i . Whereas PC1 showed negative association with T_{CW} and percent change in g_{sw} and mean annual precipitation. Thus, PC1 delineates the C_4 grasses into those with greater CO_2 response of g_m (higher PC1; greater enzyme activity and lower T_{CW}) and those with lower CO_2 response of g_m (lower PC1; lower enzyme activity and greater T_{CW}). Alternatively, PC2 explains about 18 % of the total variation

in the C_4 grasses and is positively associated with K_m and percent change in A_{net} and TE_i and negatively associated with T_{CW} and k_{CA} . PC2 tends to separate the C_4 grasses into those showing greater K_m values, lower T_{CW} and lesser decrease in A_{net} and TE_i at low CO_2S .

Discussion

Changes in g_m in response to CO_2 have been well studied for C_3 species. In general C_3 - g_m increases under short-term decreases in pCO_2 (Flexas *et al.*, 2007; Bunce, 2010; Douthe *et al.*, 2011), though not always (von Caemmerer & Evans, 1991; Loreto *et al.*, 1992; Tazoe *et al.*, 2009). The main candidates suggested to affect the CO_2 response of C_3 - g_m include chloroplast movement and hence changes in Sc , changes in activities of carbonic anhydrase (Evans *et al.*, 2009; Momayyezi & Guy, 2017) and the facilitation effect of CO_2 -permeable aquaporins (Uehlein *et al.*, 2008; Flexas *et al.*, 2012; Kaldenhoff, 2012). However, the C_3 - g_m response to pCO_2 may also result from systematic errors associated with use of methods like gas exchange, chlorophyll fluorescence and discrimination against ^{13}C or the use of oversimplified models (Pons *et al.*, 2009; Yin & Struik, 2009; Tholen & Zhu, 2011; Gu & Sun, 2014). For instance, the apparent response of C_3 - g_m to short-term changes in pCO_2 has partly been attributed to changes in photorespiratory and non-photorespiratory release of CO_2 to the total CO_2 pool in the leaf particularly under low pCO_2 . Ignoring these CO_2 pools while estimating C_3 - g_m also overlooks the effects of spatial distribution of mitochondria and chloroplast on pathlength of CO_2 movement. Inaccurate measurements of day respiration or estimates of the Rubisco fractionation factor in the $\Delta^{13}C$ method and measurements conducted at low $[O_2]$ have also been suggested as potential sources of artefacts in determining the C_3 - g_m response to pCO_2 (Pons *et al.*, 2009; Gu & Sun, 2014).

Alternatively, in C_4 species, the photorespiratory and non-photorespiratory release of CO_2 is relatively low and may not contribute significantly to estimates of g_m even at low pCO_2 (Cousins *et al.*, 2020). Also, in current study we estimated g_m in diverse C_4 grasses by using a method based on modelling of $\Delta^{18}O$ that considers the contribution of respiratory fluxes (Ogee *et al.*, 2018). Comparing many diverse C_4 species, as done in current study, using a method that is not subject to same limitations as those previously used for C_3 species, is a reasonable approach to identify CO_2 responses of C_4 - g_m . Here, we discuss the CO_2 response of g_m for 16 diverse C_4 grasses and its relationships with leaf anatomical and biochemical traits.

CO₂ response of g_m varied among diverse C₄ grasses

Very few studies have investigated the CO₂ response of g_m in C₄ species, primarily maize and *Setaria viridis* (Osborn *et al.*, 2017; Kolbe & Cousins, 2018; Ubierna *et al.*, 2018). In general, these studies reported an increase in C₄- g_m with a decrease in pCO_2 . Similarly, we demonstrate that g_m increases with a decrease in pCO_2 across the 16 diverse C₄ grasses. However, compared to previous studies, the magnitude of increase in g_m varied greatly across the 16 C₄ grasses (+13% to +250%; Fig. 1). In the following sections we discuss the potential factors that could explain this variability in CO₂ response of C₄- g_m .

CO₂ response of C₄- g_m is related with T_{CW} and photosynthetic capacity

g_m in C₃ and C₄ species is constrained by several anatomical and biochemical parameters like leaf thickness, stomatal density, T_{CW} , S_{mes} , S_c , CA activities and aquaporins (Cousins *et al.*, 2020; Evans, 2021). However, the potential implication of leaf anatomy and biochemistry for CO₂ response of g_m has not been studied for C₄ species. Most of the anatomical parameters remain unchanged under short-term changes in environmental conditions like pCO_2 (Evans *et al.*, 2009; Terashima *et al.*, 2011). Hence, the only anatomical trait suggested to influence CO₂ response of g_m is chloroplast movement which can affect g_m by changing S_c (Terashima *et al.*, 2006; Tholen *et al.*, 2008; Carriqui *et al.*, 2018). However, chloroplast movement is unlikely to explain the variability of CO₂ response of C₄- g_m observed in the current study, because S_{mes} and not S_c is a more accurate determinant of C₄- g_m (Barbour *et al.*, 2016; Pathare *et al.*, 2020a). In contrast to S_c , S_{mes} should remain unchanged under short-term changes in pCO_2 . Hence, though we observed a positive relationship between CO₂ response of C₄- g_m and S_{mes} (Fig. 3b), S_{mes} may provide only a partial explanation for this variable response. Other anatomical traits like stomatal density and leaf thickness also showed a positive relationship with CO₂ response of C₄- g_m . However, like S_{mes} , stomatal density and leaf thickness will also remain unchanged under short-term changes in pCO_2 and may provide only a partial explanation for variable CO₂ response of C₄- g_m .

T_{CW} could account for >50% of the total resistance to CO₂ diffusion inside leaves (Evans *et al.*, 2009). Hence, we investigated the influence of T_{CW} on variability of CO₂ response of C₄- g_m . In general, C₃ species with relatively lower T_{CW} show greater g_m (Onoda *et al.*, 2017; Veromann-Jürgenson *et al.*, 2017; Evans, 2021). We did not observe a strong relationship between g_m and T_{CW} for the C₄ grasses under ambient CO₂ levels (34 Pa) (Pathare *et al.*, 2020a). However, the

CO₂ response of C₄-g_m was related to T_{CW} in current study (Fig. 3a). Particularly, C₄ grasses with relatively lower T_{CW} showed greater increase in g_m at low pCO₂. It's unclear why g_m of C₄ grasses with lower T_{CW} is more responsive to changes in pCO₂. Like other anatomical traits, T_{CW} is unlikely to change under short-term changes in pCO₂ and cannot be the reason for observed variable CO₂ response of C₄-g_m. However, T_{CW} may still provide partial explanation for variability in CO₂ response of C₄-g_m. Resistances to CO₂ diffusion through liquid phase are greater compared to gaseous phase (Evans *et al.*, 2009). CO₂ must dissolve in the water-filled pores of the mesophyll cell walls and then diffuse to the plasma membrane and eventually to the site of CO₂ fixation. Because cell walls represent a significant proportion of liquid phase resistance (Evans *et al.*, 2009), C₄ species with lower T_{CW} may have the potential to achieve a greater change in g_m in response to changing pCO₂. However, the influence of lower T_{CW} on variability of CO₂ response of C₄-g_m may also be augmented by the facilitation effect of CO₂ transporting aquaporins and proteins of photosynthetic machinery that are involved in drawdown and fixation of CO₂ (Parkhurst, 1994; Wright *et al.*, 2004; Evans *et al.*, 2009).

The role of CO₂-permeable aquaporins in enhancing g_m has been well characterized in C₃ species (Uehlein *et al.*, 2008). Only recently, it has been demonstrated that overexpressing a CO₂-permeable aquaporin in plasma membrane of *Setaria viridis* (C₄ grass), can enhance C₄-g_m (Ermakova *et al.*, 2021). We did not investigate the role of aquaporins in affecting CO₂ response of C₄-g_m. However, we observed a greater CO₂ response of g_m in C₄ grasses with relatively lower T_{CW}. This indicates that g_m in plants with lower T_{CW} may be more influenced by aquaporins (Evans, 2021). There is still a need to further investigate role of aquaporins in variable CO₂ response of C₄-g_m.

Here, we investigated the relationship between CO₂ response of C₄-g_m and photosynthetic capacity as indicated by A_{max}, N_{area} and activities of key C₄ photosynthetic enzymes (PEPC, CA and Rubisco). CO₂ response of C₄-g_m showed a positive, but non-significant, relationship with A_{max}, N_{area} and PEPC, Rubisco and CA activities (Fig. 4). Thus, photosynthetic capacity alone is not strongly related to CO₂ response of C₄-g_m. Instead, greater photosynthetic capacity in combination with relatively lower T_{CW} are associated with a greater CO₂ response of C₄-g_m (Fig. 5). Lower T_{CW} may have decreased the resistance to movement of CO₂ into the mesophyll cells (Evans, 2021; Flexas *et al.*, 2021). Whereas greater photosynthetic capacity may have increased the demand for CO₂ and hence the necessity of maintaining a greater CO₂ supply through an increase in g_m at low pCO₂ (Wright *et al.*, 2004; Evans *et al.*, 2009). Furthermore, the enzyme

CA catalyzes the conversion of $\text{CO}_2/\text{HCO}_3^-$ in the cytosol and ensures sufficient HCO_3^- substrate supply to PEPC (Studer *et al.*, 2014; DiMario *et al.*, 2018). Because CO_2 diffuses much faster in liquid phase in HCO_3^- form, greater CA activities could have maintained a rapid supply of HCO_3^- substrate to PEPC and further enhanced g_m at low $p\text{CO}_2$ in C_4 grasses with lower T_{CW} .

We also investigated the relationship between the CO_2 response of $\text{C}_4\text{-}g_m$ and K_m - a kinetic constant indicating PEPC's affinity for HCO_3^- (DiMario *et al.*, 2021). In general, lower K_m values (or high affinity of PEPC for HCO_3^-) are expected to provide a selective advantage by maintaining high rates of C_4 photosynthesis, particularly under conditions like drought when CO_2 availability is low due to restricted stomatal conductance. Here, we observed that K_m alone did not show a strong relationship with CO_2 response of $\text{C}_4\text{-}g_m$ (Fig. 4d). However, after accounting for T_{CW} , we observed that T_{CW}/K_m shows a significant negative relationship with CO_2 response of $\text{C}_4\text{-}g_m$ (Fig. 5c), wherein, species with relatively lower T_{CW} and higher K_m (or lower affinity of PEPC for HCO_3^-) exhibited a greater CO_2 response of g_m (Fig. 5c). This contrasts the general expectation and could be explained by the lower T_{CW} leading to higher g_m and with the corresponding greater CA activities lead to higher HCO_3^- in mesophyll cells under low C_i conditions. The higher HCO_3^- concentration in the mesophyll cells of species with lower T_{CW} and greater CA can reduce the selective pressure on PEPC for lower K_m values.

Relationship of CO_2 response of g_m with CO_2 response of g_{sw} and TE_i

Previously, we demonstrated that $\text{C}_4\text{-}g_m$ is positively related to TE_i under ambient $p\text{CO}_2$ (Pathare *et al.*, 2020a). Our current study further suggests that for C_4 grasses, g_m may also influence TE_i under short-term changes in $p\text{CO}_2$. In general, both g_m and g_{sw} increased (Fig. 1 and S2) and TE_i decreased at low $p\text{CO}_2$ (Fig. S4). However, the magnitude of increase in g_m at low $p\text{CO}_2$ was greater (values from 13% to 250%) compared to increase in g_{sw} (values from 40% to 80%). Also, C_4 grasses showing greatest increase in g_m at low $p\text{CO}_2$ also showed the lowest increase in g_{sw} (Fig. 2b). Consequently, though TE_i decreased at low $p\text{CO}_2$, the decrease was less in the species showing greater CO_2 response of g_m (Fig. 2a). Such species with greater CO_2 response of g_m could have a particular advantage, in terms of maintaining TE_i under low CO_2 conditions like drought, compared to species whose g_m was less responsive to changes in $p\text{CO}_2$. Indeed, we observed that C_4 grasses with a greater CO_2 response of g_m are generally adapted to relatively drier habitats (Fig. S5).

Conclusions

We demonstrated that C_4 - g_m increases with decrease in pCO_2 and the magnitude of this increase in g_m varies greatly among the 16 diverse C_4 grasses. Also, CO_2 response of C_4 - g_m is a composite trait that seems to be influenced by many leaf anatomical and biochemical parameters. The greatest increase in g_m at low pCO_2 was observed in C_4 grasses with lower T_{CW} and greater photosynthetic capacities. These C_4 grasses with a greater CO_2 response of g_m were also able to maintain their TE_i under low pCO_2 , which may be advantageous under low CO_2 conditions like drought. Our study advances understanding about CO_2 response of g_m in diverse C_4 species and identifies the key leaf anatomical and biochemical traits related to this response. This understanding is essential for improving C_4 photosynthetic models (von Caemmerer, 2021) and in attempts to improve water-use efficiency of C_4 crops through modification of g_m (von Caemmerer & Furbank, 2016).

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Author contributions

V.S.P and A.B.C designed the experiment. V.S.P, R.J.D and N.K performed the measurements and analyzed the data. V.S.P interpreted the data and led the writing with constructive inputs from R.J.D, N.K and A.B.C.

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Table 1: Mean $\pm$ SE ($n = 3$ to 6) values along with the corresponding letters of post-hoc Tukey's test for important leaf-level anatomical and biochemical traits measured for 16 C_4 grasses. Species code was created using first letter of genera and first three letters of species. Results of one-way ANOVA are given in Table S1. Values for T_{CW} , S_{mes} , SR, SD_{ada} , Leaf thickness, k_{CA} and N_{area} have been published in Pathare *et al.*, 2020a, whereas values for K_m have been published in DiMario *et al.*, 2021.

Species	Species code	T_{CW}	S_{mes}	SR	SD_{ada}	Leaf thickness	PEPC activity	Rubisco activity	k_{CA}	K_m	A_{max}	N_{area}
<i>Chloris gayana</i>	cgay	0.09 ± 0.012 a	9.3 ± 0.5 bcf	0.62 ± 0.08 c	106.2 ± 6.74 fhi	153 ± 9.4 bc	112 ± 13 bf	32 ± 3 bh	19 ± 1 cdeg	29 ± 0.8 ad	35.6 ± 1 abc	1.81 ± 0.16 abcd
<i>Danthoniopsis dinteri</i>	ddim	0.11 ± 0.005 ab	18.6 ± 1.3 a	1.45 ± 0.08 ab	142.9 ± 4.5 abc	241 ± 1.7 a	156 ± 8 bc	81 ± 4 a	49 ± 4 ab	33 ± 1.4 abcd	42.4 ± 1.1 a	2.25 ± 0.07 a
<i>Digitaria sanguinalis</i>	dsan	0.11 ± 0.009 abcd	8.9 ± 0.6 bc	0.52 ± 0.02 c	40.4 ± 3.26 de	155 ± 8.3 bc	721 ± 33 a	30 ± 1 b	13 ± 2 cde	44 ± 1.6 ef	33.2 ± 2.8 bcde	1.5 ± 0.09 bcd
<i>Eriachne aristidea</i>	eari	0.08 ± 0.004 a	13.1 ± 0.2 de	2.33 ± 0.11 d	130 ± 5.53 abf	216 ± 3.6 ad	348 ± 46 de	79 ± 4 ac	74 ± 6 af	36 ± 1.1 abceg	37.7 ± 2.2 abc	2.22 ± 0.09 a
<i>Echinochloa colona</i>	ecol	0.11 ± 0.006 abc	10 ± 0.3 bcdef	0.82 ± 0.02 ce	68.7 ± 4.17 dg	168 ± 3.5 bef	408 ± 11 de	50 ± 2 de	72 ± 1 af	46 ± 2.1 f	42.5 ± 0.9 a	1.71 ± 0.03 abcd
<i>Eragrostis curvula</i>	ecur	0.17 ± 0.03 cdef	10.6 ± 0.6 bcdef	1.09 ± 0.07 ef	97.8 ± 7.39 hi	176 ± 17.9 bdef	97 ± 14 f	43 ± 0 dfgh	35 ± 2 bg	34 ± 0.7 abcdg	38 ± 0.7 abc	2.22 ± 0.22 a
<i>Eleusine indica</i>	eind	0.12 ± 0.006 abcde	10 ± 0.4 bcdef	1.41 ± 0.07 abf	155.8 ± 2.91 ac	186 ± 1.8 bdef	149 ± 15 bcf	41 ± 3 bdfgh	14 ± 2 cde	33 ± 1.3 abcd	41.1 ± 0.5 ab	2.05 ± 0.14 abc
<i>Eriochloa sericea</i>	eser	0.1 ± 0.001 abc	11.3 ± 0.4 bcdef	1.41 ± 0.11 af	89.8 ± 2.55 gh	184 ± 2.8 bdef	496 ± 16 ad	59 ± 2 ce	86 ± 2 f	41 ± 0.9 efg	35.5 ± 2.8 abc	2.08 ± 0.1 ac
<i>Heteropogon contortus</i>	hcon	0.18 ± 0.004 ef	9.9 ± 0.3 bcdf	0.56 ± 0.02 c	53.9 ± 2.98 d	119 ± 8.5 c	116 ± 21 bf	36 ± 2 bfg	9 ± 1 c	35 ± 1.2 abcdg	25.6 ± 2.7 de	1.24 ± 0.07 d
<i>Ischaemum afrum</i>	iafr	0.1 ± 0.012 a	12.4 ± 0.3 def	0.81 ± 0.03 ce	157.3 ± 10.11 c	153 ± 5.3 bc	323 ± 28 de	48 ± 2 deg	25 ± 1 deg	39 ± 0.9 cefg	33.6 ± 1.6 bcd	1.43 ± 0.08 d
<i>Paspalum dilatatum</i>	pdil	0.21 ± 0.005 f	8.5 ± 0.3 b	0.68 ± 0.05 c	52 ± 3.62 d	150 ± 9.7 bc	185 ± 8 cg	38 ± 1 bdfgh	15 ± 3 cde	31 ± 0.9 abd	30.6 ± 0.4 cde	1.39 ± 0.1 d
<i>Paspalum macrophyllum</i>	pmac	0.18 ± 0.022 def	9.8 ± 0.4 bcdf	0.12 ± 0.01 g	12 ± 1.04 e	159 ± 6.2 bce	162 ± 8 bc	30 ± 2 b	12 ± 1 cd	37 ± 2.2 bceg	25.3 ± 1.1 e	1.47 ± 0.05 bd
<i>Panicum maximum</i>	pmax	0.12 ± 0.005 abcd	11.9 ± 0.2 cdef	1.76 ± 0.12 b	111.8 ± 5.14 fhi	161 ± 2.4 bce	155 ± 9 bcf	44 ± 3 df	69 ± 5 af	30 ± 2.7 abd	38.1 ± 0.9 abc	1.72 ± 0.01 abcd
<i>Panicum miliaceum</i>	pmil	0.11 ± 0.001 abc	11.8 ± 1.4 cdef	1.31 ± 0.04 af	88.6 ± 4.52 gh	212 ± 11.4 ad	199 ± 17 cg	31 ± 2 b	78 ± 3 f	30 ± 1.1 abd	38.6 ± 0.4 abc	1.77 ± 0.04 abcd
<i>Panicum virgatum</i>	pvir	0.17 ± 0.03 bcdef	13.6 ± 1.3 e	1.28 ± 0.04 af	117.3 ± 7.89 bfi	210 ± 12.9 adf	296 ± 38 eg	62 ± 5 ace	165 ± 22 h	29 ± 0.2 d	36.9 ± 1.5 abc	2.27 ± 0.22 a
<i>Urochloa dictyoneura</i>	udic	0.08 ± 0.003 a	9.9 ± 0.6 bcdf	1.11 ± 0.03 ef	129 ± 5.08 abf	201 ± 4.6 adef	154 ± 8 bcf	34 ± 2 bfh	29 ± 2 beg	46 ± 1.7 f	37.4 ± 0.6 abc	1.29 ± 0.05 d

Figure 1. Response of mesophyll conductance (g_m) to changes in intercellular pCO_2 in 16 diverse C_4 grasses measured in current study. Measurements were performed at constant light (photosynthetic photon flux density (PPFD) = $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and leaf temperature (25°C). Values in each panel represent mean $\pm$ SE with $n = 3-6$. Response of g_m to CO_2 S for each species is plotted in separate panel. Species code has been indicated first letter of genera and first three letters of species (see Table 1 for full names of species). p values from one-way ANOVA along with Tukey's letters are shown in Fig. S1.

Figure 3. Relationship of percent increase in g_m with (a) mesophyll cell wall thickness (T_{CW}) (b) mesophyll surface area exposed to intercellular air spaces (S_{mes}) (c) stomatal ratio (SR), (d) stomatal density adaxial (SD_{ada}) and (e) leaf thickness among the 16 C_4 grasses measured in current study. Significance of Pearson correlation coefficients: ns, non-significant, +, marginally significant, *, $p \leq 0.05$, **, $p \leq 0.01$ and ***, $p \leq 0.001$. Each circle represents mean value for each species ($n = 3-6$). Species names are indicated by codes given in Table 1.

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749 **Figure 4.** Relationship of percent increase in g_m with (a) PEPC activity (b) Rubisco activity (c)
750 CA activity expressed as k_{CA} , (d) PEPC's affinity for HCO_3^- (K_m), (e) maximum photosynthetic
751 capacity (A_{max}) and (f) leaf N content (N_{area}) among the 16 C_4 grasses measured in current study.
752 Significance of Pearson correlation coefficients: ^{ns}, non-significant, +, marginally significant, *,
753 $p \leq 0.05$, **, $p \leq 0.01$ and ***, $p \leq 0.001$. Each circle represents mean value for each species ($n =$
754 3-6). Species names are indicated by codes given in Table 1.

Figure 5. Relationship of percent increase in g_m with ratio of mesophyll cell wall thickness (T_{CW}) to (a) PEPC activity (b) Rubisco activity (c) PEPC's affinity for HCO_3^- (K_m), (d) CA activity expressed as k_{CA} , (e) maximum photosynthetic rates (A_{max}) and (f) leaf N content (N_{area}) among the 16 C_4 grasses measured in current study. Significance of Pearson correlation coefficients: ^{ns}, non-significant, +, marginally significant, *, $p \leq 0.05$, **, $p \leq 0.01$ and ***, $p \leq 0.001$. Each circle represents mean value for each species ($n = 3-6$). Species names are indicated by codes given in Table 1.