

C₄ grasses adapted to low precipitation habitats show traits related to greater mesophyll conductance and lower leaf hydraulic conductance

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Abstract

In habitats with low water availability, a fundamental challenge for plants will be to maximize photosynthetic C-gain whilst minimizing transpirational water-loss. This tradeoff between C-gain and water-loss can in part be achieved through the coordination of leaf-level photosynthetic and hydraulic traits. To test the relationship of photosynthetic C-gain and transpirational water-loss we grew under common growth conditions 18 C₄ grasses adapted to habitats with different mean annual precipitation (MAP) and measured leaf-level structural and anatomical traits associated with mesophyll conductance (g_m) and leaf hydraulic conductance (K_{leaf}). The C₄ grasses adapted to lower MAP showed greater mesophyll surface area exposed to intercellular air spaces (S_{mes}) and adaxial stomatal density (SD_{ada}) which supported greater g_m . These grasses also showed greater leaf thickness and vein-to-epidermis distance which may lead to lower K_{leaf} . Collectively, these leaf traits associated with g_m and K_{leaf} scaled positively with photosynthetic rates (A_{net}) and leaf-level water-use efficiency (WUE) with low MAP adapted grasses exhibiting greater A_{net} and WUE. In summary, we identify a suite of leaf-level traits that appear important for adaptation of C₄ grasses to habitats with low MAP and may be useful to identify C₄ species showing greater A_{net} and WUE in drier conditions.

Introduction

C₄ photosynthesis has evolved independently in multiple grass lineages (Grass Phylogeny workshop 2012) thus leading to remarkable structural, anatomical and physiological trait diversity (Christin *et al.*, 2013). Studies suggest that this trait diversity among the C₄ species could be attributed to their adaptation to different environmental variables like temperature, fire frequency and precipitation (Edwards & Smith, 2010; Visser *et al.*, 2012; Zhou *et al.*, 2018). In general, C₄ species mostly occupy the lower latitudes where light availability and temperature likely do not strongly limit photosynthesis and growth (Pearcy & Ehleringer, 1984). Instead, precipitation may be an important factor affecting trait diversity in C₄ species; particularly, in traits associated with photosynthetic C-gain and transpirational water-loss (Edwards & Still, 2008; Osborne & Sack, 2012; Zhou *et al.*, 2018). During the adaption to habitats with low water availability, a fundamental challenge for plants will be to maintain photosynthetic C-gain while minimizing transpirational water-loss associated with high evaporative demand. This tradeoff could be achieved partly through coordination of leaf-level photosynthetic and hydraulic traits (Brodribb *et al.*, 2007; Nardini & Luglio, 2014; de Boer *et al.*, 2016). However, the extent of variation and coordination among these traits, particularly those associated with internal CO₂-diffusion conductance (g_m) and leaf hydraulic conductance (K_{leaf}), has not been well studied in C₄ species adapted to habitats with varying water availabilities (Osborne & Sack, 2012; Liu & Osborne, 2015; Taylor *et al.*, 2018). Although this type of trait variation and coordination has been studied

in C_3 plants there could be significant differences in C_4 plants due to their unique anatomy and physiology (Kocacinar & Sage, 2003; Osborne & Sack, 2012; Ocheltree *et al.*, 2016; Zhou *et al.*, 2018).

Species adapted to the drier habitats are known to exhibit several leaf-level structural and anatomical traits like greater leaf mass per area and leaf thickness, amphistomatous leaves, greater stomatal densities and values for mesophyll traits like mesophyll surface area exposed to intercellular air spaces (S_{mes}) and chloroplast coverage of S_{mes} (S_c) (Wright *et al.*, 2001; Galmés *et al.*, 2012; Ivanova *et al.*, 2018b). These parameters are not just anatomical features but important traits that could influence photosynthetic C-gain in species adapted to drier habitats. For instance, greater stomatal densities and amphistomaty (stomata on both leaf surfaces), associated with greater leaf thickness in drier habitats, may enhance fine regulation of water-use and help reduce the effective leaf thickness by decreasing the CO_2 -diffusion pathlength (Galmés *et al.*, 2012; Muir, 2018; Muir, 2019; Pathare *et al.*, 2020). Furthermore, mesophyll traits like S_{mes} and S_c - the parameters that characterize exchange surfaces for CO_2 , negatively correlated with water availability in the C_3 species of European steppe plant communities and were suggested as indicators of increasingly drought adapted steppe plants (Ivanova *et al.*, 2018a; Ivanova *et al.*, 2018b). These structural and anatomical adaptations could help maximize internal CO_2 -diffusion conductance (g_m), at a given stomatal conductance (g_{sw}), thus leading to greater photosynthetic rates (A_{net}) as well as leaf-level water-use efficiency (WUE) in species adapted to drier habitats (Flexas *et al.*, 2008; Flexas *et al.*, 2013; Ivanova *et al.*, 2018a; Ivanova *et al.*, 2018b). However, very few studies, mostly based on C_3 species, have investigated the leaf-level structural and anatomical traits associated with g_m that could be a characteristic of plant adaptation to drier habitats (Ivanova *et al.*, 2018a; Ivanova *et al.*, 2018b). Alternatively, even though C_4 species can successfully occupy drier and warmer habitats and form grasslands over vast areas globally, there is a little information about leaf-level structural and anatomical traits that influence photosynthetic C-gain and water-use in these species. Specifically, we are unaware of any studies that have investigated the relationship of g_m and associated anatomical traits in diverse C_4 species from habitats with different water availability. In a previous study (Pathare *et al.*, 2020) we investigated the structural and anatomical determinants of g_m in diverse C_4 grasses and found that, leaf thickness, adaxial stomatal densities (SD_{ada}), stomatal ratio (SR) and S_{mes} had a positive effect on g_m . In the current study, our aim is to determine if the variation in above traits among the C_4 species could be related to adaptation to habitats with different water availabilities. Our first hypothesis (H1) is that, C_4 grasses adapted to lower MAP will show greater g_m and leaf anatomical traits associated with greater g_m in order to maximize photosynthetic C-gain.

Though we hypothesized a greater g_m in C_4 grasses adapted to low MAP (H1), one would expect an increase in water cost relative to photosynthetic C-gain, because g_m and associated traits have been shown to scale positively with leaf hydraulic conductance (K_{leaf}) in C_3 species (Flexas *et al.*, 2013; Xiong *et al.*, 2015; Xiong *et al.*, 2017; Drake *et al.*, 2019). K_{leaf} is an important trait associated with leaf water transport and represents the conductance to flow of water from the leaf petiole through the xylem, then through the bundle sheath and finally through the mesophyll to the site of evaporation (Sack & Holbrook, 2006; Noblin *et al.*, 2008; Buckley, 2015; Buckley *et al.*, 2015). Though K_{leaf} is partitioned between the xylem (K_x) and the outside xylem pathways (K_{ox}), changes to K_{ox} are expected to have the largest effects on K_{leaf} (Buckley *et al.*, 2015; Scoffoni *et al.*, 2017; Xiong & Nadal, 2019). Consequently, leaf-level anatomical traits that influence K_{ox} such as leaf thickness, vein-to-epidermis distance (VED), vein length per unit of leaf area (VLA) and bundle sheath and mesophyll traits are expected to have a significant effect on K_{leaf} (Griffiths *et al.*, 2013; Sack *et al.*, 2013; Buckley *et al.*, 2015). For instance, greater leaf thickness and VED, if associated with low VLA, may increase the length of post-venous water path thus leading to lower K_{leaf} . Alternatively, greater VLA in thinner leaves may increase K_{leaf} by providing additional parallel flow paths through the vein system and decreasing the horizontal pathlength from veins to sites of evaporation (Brodribb *et al.*, 2007; Sack & Scoffoni, 2013; Buckley *et al.*, 2015; Drake *et al.*, 2019). Furthermore, greater bundle sheath (BS) surface area ratio, lower BS cell wall thickness (BS_{CW}) and BS exposed to intercellular air spaces (BS_{ias}) and greater S_{mes} may all enhance K_{leaf} (Buckley *et al.*, 2015; Caringella *et al.*, 2015; Xiong *et al.*, 2017; Scoffoni *et al.*, 2018). Investigating the coordination of above traits related to water-use with traits related to C-gain will provide insights into the water cost associated with photosynthetic C-gain in C_4 species adapted to habitats

with varying water availabilities.

Previous studies on C_3 species have shown a strong positive linkage of K_{leaf} with g_{sw} and hence A_{net} , suggesting the coordinated evolution of these traits (Brodribb *et al.*, 2007; Brodribb & Feild, 2010; Flexas *et al.*, 2013; Scoffoni *et al.*, 2016). Additionally, the few studies that address the coordination of K_{leaf} and g_{m} show that these two traits scale positively with each other in C_3 species as they share some structural and anatomical traits that form the mechanistic basis for their coordination independent of g_{sw} (Flexas *et al.*, 2013; Xiong *et al.*, 2015; Xiong *et al.*, 2017) but see (Th eroux-Rancourt *et al.*, 2014; Loucos *et al.*, 2017; Wang *et al.*, 2018). For example, S_{mes} positively correlates with both g_{m} and K_{leaf} (Flexas *et al.*, 2013; Xiong *et al.*, 2015; Xiong *et al.*, 2017), since greater S_{mes} increases the number of parallel pathways for CO_2 -diffusion inside mesophyll cells (Evans *et al.*, 2009) as well as the evaporating surface area for water thus increasing g_{m} and K_{leaf} respectively (Sack & Scoffoni, 2013; Xiong *et al.*, 2017). In summary, the positive correlation of K_{leaf} with g_{m} implies a greater water cost associated with greater C-gain, which could be detrimental in drier conditions where using water efficiently will be important for plant growth and fitness. Hence, a safer strategy for plants is to maintain lower K_{leaf} in drier conditions at the cost of A_{net} and growth rates (Sinclair *et al.*, 2008; Nardini & Luglio, 2014; Scoffoni *et al.*, 2016). However, these generalizations are mostly based on studies of C_3 species. It is unclear if these results would apply to C_4 grasses that are adapted to relatively drier habitats and may show different coordination between the traits associated with photosynthetic C-gain and transpirational water loss (Kocacinar & Sage, 2003; Ocheltree *et al.*, 2016; Zhou *et al.*, 2018). For example, the evolution of C_4 species from their C_3 ancestors is associated with increases in bundle sheath size and vein densities leading to greater K_{leaf} (Christin *et al.*, 2013; Griffiths *et al.*, 2013). However, it has been proposed that once the carbon concentrating mechanism in C_4 species evolved, selection for traits leading to greater K_{leaf} would not only be lessened but inverted leading to greater drought tolerance and leaf-level WUE during adaptation to drier habitats (Zhou *et al.*, 2018). Consequently, in contrast to C_3 species, maintaining greater K_{leaf} in order to achieve higher A_{net} may not be necessary in C_4 species and K_{leaf} may be uncoupled from g_{sw} , A_{net} (Kocacinar & Sage, 2003; Ocheltree *et al.*, 2016) and potentially g_{m} . However, to our knowledge, there have been no previous studies on the correlation of g_{m} with K_{leaf} or traits associated with K_{leaf} in C_4 species adapted to habitats with diverse MAP. Building knowledge upon previous evidences, we hypothesized (H2) that C_4 species adapted to habitats with low MAP will show traits associated with lower K_{leaf} that will maximize photosynthetic C-gain at a given water loss.

To test the above hypotheses, we selected 18 C_4 grasses that varied significantly in structural and anatomical traits (Pathare *et al.*, 2020). The grasses were grown under common growth conditions and abundant water and nutrient supply which avoids the influence of environmental conditions on traits and thus helps identify the differences that could be a result of species adaptation to their habitat of evolution or common occurrence (Reich *et al.*, 2003). We measured important leaf-level structural and anatomical traits associated with photosynthetic C-gain and g_{m} and transpirational water-loss and K_{leaf} in 18 diverse C_4 grasses. There is a significant knowledge gap about how C_4 - g_{m} variability relates with habitat climate variables like MAP largely because of the lack of techniques to estimate C_4 - g_{m} in field as well as laboratory conditions. However, the recent developments provide the opportunity to estimate C_4 - g_{m} under laboratory conditions and thus investigate the relationship of habitat climate variables with g_{m} in diverse C_4 species. Here, we use a recently developed method, based on modeling of leaf oxygen isotope discrimination during photosynthesis, to estimate g_{m} in 18 diverse C_4 grasses (Barboure *et al.*, 2016; Ubierna *et al.*, 2017; Ogee *et al.*, 2018).

Material and Methods

Plant material and growth conditions

Eighteen C_4 grasses (Table 1) representing the three classical biochemical subtypes and 8 evolutionary lineages were selected for this study. However, the aim of our study was not to look at subtype or lineage effect but to maximize leaf physiological, structural and anatomical trait diversity. Each species was given a unique identification number (Table S1) for presentation in Fig. 1-5, 7 and S2-S5.

As previously described by Pathare *et al.*, (2020) plants were 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 6-7 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). Plants were supplemented with Spring 330 iron chelate (BASF, Ludwigshafen, Germany) and Scott-Peters Soluble Trace Element Mix (The Scotts Co., Marysville, OH, USA) once a week at concentrations of 10 mg L⁻¹. Pot locations were randomized daily within the growth chamber.

Habitat mean annual precipitation and mean annual temperature

The global distribution data for the geo-referenced species was extracted from the Global Biodiversity Information Facility (GBIF; <http://www.gbif.org/>) site using the *gbif* function in R package (version 3.5.2) *dismo* (Hijmans & van Etten, 2012). Values for mean annual temperature (MAT) and mean annual precipitation (MAP) from 1970 to 2000 for all geo-referenced localities for each species were extracted from the WorldClim dataset (<http://www.worldclim.org/>) using the *extract* function in R package *raster* (Hijmans & van Etten, 2012). The values were then averaged as the MAT and MAP value for a given species.

Measurement of physiological traits and mesophyll conductance

The measurements of net photosynthetic rates (A_{net}), stomatal conductance to water vapor (g_{sw}), intercellular CO₂ concentrations (C_i), transpiration (E), intrinsic WUE ($A_{\text{net}}/g_{\text{sw}}$), instantaneous WUE (A_{net}/E) and mesophyll conductance to CO₂ (g_m) were previously described in Pathare *et al.*, (2020). Briefly, 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). 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 $\sim 250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and air temperature was maintained at 25 °C. In current study, g_m was estimated using the method described by Ogee *et al.*, (2018) as discussed in Pathare *et al.*, (2020). 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 .

Measurement of structural and anatomical traits associated with g_m and K_{leaf}

Light and electron microscopy techniques were used to measure important structural and anatomical traits listed in Table 1. The details of sample preparation for light and electron microscopy and measurements were presented in Pathare *et al.*, (2020). Light microscopy images of leaf cross sections were used to measure average leaf thickness (calculated as average of maximum and minimum leaf thickness), interveinal distance (IVD), vein-to-adaxial epidermis distance (VED_{ada}), vein-to-abaxial epidermis distance (VED_{aba}), average VED (calculated as (VED_{ada} + VED_{aba}) / 2) and 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. Portion of BS cell walls exposed to IAS (BS_{ias}) was calculated as a percentage from the total BS cell wall length. BS area ratio was calculated as a percentage using BS area and mesophyll area (BS area / [BS area + Mesophyll area]) for each species (Griffiths *et al.*, 2013). The mesophyll surface area exposed to IAS per unit leaf area (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. Mesophyll and BS cell wall thickness (M_{CW} and BS_{CW}) was measured from TEM micrographs using at least 15 images for each leaf. Total leaf vein length per unit leaf area (VLA) was calculated from the total number of veins per section length and expressed per mm² considering parallel venation in grasses.

Images of the adaxial and abaxial epidermal surfaces, captured on four leaves (each from a different replicate; $n = 4$) per species under the low-vacuum mode with a FEI Scanning Electron Microscope Quanta 200F (FEI Co., Field Emission Instruments), were used to measure the stomatal number on each surface of leaf and expressed per mm^2 as adaxial stomatal density (SD_{ada}) and abaxial stomatal density (SD_{aba}). The SR was calculated as ratio of the SD on the adaxial and abaxial surfaces.

Statistical analysis

Statistical analyses were performed using R software (version 3.5.2, R Foundation for Statistical Computing, Vienna, Austria). Regression analysis were performed, using the mean values of traits for each species, in order to examine the relationships between key variables of interest among the 18 C_4 grasses. Particularly, we investigated the relationships of leaf structural and anatomical traits associated with g_m and photosynthetic C-gain and K_{leaf} and transpirational water-loss with habitat MAP and MAT. One-way ANOVA with posthoc Tukey's test was used to examine differences in leaf-level physiological, structural, anatomical and biochemical traits among the 18 diverse C_4 grasses (Refer Table S2, S3 and Pathare *et al.*, 2020). For the one-way ANOVA, values of P [?] 0.05 were considered to be statistically significant. Results of one-way ANOVA for traits used in the current study are given in Table S2 and S3 in current manuscript and in Pathare *et al.*, 2020. In addition, we used a principal component analysis (PCA) to identify the major axes of variation among the important leaf-level traits associated with g_m and K_{leaf} (Table 1). 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 (PCs) had eigenvalues > 1 (Table S4) and were retained according to Kaiser's rule (Kaiser, 1960). For each trait, factor loadings > 0.5 in absolute value were considered important.

Results

Effects of habitat MAP and MAT on leaf-level traits of C_4 grasses

We investigated the relationship of leaf-level traits, affecting g_m and K_{leaf} and hence photosynthetic C-gain and transpirational water-loss respectively, with two main habitat climate variables- mean annual temperature (MAT) and mean annual precipitation (MAP). Anatomical traits associated with g_m and K_{leaf} that were measured in current study are shown in Fig. S1. Mean values along with SE for the leaf-level traits measured in the current study are given in Table S2 and Pathare *et al.*, 2020. Mean values along with SE for MAP and MAT are shown in Fig. S6. None of the measured photosynthetic C-gain or transpirational water-loss traits in current study related with MAT (Table 1). However, MAP showed a significant relationship with many important leaf-level traits associated with photosynthetic C-gain or transpirational water-loss (Table 1, Fig. S2, S3 and S4). Specifically, there was a significant negative relationship between MAP and S_{mes} ($R^2 = -0.50$, $P < 0.01$), S_c ($R^2 = -0.60$, $P < 0.001$), SD_{ada} ($R^2 = -0.20$, $P = 0.06$), SR ($R^2 = -0.21$, $P = 0.05$), N_{area} ($R^2 = -0.21$, $P = 0.05$), leaf thickness ($R^2 = -0.35$, $P = 0.037$), average VED ($R^2 = -0.45$, $P = 0.01$), VED_{ada} ($R^2 = -0.45$, $P = 0.01$) and BS_{CW} ($R^2 = -0.39$, $P = 0.021$). Whereas, there was a significant positive relationship between BS_{ias} and MAP ($R^2 = 0.27$, $P = 0.026$). We also investigated the relationship of functional traits like g_m and g_{sw} with MAP for the 18 C_4 grasses. There was no significant relationship between g_{sw} and MAP (Table 1). However, there was a strong negative relationship between g_m and MAP ($R^2 = -0.43$, $P = 0.015$).

Relationships of leaf structural and anatomical traits

Across all the C_4 grasses, g_m was positively correlated with leaf-level traits like IVD ($R^2 = 0.18$, $P = 0.07$, Fig. 1a), leaf thickness ($R^2 = 0.45$, $P < 0.01$, Fig. 1b) and average VED ($R^2 = 0.44$, $P < 0.01$, Fig. 1c). Similarly, A_{net} was positively correlated with IVD ($R^2 = 0.46$, $P = 0.01$, Fig. 2a), leaf thickness ($R^2 = 0.15$, $P = 0.1$, Fig. 2b) and average VED ($R^2 = 0.22$, $P = 0.05$, Fig. 2c). In our previous study on these C_4 grasses (Pathare *et al.*, 2020), we showed that g_m also scaled positively with S_{mes} ($R^2 = 0.63$, $P < 0.001$, Fig. S5a), SD_{ada} ($R^2 = 0.47$, $P = 0.01$, Fig. S5c), SR ($R^2 = 0.26$, $P = 0.04$, Fig. S5d) and A_{net} ($R^2 = 0.26$, $P = 0.03$, Fig. S5g), but showed no relationship with M_{CW} , SD_{aba} and g_{sw} (Fig. S5b,e and f). Here, we further investigated the relationship of traits related to g_m like SD_{ada} , SR and S_{mes} with traits related

to K_{leaf} like IVD, leaf thickness and VED. Particularly, SD_{ada} positively correlated with IVD ($R^2 = 0.45, P = 0.01$, Fig. 3a), leaf thickness ($R^2 = 0.36, P < 0.01$, Fig. 3b) and average VED ($R^2 = 0.38, P < 0.01$, Fig. 3c). Similarly, SR positively correlated with IVD ($R^2 = 0.55, P < 0.01$, Fig. 4a), leaf thickness ($R^2 = 0.45, P < 0.01$, Fig. 4b) and average VED ($R^2 = 0.53, P < 0.001$, Fig. 4c). Whereas, S_{mes} positively correlated with leaf thickness ($R^2 = 0.56, P < 0.001$, Fig. 5b) and average VED ($R^2 = 0.45, P < 0.01$, Fig. 5c).

Principal component analysis

A PCA, using MAP and leaf-level structural and anatomical traits associated with g_m and K_{leaf} , was performed to complement the trait-to-trait comparisons. The first two major axes (PC1 and PC2) along with the average position of 18 C_4 grasses in PC1-PC2 space are shown in Fig. 6. The first three axes with eigenvalues and scores are shown in Table S4. PC1 explained about 50.56 % of the total variation in the C_4 grasses. PC1 scaled positively with g_m , SD_{ada} , S_{mes} , SR, N_{area} , IVD, leaf thickness and average VED but negatively with total VLA, BS_{ias} and MAP. Thus, PC1 delineated the C_4 grasses into those which occupy relatively drier habitats and show traits associated with greater g_m and hence photosynthetic C-gain (higher score on PC1) from those which occupy relatively wetter habitats and show traits associated with greater K_{leaf} and water-loss (lower score on PC1). PC2 explained about 16 % of the total variation and scaled positively with MAP and BS area ratio but negatively with total VLA and BS_{CW} . PC3 explained 10.25% of total variation and scaled positively only with MAT. Thus, MAT was unrelated to any of the leaf-level structural and anatomical traits measured in the current study. Together, the first three axes explained about 77% of the total variation. To test if in addition to greater g_m species adapted to drier habitats also exhibit greater A_{net} and leaf-level WUE, we looked at the relationship of the PC1 with A_{net} (Fig. 7a) and two measures of leaf-level WUE, that is, instantaneous water-use efficiency (A_{net}/E , Fig. 7b) and intrinsic of water-use efficiency ($A_{\text{net}}/g_{\text{sw}}$, Fig. 7c). A higher score on PC1 indicates presence of a ‘suite’ of traits associated with greater g_m and photosynthetic C-gain and lower K_{leaf} and transpirational water-loss. There was a significant positive relationship between A_{net} and PC1 ($R^2 = 0.56, P = 0.002$, Fig. 7a). Furthermore, A_{net}/E showed a significant positive relationship with PC1 ($R^2 = 0.25, P = 0.035$, Fig. 7b). However, A_{net}/g_s did not relate with PC1. In summary, C_4 grasses with greater score on PC1 also showed greater A_{net} and A_{net}/E .

Discussion

Using 18 diverse C_4 grasses grown under common growth conditions, we investigated the relationship of growth habitat MAP and MAT with leaf-level structural and anatomical traits associated with g_m and K_{leaf} , which in turn could influence photosynthetic C-gain and water-loss. Many of the measured traits correlated with MAP but not with MAT (Table 1), which supports the previous expectations that, precipitation may be more important than temperature in affecting trait variability and potentially diversification in the C_4 species (Edwards & Still, 2008; Osborne & Sack, 2012; Liu *et al.*, 2019). Furthermore, our study provides insights into the possible relationships between g_m and leaf anatomical traits related to K_{leaf} in diverse C_4 grasses and suggest that C_4 grasses adapted to lower MAP exhibited traits associated with greater g_m and lower K_{leaf} .

C_4 grasses adapted to low MAP show traits associated with greater g_m and photosynthetic C-gain

A maximum A_{net} for a given rate of transpirational water-loss, through coordination of leaf-level photosynthetic and hydraulic traits, will in part determine species WUE and fitness during adaptation to drier growth habitats. A key question posed by earlier studies is how species adapted to drier habitats maintain similar or even higher A_{net} , at a given g_{sw} , during periods of active photosynthesis than species from more humid habitats (Wright *et al.*, 2001; Reich *et al.*, 2003). A greater A_{net} , at a given g_{sw} , can be achieved by increasing g_m through selection for leaf mesophyll traits like S_{mes} and S_c (Ivanova *et al.*, 2018a; Ivanova *et al.*, 2018b). In the current study, greater values for mesophyll traits like S_{mes} and S_c (Table 1, Fig. 6) were observed in C_4 grasses adapted to lower MAP, as shown recently for C_3 species (Ivanova *et al.*, 2018a; Ivanova *et al.*, 2018b). S_{mes} and S_c have been demonstrated to be important determinants of g_m in C_3

(Muire *et al.* , 2014; Peguero-Pina *et al.* , 2017) and C₄ species (Pathare *et al.* , 2020) . For C₄ species, S_{mes} is a more accurate determinant of g_m than S_c as the first site of CO₂ fixation is in mesophyll cytosol and not the mesophyll chloroplast (Barbour *et al.* , 2016; Pathare *et al.* , 2020). If all else remains constant, then a greater S_{mes} increases the number of parallel pathways for CO₂ diffusion inside leaves leading to higher g_m under high light and low water availability (Terashima *et al.* , 2001; Ivanova *et al.* , 2018a; Ivanova *et al.* , 2018b). Indeed, along with greater S_{mes}, we also observed greater values for g_m in the C₄ grasses adapted to low MAP (Table 1, Fig. 6). Our findings thus support the previous work on C₃ plants suggesting the importance of mesophyll traits for plants adapted to drier habitats (Ivanova *et al.* , 2018b). Also, for the first time we show that C₄ grasses adapted to low MAP also exhibit mesophyll traits that lead to greater g_m -an important trait that could help achieve greater A_{net} at a given g_{sw} (Flexas *et al.* , 2016; Cousins *et al.* , 2020; Pathare *et al.* , 2020).

In addition to greater g_m and S_{mes}, C₄ grasses adapted to low MAP also showed greater SD_{ada}, SR and N_{area} (Table 1, Fig. 6). Greater SD_{ada} and SR mostly occur in species adapted to conditions with high CO₂ demand, like high light and low water, where, they are proposed to decrease the effective leaf thickness and hence CO₂ diffusion pathlength thus increasing g_m and supporting higher A_{net} (Parkhurst, 1978; Mott & O’Leary, 1984; Muir, 2018). We recently demonstrated that greater SD_{ada} and SR in C₄ grasses were associated with greater leaf thickness and lead to greater g_m and A_{net} as a result of increase in S_{mes} (Pathare *et al.* , 2020) . The current study further supports the well-established positive link of SD_{ada} and SR with habitat MAP (Mott & O’Leary, 1984; Bucher *et al.* , 2017) and suggests that a greater SD_{ada} and SR in drier habitats could be a strategy used by C₄ grasses to facilitate greater g_m.

C₄ grasses adapted to low MAP show leaf anatomical traits associated with lower K_{leaf} and transpirational water-loss

Our results demonstrate that C₄ grasses adapted to low MAP show greater g_m and associated traits like S_{mes}, S_c, SD_{ada} and SR, thus supporting the first hypothesis. However, these traits have also been associated with greater K_{leaf} in C₃ species (Brodribb & Holbrook, 2004; Xiong *et al.* , 2017; Drake *et al.* , 2019), which could imply higher water costs in drier habitats. However, C₄ grasses adapted to low MAP also showed greater leaf thickness and VED (Table 1, Fig. 6), which indicates deeper vein placement and an increase in pathway for water movement outside the xylem (Brodribb *et al.* , 2007; McKown *et al.* , 2014; Buckley *et al.* , 2015). Additionally, in these C₄ grasses, an increase in leaf thickness was correlated with an increase in IVD i.e. a decrease in total VLA (Table 1, Fig. 6). This may reduce the parallel water flow pathways outside xylem thus decreasing K_{ox} and hence K_{leaf} (Buckley *et al.* , 2015). Together, these anatomical traits suggest that K_{leaf} would be lower in C₄ grasses adapted to low MAP. Furthermore, species adapted to low MAP also showed lower BS_{ias} and higher BS_{CW}-traits that may lower K_{ox} and hence K_{leaf} (Griffiths *et al.* , 2013; Buckley *et al.* , 2015; Scoffoni *et al.* , 2017). In summary, though C₄ grasses adapted to habitats with relatively low MAP exhibit traits associated with greater g_m and photosynthetic C-gain, they also possess traits associated with lower K_{leaf} and water-loss. This supports our second hypothesis as well as the previous expectation of selection for traits associated with lower K_{leaf} in C₄ species during adaptation to drier conditions (Zhou *et al.* , 2018). Thus, for C₄ grasses the greater photosynthetic C-gain in drier habitats may not be associated with greater leaf-level water-loss.

Coordination of traits associated with g_m and K_{leaf}

Our findings on the coordination of traits associated with photosynthetic C-gain and transpirational water-loss in C₄ grasses contrast some of those reported previously for C₃ species. For instance, C₄ grasses adapted to drier habitats exhibit traits associated with greater g_m and lower K_{leaf} (Fig. 6). Also, g_m, A_{net} and traits associated with g_m like SD_{ada}, SR and S_{mes} (Pathare *et al.* , 2020) scaled positively with traits like IVD, leaf thickness and VED (Fig. 1-5) which are known to be important determinants of K_{leaf} (Sack *et al.* , 2013; Buckley *et al.* , 2015) These results suggest that K_{leaf} may be negatively related to g_m and hence A_{net} for the C₄ grasses belonging to habitats with diverse MAP. This finding contrasts the previous reports of a positive relationship of K_{leaf} with g_m and A_{net} observed in C₃ species (Sack & Holbrook, 2006; Flexas *et al.* , 2013; Xiong *et al.* , 2017; Drake *et al.* , 2019) and could be partly explained by the carbon concentrating

mechanism of C_4 species that maintains high A_{net} at relatively low g_{sw} compared to C_3 species (Ocheltree *et al.*, 2016) and the relationship of g_{m} and K_{leaf} with leaf thickness. Specifically, greater leaf thickness in C_4 grasses from drier habitats was associated with greater VED and lower total VLA (Fig. 6) which may imply a lower K_{leaf} and also an increase in space available for photosynthetic tissue (Brodribb *et al.*, 2007; McKown *et al.*, 2014; Zwieniecki & Boyce, 2014; Buckley *et al.*, 2015). Also, in these C_4 grasses, greater leaf thickness was associated with a greater SD_{ada} , SR and S_{mes} implying a greater g_{m} and A_{net} (Muir, 2018). Consequently, we predict a negative relationship of K_{leaf} with g_{m} and hence A_{net} in these C_4 grasses. The negative relationship of SD_{ada} with total VLA (Fig. 6) also contrasts the previous reports for C_3 species (Drake *et al.*, 2019) and suggests that, for the C_4 grasses the presence of a greater number of stomata may not be associated with greater investment in leaf water transport tissue and hence K_{leaf} , though it is associated with a greater g_{m} and A_{net} (Pathare *et al.*, 2020).

Species adapted to drier habitats are known to employ a safer xylem strategy, wherein, resistance to cavitation is achieved by maintaining lower K_{leaf} , which however comes at the cost of A_{net} thus leading to the safety versus efficiency trade-off (Zimmermann, 1983; Meinzer *et al.*, 2010). However, C_4 grasses adapted to low MAP exhibited traits associated with lower K_{leaf} , but greater g_{m} and A_{net} (Table 1, Fig. 6). Previous studies have also observed a decoupling between K_{leaf} and A_{net} for the C_4 grasses (Kocacinar & Sage, 2003; Ocheltree *et al.*, 2016). Our results along with these previous findings suggest that maintaining a greater K_{leaf} in order to achieve greater A_{net} may not be a necessity for C_4 grasses and that the safety-versus efficiency trade-off may not apply to the C_4 grasses which can achieve greater g_{m} and A_{net} in drier habitats whilst maintaining a lower K_{leaf} .

Conclusions

Leaf adaptation to climate may not be limited to a single or even a few traits but to a suite of traits representing a meaningful ‘syndrome’ that may be helpful for understanding vegetation response to climate change without detailed analysis of each species (Wright *et al.*, 2001; Reich *et al.*, 2003). Our results suggest that C_4 grasses adapted to low MAP exhibit greater SD_{ada} , SR, S_{mes} and g_{m} , which allow for greater photosynthetic C-gain. Additionally, C_4 grasses adapted to low MAP also exhibit greater average leaf thickness, average VED and BS_{CW} but lower BS_{ias} which may lead to lower K_{leaf} and transpirational water-loss. Taken together, this entire suite of traits (collectively represented by PC1) was positively correlated with A_{net} and A_{net}/E (Fig. 7) indicating that C_4 grasses adapted to relatively drier habitats exhibited greater photosynthetic C-gain and leaf-level WUE. This ‘syndrome’ of C_4 grasses appears important for adaptation to drier habitats and could be useful to identify or screen for agriculturally important C_4 grasses with greater productivities and leaf-level WUE (Sack *et al.*, 2016). Furthermore, a negative relationship of K_{leaf} with g_{m} , predicted in current study for C_4 grasses belonging to habitats with diverse MAP, warrants further investigation as it could have important implications for modelling the carbon and water fluxes of grasslands (De Kauwe *et al.*, 2015; Knauer *et al.*, 2019a; Knauer *et al.*, 2019b).

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Author contributions

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

Conflict of interest

The authors declare that they have no conflict of interest

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Figure legends

Figure 1. Relationship of mesophyll conductance (g_m) with (a) interveinal distance (IVD), (b) leaf thickness and (c) average vein-to-epidermis distance (VED) for the 18 C_4 grasses measured in current study. Numbers correspond to species listed in Table S1. Regression coefficient (R^2) is shown when P [?] 0.001 (***), P [?] 0.01 (**), P [?] 0.05 (*) and P [?] 0.1 (+). Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2).

Figure 2. Relationship of net photosynthetic rates (A_{net}) with (a) interveinal distance (IVD), (b) leaf thickness and (c) average vein-to-epidermis distance (VED) for the 18 C_4 grasses measured in current study. Numbers correspond to species listed in Table S1. Regression coefficient (R^2) is shown when P [?] 0.001 (***), P [?] 0.01 (**), P [?] 0.05 (*) and P [?] 0.1 (+). Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2).

Figure 3. Relationship of adaxial stomatal density (SD_{ada}) with (a) interveinal distance (IVD), (b) leaf thickness and (c) average vein-to-epidermis distance (VED) for the 18 C_4 grasses measured in current study. Numbers correspond to species listed in Table S1. Regression coefficient (R^2) is shown when P [?] 0.001 (***), P [?] 0.01 (**), P [?] 0.05 (*) and P [?] 0.1 (+). Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2).

Figure 4. Relationship of ratio of adaxial to abaxial stomatal density (stomatal ratio; SR) with (a) interveinal distance (IVD), (b) leaf thickness and (c) average vein-to-epidermis distance (VED) for the 18 C_4 grasses measured in current study. Numbers correspond to species listed in Table S1. Regression coefficient (R^2) is shown when P [?] 0.001 (***), P [?] 0.01 (**), P [?] 0.05 (*) and P [?] 0.1 (+). Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2).

Figure 5. Relationship of mesophyll surface area exposed to intercellular air spaces (S_{mes}) with (a) interveinal distance (IVD), (b) leaf thickness and (c) average vein-to-epidermis distance (VED) for the 18 C_4 grasses measured in current study. Numbers correspond to species listed in Table S1. Regression coefficient (R^2) is shown when P [?] 0.001 (***), P [?] 0.01 (**), P [?] 0.05 (*) and P [?] 0.1 (+). Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2).

Figure 6. Principal component analysis biplot showing major axes of variation in important leaf-level physiological, structural and anatomical traits among 18 diverse C_4 grasses. Eigenvalues and factor loadings for first three principal components (PCs) are shown in Supporting Information Table S4. The arrows are the vectors showing the correlation (across the C_4 grasses) between a trait and the PCs. The position of species in PC space is shown in blue circles. Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2). Species names correspond to the description in Table S1. Total VLA, vein length per unit leaf area; BS_{ias} , BS exposed to intercellular air spaces; MAP, mean annual precipitation; MAT, mean annual temperature; BS_{CW} , BS cell wall thickness; BS exposed to intercellular air spaces; BS area ratio (calculated as (BS area / [BS area + Mesophyll area])); g_m , mesophyll conductance to CO_2 diffusion estimated by Ogee *et al.* (2018); SD_{ada} , adaxial stomatal density; S_{mes} , total mesophyll cell surface area exposed to intercellular air space per unit of leaf surface area; SR, stomatal ratio; N_{area} , leaf N content expressed on area basis; IVD, interveinal distance; VED, average vein-to-epidermis distance.

Figure 7. Relationship of (a) net photosynthetic rates (A_{net}), (b) instantaneous water-use efficiency (A_{net}/E) and (c) intrinsic water-use efficiency (A_{net}/g_s) with principal component 1 (PC1, where higher score on PC1 indicates greater g_m and lower K_{leaf} in drier habitats) for the 18 C_4 grasses. Numbers correspond to species listed in Table S1. Regression coefficient (R^2) is shown when P [?] 0.001 (***), P [?] 0.01

(**), P [?] 0.05 (*) and P [?] 0.1 (+). Points are mean values with $n = 3-6$ per species (Mean \pm SE values are given in Pathare *et al.*, 2020 and Table S2).

Supporting information

Table S1 . 18 C_4 grasses, along with biochemical subtype and evolutionary lineage, used in the current study (Adapted from Pathare *et al.*, 2020).

Table S2 . Mean \pm SE ($n = 3$ to 6) values along with the corresponding letters of post-hoc Tukey's test for important leaf level traits measured in 18 C_4 grasses.

Table S3 . Results of one-way ANOVA with species as main effects for the traits measured in 18 C_4 grasses.

Table S4 . Component loadings for important leaf level traits determined on 18 diverse C_4 grasses.

Fig. S1 Representation of the anatomical traits associated with mesophyll conductance to CO_2 (g_m) and leaf hydraulic conductance (K_{leaf}) measured in current study.

Fig. S2 Relationship of mean annual precipitation with (a) mesophyll surface area exposed to intercellular air space (S_{mes}), (b) extent of S_{mes} covered by chloroplast (S_c), (c) mesophyll conductance (g_m), (d) mesophyll cell wall thickness (M_{CW}), (e) adaxial stomatal density (SD_{ada}) and (f) abaxial stomatal density (SD_{aba}) for the 18 C_4 grasses.

Fig. S3 Relationship of mean annual precipitation with (a) stomatal ratio (SR), (b) N content per unit leaf area (N_{area}), (c) stomatal conductance to water (g_{sw}), (d) leaf thickness and (e) average vein to epidermis distance (VED) for the 18 C_4 grasses.

Fig. S4 Relationship of mean annual precipitation with (a) vein to adaxial epidermis distance (VED_{ada}), (b) vein to abaxial epidermis distance (VED_{aba}), (c) interveinal distance (IVD), (d) Bundle sheath cell wall thickness (BS_{CW}), (e) BS exposed to intercellular air space (BS_{ias}) and (f) BS area ratio for the 18 C_4 grasses.

Fig. S5 Relationship of mesophyll conductance to CO_2 (g_m) with (a) mesophyll surface area exposed to intercellular air spaces (S_{mes}), (b) Mesophyll (M) cell wall thickness (M_{CW}), (c) adaxial stomatal density (SD_{ada}), (d) stomatal ratio or ratio of adaxial to abaxial stomatal density (SR), (e) abaxial stomatal density (SD_{aba}), (f) stomatal conductance to water (g_{sw}) and (g) net CO_2 assimilation rates (A_{net}) for the 18 C_4 grasses.

Fig. S6 Mean (\pm SE) habitat (a) mean annual precipitation and (b) mean annual temperature for 18 C_4 grasses measured in current study. Species names shown on the x-axis correspond to the description in Table S1.

Table 1. Relations between habitat climate variables (MAP and MAT) and important anatomical, stomatal and functional traits associated with carbon gain and water use in 18 diverse C_4 grasses.

Traits	MAP	MAP	MAP	MAT
	R^2	P - value	Type	R^2
Photosynthetic C-gain	Photosynthetic C-gain	Photosynthetic C-gain	Photosynthetic C-gain	Photosyn
S_{mes}	-0.5	0.005	polynomial	0.006
S_c	-0.6	0.0009	polynomial	0.023
g_m	-0.43	0.015	polynomial	0
M_{CW}	0.04	0.4	linear	-0.05
SD_{ada}	-0.2	0.06	linear	0.06
SD_{aba}	0.002	0.84	linear	0.043
SR	-0.21	0.05	linear	0.013
N_{area}	-0.21	0.05	linear	0.02
Transpirational water-loss	Transpirational water-loss	Transpirational water-loss	Transpirational water-loss	Transpira

Traits	MAP	MAP	MAP	MAT
g_{sw}	-0.04	0.38	linear	0
Leaf thickness	-0.35	0.036	polynomial	-0.03
VED	-0.21	0.05	linear	-0.03
VED _{ada}	-0.45	0.01	polynomial	-0.03
VED _{aba}	-0.08	0.25	linear	0.009
IVD	-0.057	0.34	linear	-0.079
Total VLA	0.057	0.34	linear	-0.08
BS _{cw}	-0.39	0.021	polynomial	0
BS _{ias}	0.27	0.026	linear	0
BS area ratio (%)	0.04	0.40	polynomial	0.08

Regression coefficient (R^2), P -values and type of regression model fit are shown. R^2 is shown in bold when P [?] 0.001 (***), P [?] 0.01 (**), P [?] 0.05 (*) and P [?] 0.1 (+). S_{mes} , total mesophyll cell surface area exposed to intercellular air space per unit of leaf surface area; S_c , chloroplast coverage of S_{mes} ; g_m , mesophyll conductance to CO_2 diffusion estimated by Ogee *et al.* (2018); mesophyll cell wall thickness (M_{CW}); SD_{ada} , adaxial stomatal density; SD_{aba} , abaxial stomatal density; SR, stomatal ratio; N_{area} , leaf N content expressed on area basis; stomatal conductance to water vapor diffusion (g_{sw}); VED, average vein-to-epidermis distance; VED_{ada}, vein-to-adaxial epidermis distance; VED_{aba}, vein-to-abaxial epidermis distance; IVD, interveinal distance; total VLA, vein length per unit leaf area; BS_{CW}, BS cell wall thickness; BS_{ias}, BS exposed to intercellular air spaces; BS area ratio (calculated as (BS area / [BS area + Mesophyll area])).







