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Bundle sheath chloroplast volume can house sufficient Rubisco to avoid limiting C₄ photosynthesis during chilling

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Abstract

C₄ leaves confine Rubisco to bundle sheath cells. Thus, the size of bundle sheath compartments and the total volume of chloroplasts within them limit the space available for Rubisco. Rubisco activity limits photosynthesis at low temperatures. C₃ plants counter this limitation by increasing leaf Rubisco content, yet few C₄ species do the same. Because C₃ plants usually outperform C₄ plants in chilling environments, it has been suggested that there is insufficient chloroplast volume available in the bundle sheath of C₄ leaves to allow such an increase in Rubisco at low temperatures. We investigated this potential limitation by measuring bundle sheath and mesophyll compartment volumes and chloroplast contents, as well as leaf thickness and inter-veinal distance, in three C₄ Andropogoneae grasses: two crops (Zea mays and Saccharum officinarum) and a wild, chilling-tolerant grass (Miscanthus × giganteus). A wild C₄ Paniceae grass (Alloteropsis semialata) was also included. Despite significant structural differences between species, there was no evidence of increased bundle sheath chloroplast volume per leaf area available to the chilling-tolerant species, relative to the chilling-sensitive ones. Maximal theoretical photosynthetic capacity of the leaf far exceeded the photosynthetic rates achieved even at low temperatures. C₄ bundle sheath cells therefore have the chloroplast volume to house sufficient Rubisco to avoid limiting C₄ photosynthesis during chilling.

Keywords: Alloteropsis, bundle sheath, C₄ photosynthesis, chilling tolerance, chloroplast, cold tolerance, confocal microscopy, maize, Miscanthus, sugarcane.

Introduction

C₄ photosynthesis involves a biochemical CO₂ concentrating mechanism. In mesophyll cells, the enzyme phosphoenolpyruvate carboxylase assimilates CO₂ into oxaloacetate, which is then metabolized into further C₄ compounds that are transferred to,
and decarboxylated in, bundle sheath (BS) cells to raise [CO₂] around the enzyme Rubisco (von Caemmerer and Furbank, 2003). Rubisco then fixes this CO₂ via the Calvin–Benson cycle in the BS. In C₄ plants, Rubisco is therefore predominantly localized to the chloroplasts of BS cells, where the increased [CO₂] greatly improves photosynthetic efficiency because it effectively eliminates photorespiration, the energetically costly process initiated when O₂ instead of CO₂ is fixed by Rubisco (Hatch, 1987). The BS cells of C₄ leaves are arranged radially around veins and isolated from internal leaf air spaces by surrounding mesophyll cells (Dengler and Nelson, 1999).

Relative to the leaves of C₃ plants, C₄ leaves achieve greater overall BS tissue area via a combination of higher vein density, enlarged BS cells, and more numerous BS cells (Christin et al., 2013; Lundgren et al., 2014).

The enhanced efficiency of C₄ photosynthesis under warm conditions is evident in the high productivity of the Andropogoneae grass crops maize (Zea mays L.), sorghum (Sorghum bicolor (L.) Moench), and sugarcane (Saccharum officinarum L.). However, photosynthesis in the majority of C₄ grasses is characterized by poor chilling tolerance, limiting them to warmer environments (Long, 1983; Sage, 2002; Long and Spence, 2013). Improving chilling tolerance could therefore expand the growing region and lengthen the growth seasons of C₄ crops (Głowacka et al., 2016). Such tolerance of low temperatures has evolved many times in wild C₄ grasses, enabling them to shift their niches into cooler alpine or temperate environments (Warehammongkol et al., 2018).

The mechanisms conferring chilling tolerance to C₃ grasses have been especially well studied in the grass Miscanthus × giganteus Greff et Deu, because of its importance for cellulosic biomass production (Heaton et al., 2010). For example, Z. mays leaves developing at 14 °C have less than 10% of the photosynthetic capacity of Z. mays leaves developing at 25 °C, while leaves of M. × giganteus are unaffected by this temperature difference (Long and Spence, 2013). Another study found that M. × giganteus achieved 59% greater biomass than Z. mays by producing photosynthetically competent leaves earlier in the year and maintaining them several weeks after Z. mays senesced in side–by–side trials in the US Corn Belt (Dohlemann and Long, 2009). This growth advantage may be even more pronounced in the near future, as anthropogenic climate change may cause more frequent and intense springtime chilling events across the US Corn Belt (Kim et al., 2017). Understanding and harnessing the potential of chilling-tolerant C₄ photosynthesis could provide crucial improvements to the yield and robustness of key C₄ crops (Long et al., 2006; Zhu et al., 2010; Yin and Struik, 2017).

Chilling tolerance in C₄ grasses may be linked to leaf anatomy. Because C₄ leaves restrict Rubisco to BS cells, the space potentially available to house this enzyme is roughly halved relative to C₃ leaves, which can accommodate the enzyme in all photosynthetic cells (Pittermann and Sage, 2000). Under moderate temperatures, flux analysis points to Rubisco as a major control point on the rate of CO₂ assimilation in C₄ leaves, as it is in C₃ leaves (Furbank et al., 1997). Since catalytic rate declines with temperature, Rubisco becomes an even greater limitation under chilling, unless its amount is increased (Sage et al., 2011; Long and Spence, 2013). It has been proposed that BS chloroplast volume would limit acclimatory increases in Rubisco in C₄ plants at chilling temperatures (<15 °C), so disadvantaging them relative to their C₃ counterparts (Pittermann and Sage, 2000; Kubien et al., 2003; Kubien and Sage, 2004; Sage and McKown, 2006; Sage et al., 2011). This hypothesis is supported by the observation that leaves of chilling-tolerant C₃ plants often increase Rubisco content during acclimation, whereas this is rarely seen in C₄ leaves (Sage and McKown, 2006; Long and Spence, 2013). Net photosynthetic CO₂ uptake (Aₚ) in C₄ leaves correlates with Rubisco content (Peary, 1977) and activity (Pittermann and Sage, 2000; Kubien and Sage, 2004; Friesen and Sage, 2016) at low (<15 °C), but not high (>25 °C), temperatures. Rubisco’s flux control coefficient over photosynthetic CO₂ assimilation reaches 0.99 (i.e. near–total control) at 6 °C in Flavera bidentis L. Kuntze (Kubien et al., 2003). These observations raise important questions: does Rubisco limit photosynthesis in all C₄ plants at low temperatures, and is this limitation specifically imposed by the restricted space available in the BS to house the enzyme?

Under chilling conditions, the chilling-tolerant M. × giganteus maintains photosynthetic capacity and, unusually, maintains or slightly increases leaf Rubisco content per unit leaf area, while showing large increases in pyruvate P₄ dikinase (PPDK) expression (Naidu et al., 2003; Wang et al., 2008b; Long and Spence, 2013). Accessions of M. sacchariflorus, one of the parent species of M. × giganteus, achieved some of the highest light-saturated rates of leaf CO₂ uptake (Aₚ>16 µmol m⁻² s⁻¹) recorded for any plant grown and measured at 15 °C (Głowacka et al., 2015), showing that this species must accumulate sufficient Rubisco to support such high photosynthetic rates. Of course, there is the possibility that these Miscanthus genotypes are exceptional in providing unusually large bundle sheath chloroplast volumes.

Coinciding with the acclimation of C₄ cycle enzymes in Miscanthus, the up-regulation of key photoprotective mechanisms reduces damage to photosystem II (Farage et al., 2006). This suggests that decreased photosynthetic rates in most C₄ grasses at low temperature have multiple causes rather than arising from one inherent limitation. Indeed, comparative transcriptomics has suggested that the chilling tolerance of photosynthesis in M. × giganteus corresponds to the up-regulation of genes encoding several photosynthetic proteins (Spence et al., 2014). Miscanthus × giganteus maintains the linear relationship between operating photochemical efficiency of photosystem II and the quantum efficiency of CO₂ assimilation during chilling, suggesting that the balance of C₃ and C₄ cycles is not compromised (Naidu and Long, 2004). In total, these findings suggest that Rubisco is not the sole limitation to C₄ photosynthesis at chilling temperatures, and that any volume limitation imposed by restriction of the enzyme to the bundle sheath can be overcome, at least in the case of M. × giganteus and related species (Long and Spence, 2013).

Because most Rubisco in C₄ leaves is confined to BS chloroplasts, a measure of the total volume of chloroplasts in the BS is required to determine if there is enough space available to increase Rubisco content in C₄ leaves. However, most attempts at chloroplast quantification have not documented 3D measurements, but rather chloroplast counts and 2D planar...
area (Pyke and Leech, 1987; Brown and Hattersley, 1989; Stata et al., 2014, 2016). With confocal laser scanning microscopy, it is possible to measure chloroplast volume directly from an optically produced 3D image (Park et al., 2009; Coate et al., 2012). Chloroplast measurements have previously been made on fixed, dehydrated samples in accordance with TEM imaging procedures (Sage and Williams, 1995). While this method is adequate for relative comparisons of chloroplast size and number between plant taxonomic clades or functional types (Stata et al., 2016; Stata et al., 2014), it may distort chloroplast shape and prevent accurate estimation of absolute chloroplast volume in vivo. Cryo-sectioning and analysis of fresh plant material may prevent this type of distortion.

To test the hypothesis that BS chloroplast volume restricts the capacity for Rubisco to the extent that it would limit photosynthesis in C₄ grasses, chloroplast volume and associated leaf anatomical characteristics were measured, and used to calculate the amount and activity of Rubisco that could be supported on a leaf area basis. The focus of the study was on grasses of the Andropogoneae: since M. x giganteus appears to escape the low temperature limitation observed in most C₄ grasses, its BS chloroplast volumes were compared to two chilling-intolerant crop species of the same tribe (Z. mays and S. officinarum). The unrelated, non-Andropogoneae, non-crop and chilling-intolerant C₄ grass (Alloteropsis semialata J. Presl) was also included in the study (Osborne et al., 2008).

Materials and methods

Plant material

Measurements were taken on Z. mays cv. FR1064, S. officinarum hybrid cultivar cv. CP888-1762, a C₄ lineage of A. semilata originating from South Africa (Osborne et al., 2008), and the ‘Illinois’ clone of M. x giganteus. Miscanthus × giganteus was grown in the field and the other species were grown in a controlled-environment greenhouse, maintained between 25 and 30 °C with high pressure sodium lamps ensuring an average photon flux of 450 µmol m⁻² s⁻¹ over a 12 h photoperiod.

Miscanthus × giganteus was grown at the University of Illinois Agricultural Research Station farm near Champaign, IL, USA (40°09’2’’N, 88°14’22’’W, 228 m above sea level). Soils at this site are deep Drummer/Flanagan series (a fine silt, mixed, mesic Typic Endoaquoll) with high organic matter typical of the central Illinois Corn Belt. Fertilizer was not applied. As in previous studies, the youngest fully expanded leaf of M. × giganteus plants, as judged by ligule emergence, was sampled in July (Dohleman et al., 2012; Arundale et al., 2014a,b; Pignon et al., 2017). Alloteropsis semialata and Z. mays seeds were germinated on moist filter paper in a growth chamber maintained at 25 °C with an average photon flux of 200 µmol m⁻² s⁻¹. They were then transferred to pots of soil-less cultivation medium (LC1 Sunshine Mix, Sun Gro Horticulture, Agawam, MA, USA), with additional coarse sand and perlite mixed into pots for A. semilata. Single stem segments of S. officinarum were planted directly into pots of a second soil-less cultivation medium (Metromix 900; SunGro Horticulture). All pots were watered daily to field capacity. Zea mays was initially fertilized with granulated fertilizer (Osomocote Pus 15/9/12, The Scotts Company LLC, Marysville, OH, USA) followed by general nutrient solution (Peter’s Excel 15-5-5, Everris NA Inc., Dublin, OH, USA) and iron chelate supplement (Sprint 330, BASF Corp, NC, USA) added to the watering regime once every week. Alloteropsis semialata and S. officinarum were fertilized with granulated fertilizer (Osomocote Classic 13/13/13, The Scotts Company LLC), and A. semilata supplemented with iron chelate (Sprint 330, BASF Corp.). Plants were grown until at least the fifth leaf was fully expanded, as judged by ligule emergence, and the youngest fully expanded leaf was sampled.

Sample preparation and measurement

On sampling, leaves were immediately immersed in a glycol and resin based cryostat embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek, Torrance, CA, USA), which provides solid sectioning support on dry ice. Transverse sections of 40 µm were cut (Leica CM3050 S, Leica Biosystems, Wetzlar, Germany) and mounted on glass slides. Slides were then immersed for 15 min in a cell membrane and wall dye solution (FM 1–43FX, Thermo Fisher Scientific, Waltham, MA, USA), and diluted to 3.6 mM in dimethylsulfoxide (Thermo Fisher Scientific) and water, in order to image cell walls. Samples were imaged with a confocal laser-scanning microscope (LSM 780, Carl Zeiss AG, Oberkochen, Germany). Images were acquired through a ×63 oil-immersion objective (×63 Plan-Apochromat, Carl Zeiss AG) for M. × giganteus. It was determined that reduced magnification could be used to widen the field of view while still providing accurate estimates of chloroplast volume. Therefore a ×40 oil-immersion objective (×40 Plan-Apochromat, Carl Zeiss AG) was used for Z. mays, S. officinarum, and A. semilata. The fluorescence of dye-labelled cell walls was analysed by excitation at 555 nm, and emission was detected at a bandpass of 405–630 nm. Chlorophyll was excited at 633 nm, and its fluorescence emission was detected at a bandpass of 630–700 nm. Serial optical sections were obtained at 1-µm depth intervals, i.e. in the z-axis (Zen software, Carl Zeiss AG). Although sampling depth (8–15 µm in the z-axis) was insufficient to capture whole BS cells, each leaf section contained a random sampling of cells, which avoided the risk of biasing measurements due to non-homogeneous chloroplast distribution through the length of the cell. Supplementary Video S1 at JXB online illustrates how the delineation of BS and mesophyll compartments, and the chloroplasts within them, was achieved within a 3D optical section. BS and mesophyll compartments were identified from the fluorescence of dye-labelled cell walls, using image segmentation software (IMARIS 7.0.0 software, BitPlane, Inc., Zürich, Switzerland). These segments were used to determine the volume of BS (vol BS) and mesophyll (vol M) per leaf unit area. The chlorophyll fluorescence signal within the BS and mesophyll was then used to determine total chloroplast volume per unit leaf area within each compartment (vol BS,sp and vol M,sp respectively) and the percentage occupancy of each compartment by chloroplasts (% BS,sp and % M,sp respectively). Although chlorophyll fluorescence from out-of-focus planes was typically visible in individual optical slices, the surface-finding algorithm of the image segmentation software was able to accurately delineate chloroplast volumes when processing the overall 3D optical section. As a result, individual 2D slices appear to overestimate chloroplast content of cells, but the 3D sections actually used to produce measurements do not; this can be seen by comparing Fig. 1C with Supplementary Videos S1. Leaf thickness was measured in a single location per image, across the mesophyll between two veins, and inter-venal distance (IVD) was measured as the average distance between the centers of all the adjacent vascular bundles visible in each image.

Calculating photosynthetic capacity

An important goal of this study was to determine the theoretical maximum amount of Rubisco that C₄ BS chloroplasts could contain, in order to calculate the corresponding theoretical maximum level of Rubisco-limited photosynthetic CO₂ uptake (A max sat), that could be achieved by a given leaf. Calculated values for A max sat could then be compared to achieved values for light-saturated photosynthetic CO₂ uptake (A sat). Because A max sat is a measure of theoretical, and not achieved, Rubisco-limited CO₂ uptake, factors such as leaf N content and incident light intensity could be ignored. Instead, A sat was determined from the volume of BS chloroplasts available for Rubisco investment (vol BS,sp), the amount of Rubisco that could be contained within these chloroplasts, and the carboxylation activity of Rubisco. Although there is evidence of C₄ subspecies of A. semilata expressing Rubisco in chloroplasts outside of the BS (Ueno and Sentoku, 2006), here it was assumed in all species that only BS chloroplasts contained Rubisco.

vol BS,sp was determined experimentally in this study as described above. A Rubisco carboxylation rate per site at 25 °C (k cat) of 3.3 mol CO₂ mol⁻¹ s⁻¹ had been determined previously for both Z. mays and
and M. × giganteus (Wang et al., 2008a). This value was reduced by 15%, reflecting the Rubisco activation state at 25 °C of 85%, reported for M. × giganteus (Wang et al., 2008a). This gives an estimated carboxylation rate of 41.6 μmol CO$_2$ g$^{-1}$ Rubisco s$^{-1}$ at 25 °C. Rubisco content per unit chloroplast volume was assumed to be 2.2 × 10$^3$ g Rubisco m$^{-3}$ chloroplast based on measurements for mesophyll chloroplasts of several genotypes of the hexaploid bread wheat *Triticum aestivum* L. (Pyke and Leech, 1987). Combining the carboxylation rate per gram Rubisco calculated with a molecular mass of 540 kDA, with the grams of Rubisco per unit volume of chloroplast, leads to a theoretical maximal photosynthetic rate of 9.2 mol CO$_2$ m$^{-3}$ chloroplast s$^{-1}$ at 25 °C. In the Results, this factor is combined with measured BS chloroplast volume (vol$_{BS,cp}$) to determine the potential photosynthetic rate that could theoretically be supported given the measured chloroplast volume ($A_{mes,cp}$).

To extend this estimation to temperatures below 25 °C, an Arrhenius function was used based on the activation energy ($E_a$) of 78 kJ mol$^{-1}$ determined for Rubisco in the C$_4$ grass *Setaria viridis* (L.) P.Beauv. (Boyd et al., 2015). To compare this estimation with achieved photosynthesis values, the literature was reviewed to identify values for light-saturated net leaf CO$_2$ uptake ($A_{n,s}$) at moderate and chilling temperatures in all four species: *Z. mays* (Long, 1983; Naidu et al., 2003; Naidu and Long, 2004; Glowacka et al., 2016), *S. officinarum* (Spitz, 2015; Glowacka et al., 2016), *A. semialata* (Osborne et al., 2008), and *M. × giganteus* (Naidu et al., 2003; Naidu and Long, 2004; Glowacka et al., 2014, 2015, 2016; Spitz, 2015; Friesen and Sage, 2016), using values measured at different temperatures and at a photon flux ≥1000 μmol m$^{-2}$ s$^{-1}$.

### Statistical analysis

Replication was: *Z. mays* (n=7), *S. officinarum* (n=5), *A. semialata* (n=6), and *M. × giganteus* (n=6). Statistical analysis was performed on the following parameters: leaf thickness, IVD, vol$_{BS}$, vol$_{M}$, vol$_{BS,cp}$, vol$_{M,cp}$, %$_{BS}$, and %$_{M}$.

The fixed effect of species on each parameter was tested by one-way ANOVA (PROC GLM, SAS v8.02; SAS Institute Inc., Cary, NC, USA), with homogeneity of variances tested by Levene and normality of residuals tested by Shapiro–Wilk (PROC UNIVARIATE, SAS v8.02) at a $P=0.05$ threshold. A Tukey test was performed alongside the ANOVA at a $P=0.05$ threshold in order to identify significant pairwise differences between species. When no significant differences were found, the test was repeated at a $P=0.1$ threshold to reduce the risk of a type II error given the relatively low replication for each species.

### Results

The average volume of chloroplasts per unit leaf area ranged from $6 \times 10^{-6}$ to $10 \times 10^{-6}$ m$^3$ m$^{-2}$ in the BS and from $10 \times 10^{-6}$ to $14 \times 10^{-6}$ m$^3$ m$^{-2}$ in the mesophyll (Figs 1, 2, 3E, F). There was no evidence of greater BS chloroplast volume available per unit leaf area (vol$_{BS,cp}$) in the chilling-tolerant *M. × giganteus* compared with the chilling-sensitive species. On the contrary, *M. × giganteus* had the smallest BS chloroplast volume per unit
leaf area, at ca. 40% less than the wild and chilling-sensitive A. semialata. Although there were no significant differences between species in volBS, significantly greater occupancy of the BS by chloroplasts (%BS,cp) resulted in greater volBS,cp overall in A. semialata (Fig. 3C, E, G).

Across the four study-species, chloroplasts occupied 15–30% of the BS (%BS,cp), and 8–14% of the mesophyll (%M,cp) (Figs 1, 3G, H; Supplementary Video S1). Between species, %BS,cp and %M,cp were significantly highest and lowest, respectively, in A. semialata. Leaf thickness ranged from 100 to 250 µm, with veins spaced 100–140 µm apart on average (Figs 1, 3A, B). Allotropis semialata leaves at ca. 225 µm were nearly twice as thick as those of M. × giganteus at ca. 125 µm. The distance between veins (IVD) in the two crops (Z. mays and S. officinarum) was ca. 40% greater than in the two wild species (M. × giganteus and A. semialata) (Fig. 3B). Across the species, the volume of mesophyll per unit leaf area (volM) generally mirrored leaf thickness, though due to a thick epidermis the significantly greater leaf thickness of A. semialata did not result in a substantially greater volM (Fig. 3D). BS volume per unit leaf area (volBS), however, was conserved across species at ca. 40 × 10⁻⁶ m⁻² (Fig. 3C).

When the maximal theoretical photosynthetic capacity of the leaf (Amax,cp) was estimated from volBS,cp, values ranged from ca. 60 to 90 µmol m⁻² s⁻¹ at 25 °C. This was substantially greater than published values of light-saturated net photosynthetic CO₂ uptake (Aₙ₉) for these species at this temperature (Fig. 4). However, at lower temperatures Aₙ₉ was closer to Amax,cp, with Aₙ₉ being 20–90% of Amax,cp at 5 °C.

**Discussion**

In all four of the C₄ grass species studied here, the volume of BS per unit leaf area available for Rubisco (volBS) was not a limitation for observed rates of photosynthesis, even at chilling temperatures. This conclusion is based on two key findings, derived from 3D confocal microscopy and analysis of leaf structure (Fig. 2). First, the chilling-tolerant M. × giganteus (Long and Spence, 2013) has a smaller BS chloroplast volume per unit leaf area (volBS,cp) than the chilling-sensitive C₄ grasses S. officinarum, A. semialata, and Z. mays (Fig. 3). Second, the theoretical maximum level of Rubisco-restricted photosynthetic CO₂ uptake (Amax,cp) that could be achieved by each species was greater than realized levels of Aₙ₉ even at chilling temperatures (Fig. 4). This study focused on closely related C₄ grasses of the Andropogoneae clade, which contain the major C₄ crops as well as candidate bioenergy crops. Even A. semialata, which descends from a separate evolutionary lineage in the Paniceae, would not suffer from limitation of photosynthesis by volBS during chilling.

Several leaf structural characteristics, including leaf thickness, IVD, volM, %BS,cp, and %M,cp, varied significantly between species (Figs 1, 3A, B, D, G, H). Indeed, the volBS,cp was actually greatest in the chilling-sensitive A. semialata and lowest in the chilling-tolerant M. × giganteus (Fig. 3E). This clearly demonstrates that volBS,cp does not determine chilling tolerance in C₄ plants, and therefore that the volume of BS chloroplast available for leaf Rubisco investment is unlikely to meaningfully restrict C₄ photosynthesis at low temperatures.

Based on 2D leaf profiles, the percentage occupancy of the total mesophyll volume by chloroplasts varies significantly between photosynthetic types and taxonomic clades of diverse C₄ plants, with an average occupation of ca. 12.2% (Stata et al., 2014), which is similar to the 8–14% range seen here (Figs 1, 3H). In various species of the eudicot genus Flaveria that use the NADP-ME subtype of C₄ photosynthesis, chloroplasts occupied 12–18% of the total BS volume (Stata et al., 2016), which is somewhat lower than the range of 15–25% seen in our grasses (Figs 1, 3G); this may reflect differences due to taxonomy or specimen preparation. Allotropis semialata, which belongs to the Paniceae tribe, had the greatest volume of chloroplast in the BS (%BS,cp) (Figs 1, 3G, H). This may reflect this species’ need to house grana in their BS chloroplasts, while...
the other three studied grasses of the Andropogoneae tribe have little to no BS chloroplast grana (Ueno and Sentoku, 2006). *Alloteropsis semialata*'s high BS chloroplast volume may also result from the very recent development of C₄ anatomy in this species, which might not have evolved the faster Rubisco kinetics of other, older C₄ lineages and could therefore require relatively more Rubisco in the BS to compensate (Lundgren et al., 2015; Dunning et al., 2017).

While chloroplasts across the entire mesophyll tissue are available for Rubisco investment in C₃ plants, there is clearly less space available in the BS tissue of C₄ leaves. However, in the mesophyll of C₃ species, CO₂ must diffuse from the air space to Rubisco in the chloroplast, and chloroplasts must be adjacent to the cell wall to maximize mesophyll conductance to CO₂ and facilitate access of Rubisco to CO₂ (Evans and Loreto, 2000; Flexas et al., 2008). In the BS of C₄ species, CO₂ results from decarboxylation of C₄-dicarboxylates in the chloroplast or the cytosol, and the effective chloroplast volume is therefore not limited by the area of wall adjacent to air space. In effect, this can allow larger and more numerous chloroplasts,
and may explain the greater proportion of the BS cell occupied by chloroplasts, relative to mesophyll (Figs 1, 3G, H).

The comparison of \( A_{\text{max,.cp}} \) to published values for \( A_{\text{sat}} \) is directly dependent on terms used to calculate \( A_{\text{max,.cp}} \); for instance, a 20% lower value for \( k_{\text{sat}} \) will result in 20% lower \( A_{\text{max,.cp}} \). At lower temperatures this could lead to \( A_{\text{max,.cp}} \) much closer to published values for \( A_{\text{sat}} \) (Fig. 4A, B). However, the values used in this study were generally conservative. In a survey of Rubisco \( k_{\text{sat}} \) in 14 grasses using different subtypes of C\(_4\) photosynthesis (Ghanoum et al., 2005), all seven NADP-ME grasses and five of the seven NAD-ME grasses registered values greater than, and up to two times, the \( k_{\text{sat}} \) value used here; i.e. 3.3 mol CO\(_2\) mol site\(^{-1}\) s\(^{-1}\) (Wang et al., 2008a).

Another important term in the calculation of \( A_{\text{max,.cp}} \) is the Rubisco content per unit volume chloroplast. Here, we used a published value of 0.41 mol Rubisco m\(^{-3}\) chloroplast, derived from \( T. \text{aestivum} \) mesophyll chloroplasts (Pyke and Leech, 1987). This value is conservative, as it is at the lower end of the 0.4–0.5 mol Rubisco m\(^{-3}\) chloroplast range predicted from measurements in C\(_3\) chloroplasts (Jensen and Bahr, 1977). Furthermore, C\(_4\) plants generally produce larger chloroplasts than C\(_3\) plants, particularly in the BS (Brown and Hattersley, 1989; Stata et al., 2014) and these chloroplasts likely contain more Rubisco per unit volume, since NADP-ME C\(_4\) grasses, including \( Z. \text{mays}, S. \text{officinarum}, \) and \( M. \times \text{giganteus}, \) typically show few or no stacked thylakoids in the BS. This arrangement leaves more space available for stroma, and therefore Rubisco, in comparison with bread wheat chloroplasts (Furbank, 2011; Voznesenskaya et al., 2006, 2007; Bellasio and Griffiths, 2014).

Despite the use of conservative terms to calculate \( A_{\text{max,.cp}} \), this parameter was greater than published light-saturated photosynthetic rates (\( A_{\text{sat}} \)) for all four studied species (Fig. 4) (Long, 1983; Naidu et al., 2003; Naidu and Long, 2004; Osborne et al., 2008; Glowacka et al., 2014, 2015, 2016; Spitz, 2015; Friesen and Sage, 2016). This was even true at low temperatures, where Rubisco has been predicted to be a strong limitation to \( C_4 \) photosynthesis (Parcy, 1977; Pittermann and Sage, 2000; Kubien et al., 2003; Kubien and Sage, 2004). Therefore, we conclude that while the quantity of Rubisco may be limiting, this is not an inherent result of the smaller proportion of cells that can contain the enzyme in \( C_4 \) leaves with Kranz anatomy. Further supporting our conclusion that BS chloroplast space does not limit Rubisco comes from the fact that Rubisco content does increase in \( M. \times \text{giganteus} \) on chilling (Long and Spence, 2013). Additional evidence comes from a recent transgenic up-regulation of Rubisco content by >30% above wild type in leaves of \( Z. \text{mays} \) (Salesse et al., 2018).

Based on genetic diversity, the assumed origin of the \( C_4 \) grass tribe Andropogoneae is tropical Southeast Asia (Harley, 1958; Arthan et al., 2017). Tropical origins are common across

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**Fig. 4.** Comparison of theoretical maximum versus achieved leaf photosynthetic carboxylation rates at different temperatures. (A) Symbols indicate published rates of net \( CO_2 \) uptake (\( A_{\text{sat}} \)) measured on leaves at different temperatures. Lines show estimated leaf maximal photosynthetic capacity (\( A_{\text{max,cp}} \)) calculated from bundle sheath chloroplast volume per unit leaf area. (B) Measurements of \( A_{\text{sat}} \) expressed as a percentage of \( A_{\text{max,cp}} \). Measurements were obtained for \( Z. \text{mays} \) (Long, 1983; Naidu et al., 2003; Naidu and Long, 2004; Glowacka et al., 2016), \( S. \text{officinarum} \) (Spitz, 2015; Glowacka et al., 2016), \( A. \text{semalata} \) (Osborne et al., 2008), \( M. \times \text{giganteus} \) (Greef et al., 2015), and \( M. \text{spicata} \) (Naidu et al., 2003; Naidu and Long, 2004; Glowacka et al., 2014, 2015, 2016; Spitz, 2015; Friesen and Sage, 2016) at different temperatures and at an incident photon flux ≥1000 \( \mu \text{mol} \text{m}^{-2}\text{s}^{-1} \).
the C₄ grass clades (Watcharamongkol et al., 2018). Radiation into temperate climates has therefore involved solving the challenges of chilling and freezing temperatures faced by all tropical plants, regardless of photosynthetic type, as well as any additional restrictions added by the C₄ cycle and associated anatomy. The literature has already addressed these additional restrictions and the evolution of chilling-tolerant C₄ photosynthesis (Long, 1983, 1999; Long and Spence, 2013).

Several C₃ grasses, including Muhlenbergia glomerata (Kubien and Sage, 2004), Spartina anglica (Long et al., 1975), and Cleistogenes squarrosa (Liu and Osborne, 2008) can achieve rates of CO₂ assimilation at chilling temperatures that equal or exceed rates achieved by temperate and even arctic/alpine C₃ grasses. Notably, the C₄ grass M. × giganteus appears exceptional in its ability to acclimate its photosynthetic apparatus to chilling temperatures. Comparison with the chilling-intolerant Z. mays suggests that chilling tolerance in M. × giganteus results from its ability to maintain and increase the expression of the enzymes PPDK and Rubisco, as well as increase leaf xanthophyll content, in particular zeaxanthin, to harmlessly dissipate excess absorbed light energy under chilling conditions and protect photosystem II from oxidative damage (reviewed in Long and Spence, 2013). Gene expression analyses suggest that these increases are part of a syndrome of acclimative changes that allow efficient C₄ photosynthesis under chilling conditions (Spence et al., 2014), and in turn the exceptional productivity achieved by M. × giganteus in temperate climates (Dohleman and Long, 2009). Therefore, while Rubisco content clearly co-limits photosynthesis in many C₃ species under chilling conditions, the findings here show that this does not directly result from restricting Rubisco to the BS in C₄ grasses.

In conclusion, while the volume of the cells that can hold Rubisco in C₄ grass leaves is lower than in their C₃ counterparts, measurements of BS chloroplast volume show that space per se does not present a physical, and in turn intrinsic, limitation on photosynthesis at chilling temperatures. Therefore, restriction of leaf Rubisco content by the volume of BS chloroplasts does not inherently limit the adaptation of C₄ grasses to cold environments.

**Supplementary data**

Video S1. Video of the full 3D image of leaf, bundle sheath cells, mesophyll cells, and chloroplasts seen in Fig. 2.

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**References**

Arthan W, McKain MR, Traiperm P, Welker CAD, Teisher JK, Kellogg EA. 2017. Phylgenomics of Andropogoneae (Panicaeae: Poaceae) of Mainland Southeast Asia. Systematic Botany 42, 418–431.

Arundale RA, Dohleman FG, Heaton EA, McGrath JM, Voigt TB, Long SP. 2014a. Yields of Miscanthus × giganteus and Panicum virgatum decline with stand age in the Midwestern USA. Global Change Biology Bioenergy 6, 1–13.

Arundale RA, Dohleman FG, Voigt TB, Long SP. 2014b. Nitrogen fertilization does significantly increase yields of stands of Miscanthus × giganteus and Panicum virgatum in multiyear trials in Illinois. Bioenergy Research 7, 408–416.

Bellasio C, Griffiths H. 2014. Acclimation of C₄ metabolism to low light in mature maize leaves could limit energetic losses during progressive shading in a crop canopy. Journal of Experimental Botany 65, 3725–3736.

Boyd RA, Gandin A, Cousins AB. 2015. Temperature responses of C₄ photosynthesis: biochemical analysis of rubisco, phosphoenolpyruvate carboxylase, and carbonic anhydrase in Setaria viridis. Plant Physiology 169, 1850–1861.

Brown RH, Hattersley PW. 1989. Leaf anatomy of C₄-C₃ species as related to evolution of C₃ photosynthesis. Plant Physiology 91, 1543–1550.

Christina PS, Osborne CP, Chatelot DS, Columbus JT, Besnard G, Hodkinson TR, Garrison LM, Vorontsova MS, Edwards EJ. 2013. Anatomical enablers and the evolution of C₄ photosynthesis in grasses. Proceedings of the National Academy of Sciences, USA 110, 1381–1386.

Coate JE, Luciano AK, Seralathan V, Minchew KJ, Owens TG, Doyle JJ. 2012. Anatomical, biochemical, and photosynthetic responses to recent allopolyploidy in Glycine dolichocarpa (Fabaceae). American Journal of Botany 99, 55–67.

Dengler NG, Nelson T. 1999. Leaf structure and development in C₃ plants. In: Sage RF, Monson RK, eds. C₃ plant biology. San Diego, CA, USA: Academic Press, 133–172.

Dohleman FG, Heaton EA, Arundale RA, Long SP. 2012. Seasonal dynamics of above- and below-ground biomass and nitrogen partitioning in Miscanthus × giganteus and Panicum virgatum across three growing seasons. Global Change Biology Bioenergy 4, 534–544.

Dohleman FG, Long SP. 2009. More productive than maize in the Midwest: how does miscanthus do it? Plant Physiology 150, 2104–2115.

Dunning LT, Lundgren MR, Moreno-Villena JJ, Namaganda M, Edwards EJ, Nosil P, Osborne CP, Christin PA. 2017. Intron reorganization and repeated co-option facilitated the recurrent emergence of C₄ photosynthesis among close relatives. Evolution 71, 1541–1555.

Evans JR, Loreto F. 2000. Acclimation and diffusion of CO₂ in higher plant leaves. In: Leegood RC, Sharkey TD, Von Caemmerer S, eds. Photosynthesis: physiology and metabolism. Dordrecht, The Netherlands: Kluwer, 321–351.

Farage PK, Blowers D, Long SP, Baker NR. 2006. Low growth temperatures modify the efficiency of light use by photosystem II for CO₂ assimilation in leaves of two chilling-tolerant C₃ species, Cyperus longus L. and Miscanthus × giganteus. Plant, Cell & Environment 29, 720–728.

Flexas J, Ribas-Carbó M, Díaz-Depejo A, Galmés J, Medrano H. 2008. Mesophyll conductance to CO₂: current knowledge and future prospects. Plant, Cell & Environment 31, 602–621.

Friesen PC, Sage RF. 2016. Photosynthetic responses to chilling in a chilling-tolerant and chilling-sensitive Miscanthus hybrid. Plant, Cell & Environment 39, 1420–1431.

Furbank RT. 2011. Evolution of the C₄ photosynthetic mechanism: are there really three C₄ acid decarboxylation types? Journal of Experimental Botany 62, 3103–3108.

Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S, Ashton AR. 1997. Genetic manipulation of key photosynthetic enzymes in the C₃ plant Flaveria bidentis. Australian Journal of Plant Physiology 24, 477–485.

Ghannoum O, Evans JR, Chow WS, Andrews TJ, Conroy JP, von Caemmerer S. 2005. Faster Rubisco is the key to superior nitrogen-use efficiency in NADP-malic enzyme relative to NAD-malic enzyme C₄ grasses. Plant Physiology 137, 638–650.

Glowacka K, Adhikari S, Peng J, Gifford J, Juvik JA, Long SP, Sacks EJ. 2014. Variation in chilling tolerance for photosynthesis and leaf extension growth among genotypes related to the C₄ grass Miscanthus × giganteus. Journal of Experimental Botany 65, 5267–5278.

Glowacka K, Ahmed A, Sharma S, Abbott T, Comstock JC, Long SP, Sacks EJ. 2016. Can chilling tolerance of C₄ photosynthesis in Miscanthus be transferred to sugarcane? Global Change Biology Bioenergy 8, 407–418.

Glowacka K, Jorgensen U, Kjeldsen JB, Kerup K, Spitz I, Sacks EJ, Long SP. 2015. Can the exceptional chilling tolerance of C₄ photosynthesis found in Miscanthus × giganteus be exceeded? Screening of a novel...
Miscanthus Japanese germplasm collection. Annals of Botany 115, 981–990.

Hartley W. 1958. Studies on the origin, evolution, and distribution of the Gramineae. II. The tribe Paniceae. Australian Journal of Botany 6, 343–357.

Hatch MD. 1987. C₄ photosynthesis – a unique blend of modified biochemistry, anatomy and ultrastructure. Biochemical and Biophysical Acta 895, 81–106.

Heaton EA, Dohleman FG, Miguez AF, et al. 2010. Miscanthus: a promising biomass crop. Advances in Botanical Research 56, 75–137.

Jensen RG, Bahr JT. 1977. Ribulose 1,5-bisphosphate carboxylase-oxygenase. Annual Review of Plant Physiology and Plant Molecular Biology 28, 379–400.

Kim J-S, Kug J-S, Jeong S-J, Huntzinger DN, Michalak AM, Schwalm CR, Wei Y, Schaefer K. 2017. Reduced North American terrestrial primary productivity linked to anomalous Arctic warming. Nature Geoscience 10, 572–576.

Kubien DS, Sage RF. 2004. Low-temperature photosynthetic performance of a C₃ grass and a co-occurring C₄ grass native to high latitudes. Plant Cell and Environment 27, 907–916.

Kubien DS, von Caemmerer S, Furbank RT, Sage RF. 2003. C₄ photosynthesis at low temperature. A study using transgenic plants with reduced amounts of Rubisco. Plant Physiology 132, 1577–1585.

Liu MZ, Osborne CP. 2008. Leaf cold acclimation and freezing injury in C₃ and C₄ grasses of the Mongolian Plateau. Journal of Experimental Botany 59, 4161–4170.

Long SP. 1983. C₄ photosynthesis at low temperatures. Plant Cell and Environment 6, 345–363.

Long SP. 1999. Environmental responses. In: Sage RF, Monson RF, eds. C₄ plant biology. San Diego, CA: Academic Press, 215–249.

Long SP, Incoll LD, Woolhouse HW. 1975. C₄ photosynthesis in plants from cool temperate regions, with particular reference to Spartina townsendii. Nature 257, 622–624.

Long SP, Spence AK. 2013. Toward cool C₄ crops. Annual Review of Plant Biology 64, 701–722.

Long SP, Zhu XG, Naidu SL, Ort DR. 2006. Can improvement in photosynthesis increase crop yields? Plant, Cell & Environment 29, 315–330.

Lundgren MR, Besnard G, Ripley BS, et al. 2015. Photosynthetic innovation broadens the niche within a single species. Ecology Letters 18, 1021–1029.

Lundgren MR, Osborne CP, Christin PA. 2014. Deconstructing Kranz anatomy to understand C₄ evolution. Journal of Experimental Botany 65, 3357–3369.

Naidu SL, Long SP. 2004. Potential mechanisms of low-temperature tolerance of C₄ photosynthesis in Miscanthus × giganteus: an in vivo analysis. Planta 220, 145–155.

Naidu SL, Moore SP, Al-Shoaiab AI, Raines CA, Long SP. 2003. Cold tolerance of C₄ photosynthesis in Miscanthus × giganteus: adaptation in amounts and sequence of C₄ photosynthetic enzymes. Plant Physiology 132, 1685–1697.

Osborne CP, Wythe EJ, Ibrahim DG, Gilbert ME, Ripley BS. 2008. Low temperature effects on leaf physiology and survivorship in the C₃ and C₄ subspecies of Alloteropsis semialata. Journal of Experimental Botany 59, 1743–1754.

Park J, Knoblauch M, Okita TW, Edwards GE. 2009. Structural changes in the vacuole and cytoskeleton are key to development of the two cytoplasmic domains supporting single-cell C₄ photosynthesis in Bnetera sinuspersici. Planta 229, 369–382.

Pearcy RW. 1977. Accelitation of photosynthesis and respiratory carbon dioxide exchange to growth temperature in Atriplex lantiformis (Torr.) Wats. Plant Physiology 59, 795–799.

Pignon CP, Jaiswal D, McGrath JM, Long SP. 2017. Loss of photosynthetic efficiency in the shade. An Achilles heel for the dense modern stands of our most productive C₃ crops? Journal of Experimental Botany 68, 335–345.

Pittermann J, Sage RF. 2000. Photosynthetic performance at low temperature of Bouteloua gracilis Lag., a high-altitude C₃ grass from the Rocky Mountains, USA. Plant Cell & Environment 23, 811–823.

Pyke KA, Leech RM. 1987. Cellular levels of ribulose 1,5-bisphosphate carboxylase and chloroplast compartment size in wheat mesophyll cells. Journal of Experimental Botany 38, 1949–1956.

Sage RF. 2002. Variation in the K₅ₛ of Rubisco in C₃ and C₄ plants and some implications for photosynthetic performance at high and low temperature. Journal of Experimental Botany 53, 609–620.

Sage RF, Kocacinar F, Kubien DS. 2011. C₄ photosynthesis and temperature. In: Raghavendra AS, Sage RF, eds. C₄ photosynthesis and related CO₂ concentrating mechanisms. Advances in Photosynthesis and Respiration, Vol. 32. Dordrecht, Netherlands: Springer, 1–410.

Sage RF, McKown AD. 2006. Is C₄ photosynthesis less phenotypically plastic than C₃ photosynthesis? Journal of Experimental Botany 57, 303–317.

Sage TL, Williams EG. 1995. Structure, ultrastructure, and histochemistry of the pollen-tube pathway in the milkwheat Asclepias exaltata L. Sexual Plant Reproduction 8, 257–265.

Sallese C, Sharwood R, Busch FA, Kromdijk J, Bardal V, Stern D. 2018. Overexpression of Rubisco subunits with RAF1 increases Rubisco content in maize. Nature Plants 4, 802–810.

Spence AK, Boddu J, Wang D, James B, Swaminathan K, Moose SP, Long SP. 2014. Transcriptional responses indicate maintenance of photosynthetic proteins as key to the exceptional chilling tolerance of C₄ photosynthesis in Miscanthus giganteus. Journal of Experimental Botany 65, 3737–3747.

Spitz I. 2015. Improving C₄ photosynthetic chilling tolerance in bioenergy crops: the search for elite breeding materials. MS Thesis, University of Illinois at Urbana-Champaign.

Stata M, Sage TL, Hoffmann N, Covshoff S, Ka-Shuo Wong G, Sage RF. 2016. Mesophyll chloroplast investment in C₃, C₄, and C₅ species of the genus Flaveria. Plant & Cell Physiology 57, 904–918.

Stata M, Sage TL, Rennie TD, Khosharev S, Sultmanis S, Khaikin Y, Ludwig M, Sage RF. 2014. Mesophyll cells of C₃ plants have fewer chloroplasts than those of closely related C₄ plants. Plant, Cell & Environment 37, 2587–2600.

Unno O, Sentoku N. 2006. Comparison of leaf structure and photosynthetic characteristics of C₃ and C₄ Alloteropsis semialata subspecies. Plant, Cell & Environment 29, 257–268.

von Caemmerer S, Furbank RT. 2003. The C₃ pathway: an efficient CO₂ pump. Photosynthesis Research 77, 191–207.

Voznesenskaya EV, Franceschi VR, Chuong SD, Edwards GE. 2006. Functional characterization of phosphoenolpyruvate carboxykinase-type C₄ leaf anatomy: immuno-, cytochemical and ultrastructural analyses. Annals of Botany 98, 77–91.

Voznesenskaya EV, Koteyeva NK, Chuong SDX, Ivanova AN, Barroca J, Craven LA, Edwards GE. 2007. Physiological, anatomical and biochemical characterisation of photosynthetic types in genus Cleome (Cleomaceae). Functional Plant Biology 34, 247–267.

Wang D, Naidu SL, Portis AR Jr, Moore SP, Long SP. 2008a. Can the cold tolerance of C₄ photosynthesis in Miscanthus × giganteus relative to Zea mays be explained by differences in activities and thermal properties of Rubisco? Journal of Experimental Botany 59, 1779–1787.

Wang D, Portis AR Jr, Moore SP, Long SP. 2008b. Cool C₄ photosynthesis: pyruvate P₄ dikinase expression and activity corresponds to the exceptional cold tolerance of carbon assimilation in Miscanthus × giganteus. Plant Physiology 148, 557–567.

Watcharamongkol T, Christin PA, Osborne CP. 2018. C₄ photosynthesis evolved in warm climates but promoted migration to cooler ones. Ecology Letters 21, 376–383.

Yin X, Struik PC. 2017. Can increased leaf photosynthesis be converted into higher crop mass production? A simulation study for rice using the crop model GECROS. Journal of Experimental Botany 68, 2345–2360.

Zhu XG, Long SP, Ort DR. 2010. Improving photosynthetic efficiency for greater yield. Annual Review of Plant Biology 61, 235–261.