Bundle sheath chloroplast volume can house sufficient Rubisco to avoid limiting C_4 photosynthesis during chilling

Charles P. Pignon[1], Marjorie R. Lundgren[2,3,4], Colin P. Osborne[5], Stephen P. Long[1,4]

[1] University of Illinois, Carl R. Woese Institute for Genomic Biology and Departments of Crop Sciences and of Plant Biology, 1206 W Gregory Drive, Urbana, IL 61801, USA.

[2] Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, 15 Vassar Street, Cambridge, MA 02139, USA

[3] Arnold Arboretum, Harvard University, 1300 Centre Street, Boston, MA 02131, USA.

[4] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[5] Department of Animal and Plant Sciences, Alfred Denny Building, University of Sheffield, Sheffield, S10 2TN, UK.

pignon2@illinois.edu

slong@illinois.edu*

*Corresponding. 217 244 0881; 217 417 2991

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Opinion paper

Running title: Chloroplast volume does not restrict C_4 photosynthesis

Highlight:

The volume of bundle-sheath chloroplasts available for Rubisco investment in the leaves of four C_4 grasses could potentially support much greater photosynthetic activity than is typically observed, even at chilling temperature.

Abstract

C_4 leaves confine Rubisco to bundle-sheath cells. Thus, the size of bundle-sheath compartments, and total volume of chloroplasts within them, limits space available for Rubisco. Rubisco activity limits photosynthesis at low temperatures. C_3 plants counter this limitation by increasing leaf Rubisco content, yet few C_4 species do the same. Because C_3 plants usually outperform C_4 plants in chilling environments, it has been suggested that there is insufficient chloroplast volume available in the bundle-sheath of C_4 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 C_4 *Andropogoneae* grasses: two crops (*Zea mays*, *Saccharum officinarum*) and a wild, chilling-tolerant grass (*Miscanthus* x *giganteus*). A wild C_4 *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. C_4 bundle-sheath cells therefore house more than enough chloroplasts to avoid Rubisco limitation to photosynthesis during chilling.

Keywords:

Cold tolerance, chilling tolerance, C_4 photosynthesis, confocal microscopy, chloroplast, maize, Miscanthus, sugarcane, *Alloteropsis*, bundle-sheath

Abbreviations

A_{sat}: light-saturated net rate of photosynthetic CO_2 assimilation in leaves (µmol m^{-2} s^{-1})

$A_{max,\ cp}$: A_{sat} that could be supported by the Rubisco that could be accommodated in theory within the measured BS chloroplast volume (µmol m^{-2} s^{-1})

BS: bundle-sheath

IVD: inter-veinal distance (µm)

M: mesophyll

vol_{BS}: bundle-sheath volume per unit leaf area (m^3 m^{-2})

$vol_{BS,\ cp}$: bundle-sheath chloroplast volume per unit leaf area (m^3 m^{-2})

vol_{M}: mesophyll volume per unit leaf area (m^3 m^{-2})

$vol_{M,\ cp}$: mesophyll chloroplast volume per unit leaf area (m^3 m^{-2})

$\%_{BS,\ cp}$: % occupancy of the bundle-sheath by chloroplasts (dimensionless)

$\%_{M,\ cp}$: % occupancy of the mesophyll by chloroplasts (dimensionless)

Introduction

C_4 photosynthesis involves a biochemical CO_2 concentrating mechanism. In mesophyll (M) cells, the enzyme phospho*enol*pyruvate carboxylase assimilates CO_2 into oxaloacetate, which is then metabolized into further C_4 compounds that are transferred to, and decarboxylated in, bundle-sheath (BS) cells to raise [CO_2] around the enzyme Rubisco (von Caemmerer and Furbank, 2003). Rubisco then fixes this CO_2 via the Calvin-Benson cycle in the BS. In C_4 plants, Rubisco is therefore predominantly localized to the chloroplasts of BS cells, where the increased [CO_2] greatly improves photosynthetic efficiency because it effectively eliminates photorespiration, the energetically costly process initiated when O_2 is fixed by Rubisco instead of CO_2 (Hatch, 1987). The BS cells of C_4 leaves are arranged radially around veins and isolated from internal leaf air spaces by surrounding M cells (Dengler and Nelson, 1999). Relative to the leaves of C_3 plants, C_4 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 C_4 photosynthesis under warm conditions is evident in the high productivity of the *Andropogoneae* grass crops maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (Lu.) Moench), and sugarcane (*Saccharum officinarum* L). However, photosynthesis in the majority of C_4 grasses is characterized by poor chilling tolerance, limiting them to warmer environments (Long, 1983; Long and Spence, 2013; Sage, 2002). Improving chilling tolerance could therefore expand the growing region and lengthen the growth seasons of C_4 crops (Glowacka *et al.*, 2016). Such tolerance of low temperatures has evolved many times in wild C_4 grasses, enabling them to shift their niches into cooler alpine or temperate environments (Watcharamongkol *et al.*, 2018).

The mechanisms conferring chilling tolerance to C_4 grasses have been especially well studied in the grass *Miscanthus* x *giganteus* Greef 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% the photosynthetic capacity of *Z. mays* leaves developing at 25 °C, while leaves of *M.* x *giganteus* are unaffected by this temperature difference (Long and Spence, 2013). Another study found that *M.* x *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 (Dohleman 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 C_4 photosynthesis could provide crucial improvements to the yield and robustness of key C_4 crops (Long *et al.*, 2006; Yin and Struik, 2017; Zhu *et al.*, 2010).

Chilling tolerance in C_4 grasses may be linked to leaf anatomy. Because C_4 leaves restrict Rubisco to BS cells, the space potentially available to house this enzyme is roughly halved relative to C_3 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 CO_2 assimilation in C_4 leaves, as it is in C_3 leaves (Furbank *et al.*, 1997). Since catalytic rate declines with temperature, Rubisco becomes an even greater limitation under chilling, unless its amount is increased (Long and Spence, 2013; Sage *et al.*, 2011).

It has been proposed that BS chloroplast volume would limit acclimatory increases in Rubisco in C_4 plants at chilling temperatures (<15 °C), so disadvantaging them relative to their C_3 counterparts (Kubien and Sage, 2004; Kubien *et al.*, 2003; Pittermann and Sage, 2000; Sage *et al.*, 2011; Sage and McKown, 2006). This hypothesis is supported by the observation that leaves of chilling tolerant C_3 plants often increase Rubisco content during acclimation, whereas this is rarely seen in C_4 leaves (Long and Spence, 2013; Sage and McKown, 2006). Net photosynthetic CO_2 uptake (A_{sat}) in C_4 leaves correlates with Rubisco content (Pearcy, 1977) and activity (Friesen and Sage, 2016; Kubien and Sage, 2004; Pittermann and Sage, 2000) at low (<15 °C), but not high (>25 °C), temperatures. Rubisco's flux control coefficient over photosynthetic CO_2 assimilation reaches 0.99 (*i.e.* near-total control) at 6 °C in *Flaveria bidentis* L. Kuntze (Kubien *et al.*, 2003). These observations raise important questions: does Rubisco limit photosynthesis in all C_4 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.* x *giganteus* maintains photosynthetic capacity and, unusually, maintains or slightly increases leaf Rubisco content per unit leaf area, while showing large increases in PPDK expression (Long and Spence, 2013; Naidu *et al.*, 2003; Wang

et al., 2008b). Accessions of *M. sacchariflorus*, one of the parent species of *M.* x *giganteus*, achieved some of the highest light-saturated rates of leaf CO_2 uptake (A_{sat} >16 µmol m^{-2} s^{-1}) recorded for any plants grown and measured at 15 °C (Glowacka *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 C_4 cycle enzymes in *Miscanthus*, the upregulation of key photoprotective mechanisms reduces damage to photosystem II (Farage *et al.*, 2006). This suggests that decreased photosynthetic rates in most C_4 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.* x *giganteus* corresponds to the up-regulation of genes encoding several photosynthetic proteins (Spence *et al.*, 2014). *M.* x *giganteus* maintains the linear relationship between operating photochemical efficiency of photosystem II and the quantum efficiency of CO_2 assimilation during chilling, suggesting that the balance of C_3 and C_4 cycles is not compromised (Naidu and Long, 2004). In total, these findings suggest that Rubisco is not the sole limitation to C_4 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.* x *giganteus* and related species (Long and Spence, 2013).

Because most Rubisco in C_4 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 C_4 leaves. However, most attempts at chloroplast quantification have not documented 3D measurements, but rather chloroplast counts and 2D planar area (Brown and Hattersley, 1989; Pyke and Leech, 1987; Stata *et al.*, 2016; Stata *et al.*, 2014). With confocal laser scanning microscopy, it is possible to measure chloroplast volume directly from an optically produced 3D image (Coate *et al.*, 2012; Park *et al.*, 2009). 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 C_4 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 C_4 grasses, its BS chloroplast volumes were compared to two chilling-intolerant crop species of the same tribe (*Z. mays*, *S. officinarum*). The unrelated, non-*Andropogoneae*, non-crop and chilling-intolerant C_4 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 complex cultivar cv. CP88-1762, a C_4 lineage of *A. semialata* originating from South Africa (Osborne *et al.*, 2008), and the "Illinois" clone of *M.* x *giganteus*. *M.* x *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 hour photoperiod.

M. x *giganteus* was grown at the University of Illinois Agricultural Research Station farm near Champaign, IL, USA (40°02'N, 5 88°14'W, 228m above sea level). Soils at this site are deep Drummer/Flanagan series (a fine silty, 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.* x *giganteus* plants, as judged by ligule emergence, was sampled in July (Arundale *et al.*, 2014a; Arundale *et al.*, 2014b; Dohleman *et al.*, 2012; Pignon *et al.*, 2017).

A. 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 soilless cultivation medium (LC1 Sunshine mix, Sun Gro Horticulture, Agawam, MA, USA), with additional coarse sand and perlite mixed into pots for *A. semialata*. Single stem segments of *S. officinarum* were planted directly into pots of a second soilless cultivation medium (Metromix 900: SunGro Horticulture, Agawam MA). All pots were watered daily to field capacity. *Z. mays* was initially fertilized with granulated fertilizer (Osmocote Plus 15/9/12, The Scotts Company LLC, Marysville, OH, USA) followed by general nutrient solution (Peter's Excel 15-5-15, Everris NA Inc, Dublin OH, USA) and iron chelate supplement (Sprint 330, BASF Corp. NC, USA) added to the watering regime once every week. *A. semialata* and *S. officinarum* were fertilized with granulated fertilizer (Osmocote Classic 13/13/13, The Scotts Company LLC, Marysville, OH, USA), and *A. semialata* 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. 40 µm transverse sections were cut (Leica CM3050 S, Leica biosystems, Wetzlar, Germany) and mounted on glass slides. Slides were then immersed for 15 minutes in a cell membrane and wall dye solution (FM 1-43FX, Thermofisher Scientific, Waltham, MA, USA), and diluted to 3.6 mM in DMSO (Thermofisher Scientific) and water, in order to image cell walls. Samples were imaged with a confocal laser-scanning microscope (LSM 700, Carl Zeiss AG, Oberkochen, Germany). Images were acquired through a 63x oil-immersion objective (63x Plan-Apochromat, Carl Zeiss AG) for *M.* x *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 40x oil-immersion objective (40x Plan-Apochromat, Carl Zeiss AG) was used for *Z. mays*, *S. officinarum*, and *A. semialata*.

The fluorescence of dye-labelled cell walls was analyzed 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.

A video illustrating how the delineation of BS and M compartments, and the chloroplasts within them, was achieved within a 3D optical section is shown in Fig. S1. BS and M 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 M (vol_{M}) per unit leaf area. The chlorophyll fluorescence signal within the BS and M was then used to determine total chloroplast volume per unit leaf area within each compartment ($vol_{BS,\,cp}$ and $vol_{M,\,cp}$, respectively) and the percent occupancy of each compartment by chloroplasts ($\%_{BS,\,cp}$ and $\%_{M,\,cp}$, 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. 1 c to Fig. S1.

Leaf thickness was measured in a single location per image, across the mesophyll between two veins, and inter-veinal 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 C_4 BS chloroplasts could contain, in order to calculate the corresponding theoretical maximum level of Rubisco-limited photosynthetic CO_2 uptake ($A_{max,\ cp}$) that could be achieved by a given leaf. Calculated values for $A_{max,\ cp}$ could then be compared to achieved values for light-saturated photosynthetic CO_2 uptake (A_{sat}). Because $A_{max,\ cp}$ is a measure of theoretical, and not achieved, Rubisco-limited CO_2 uptake, factors such as leaf N content and incident light intensity could be ignored. Instead, $A_{max,\ cp}$ was determined from the volume of BS chloroplasts available for Rubisco investment ($vol_{BS,\ cp}$), the amount of Rubisco that could be contained within these chloroplasts, and the carboxylation activity of Rubisco. Although there is evidence of C_4 subspecies of *A. semialata* 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.

$vol_{BS,\ cp}$ was determined experimentally in this study as described above. A Rubisco carboxylation rate per site at 25 °C (k_{cat}) of 3.3 mol CO_2 mol sites^{-1} s^{-1} had been determined previously for both *Z. mays* and *M.* x *giganteus* (Wang *et al.*, 2008a). This value was reduced by 15%, reflecting the Rubisco activation state at 25 °C of 85%, reported for *M.* x *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 x 10^5 g Rubisco m^{-3} chloroplast based on measurements for M 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 weight 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 ($A_{max,\ cp}$).

To extend this estimation to temperatures below 25 °C, an Arrhenius function was used based on the activation energy (*Ea*) 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 to achieved photosynthesis values, the literature was reviewed to identify values for light saturated net leaf CO_2 uptake (A_{sat}) at moderate and chilling temperatures in all four species: *Z. mays* (Glowacka *et al.*, 2016; Long, 1983; Naidu and Long, 2004; Naidu *et al.*, 2003), *S. officinarum* (Glowacka *et al.*, 2016; Spitz, 2015), *A. semialata* (Osborne *et al.*, 2008), and *M.* x *giganteus* (Friesen and Sage, 2016; Glowacka *et al.*, 2014; Glowacka *et al.*, 2016; Glowacka *et al.*, 2015; Naidu and Long, 2004; Naidu *et al.*, 2003; Spitz, 2015), 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.* x *giganteus* (n=6). Statistical analysis was performed on the following parameters: leaf thickness, *IVD*, vol_{BS}, vol_{M}, $vol_{BS,\ cp}$, $vol_{M,\ cp}$, $\%_{BS,\ cp}$, and $\%_{M,\ cp}$. 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-Wilke (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 6-10 x 10^{-6} m^3 m^{-2} in the BS and 10-14 x 10^{-6} m^3 m^{-2} in the M (Fig. 1, Fig. 2, Fig. 3 e, f). There was no evidence of greater BS chloroplast volume available per unit leaf area ($vol_{BS,\ cp}$) in the chilling-tolerant *M.* x *giganteus* compared with the chilling sensitive species. On the contrary, *M.* x *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 vol_{BS}, significantly greater occupancy of the BS by chloroplasts ($\%_{BS,\ cp}$) resulted in greater $vol_{BS,\ cp}$ overall in *A. semialata* (Fig. 3 c, e, g).

Across the four study-species, chloroplasts occupied 15-30% of the BS ($\%_{BS,\ cp}$), and 8-14% of the M ($\%_{M,\ cp}$) (Fig. 1, Fig. 3 g, h, Fig. S1). Between species, $\%_{BS,\ cp}$ and $\%_{M,\ cp}$ were significantly greatest and lowest, respectively, in *A. semialata*. Leaf thickness ranged from 100-250 µm, with veins spaced 100-140 µm apart on average (Fig. 1, Fig. 3 a, b). *A. semialata* leaves at ca. 225 µm were nearly twice as thick as those of *M.* x *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.* x *giganteus* and *A. semialata*) (Fig. 3 b). Across the species, the volume of M per unit leaf area (vol_M) 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 vol_M (Fig. 3 d). BS volume per unit leaf area (vol_{BS}), however, was conserved across species at ca. 40 m^3 m^{-2} x 10^{-6} (Fig. 3 c).

When the maximal theoretical photosynthetic capacity of the leaf ($A_{max,\ cp}$) was estimated from $vol_{BS,\ cp}$, values ranged from ca. 60-90 µmol m^{-2} s^{-1} at 25 °C. This was substantially greater than published values of light saturated net photosynthetic CO_2 uptake (A_{sat}) for these species at this temperature (Fig. 4, Table SI). However, at lower temperatures A_{sat} was closer to $A_{max,\ cp}$, with A_{sat} being 20-90% of $A_{max,\ cp}$ at 5 °C.

Discussion

In all four of the C_4 grass species studied here, the volume of BS per unit leaf area available for Rubisco (vol_{BS}) was not a limitation for observed rates of photosynthesis, even at chilling temperatures. This conclusion is based on two key findings. First, the chilling-tolerant *M.* x *giganteus* (Long and Spence, 2013) has a smaller BS chloroplast volume per unit leaf area ($vol_{BS, cp}$) than the chilling-sensitive C_4 grasses *S. officinarum, A. semialata,* and *Z. mays* (Fig. 3). Second, the theoretical maximum level of Rubisco-limited photosynthetic CO_2 uptake ($A_{max, cp}$) that could be achieved by each species was greater than realized levels of A_{sat}, even at chilling temperatures (Fig. 4). This study focused on closely related C_4 grasses of the *Andropogoneae* clade, which contain the major C_4 crops as well as candidate bioenergy crops. Even *A. semialata*, which descends from a separate evolutionary lineage in the *Paniceae*, did not suffer from limitation of chilling photosynthesis by vol_{BS}.

Several leaf structural characteristics, including leaf thickness, *IVD*, vol_M, $\%_{BS, cp}$, and $\%_{M, cp}$, varied significantly between species (Fig. 3 a, b, d, g, h). Indeed, the $vol_{BS, cp}$ was actually greatest in the chilling-sensitive *A. semialata* and lowest in the chilling-tolerant *M.* x *giganteus* (Fig. 3 e). This clearly demonstrates that $vol_{BS, cp}$ does not determine chilling tolerance in C_4 plants, and therefore that the volume of BS chloroplast available for leaf Rubisco investment is unlikely to meaningfully restrict C_4 photosynthesis at low temperatures.

Based on 2D leaf profiles, the percent occupancy of the total M volume by chloroplasts varies significantly between photosynthetic types and taxonomic clades of diverse C_4 plants, with an average occupation of ca. 12.2% (Stata *et al.*, 2014), which is similar to the 8-14% range seen here (Fig. 3 h). In various species of the eudicot genus *Flaveria* that use the NADP-ME subtype of C_4 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 (Fig. 3 g); this may reflect differences due to taxonomy or specimen preparation. *A. semialata*, which belongs to the *Paniceae* tribe, had the greatest volume of chloroplast in the BS ($\%_{BS, cp}$) (Fig. 3 g, h). This may reflect the 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). *A. semialata*'s high BS chloroplast volume may also result from the very recent development of C_4 anatomy in this species, which might not have evolved the faster Rubisco

kinetics of other, older C_4 lineages and could therefore require relatively more Rubisco in the BS to compensate (Dunning *et al.*, 2017; Lundgren *et al.*, 2015).

While chloroplasts across the entire M tissue are available for Rubisco investment in C_3 plants, there is clearly less space available in the BS tissue of C_4 leaves. However, in the M of C_3 species, CO_2 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 CO_2 and facilitate Rubisco access to CO_2 (Evans and Loreto, 2000; Flexas *et al.*, 2008). In the BS of C_4 species, CO_2 results from decarboxylation of C_4-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 M (Fig. 3 g, h).

The comparison of $A_{max,\,cp}$ to published values for A_{sat} is directly dependent on terms used to calculate $A_{max,\,cp}$: for instance, a 20% lower value for k_{cat} will result in 20% lower $A_{max,\,cp}$. At lower temperatures this could lead to $A_{max,\,cp}$ much closer to published values for A_{sat} (Fig. 4 a, b). However, the values used in this study were generally conservative. In a survey of Rubisco k_{cat} in 14 grasses using different subtypes of C_4 photosynthesis (Ghannoum *et al.*, 2005), all seven NADP-ME grasses, and 5 of the seven NAD-ME grasses, registered values greater than, and up to two times, the k_{cat} value used here; *i.e.*, 3.3 mol CO_2 mol sites^{-1} s^{-1} (Wang *et al.*, 2008a).

Another important term in the calculation of $A_{max,\,cp}$ is the Rubisco content per unit volume chloroplast. Here, we used a published value of 0.41 moles Rubisco m^{-3} chloroplast, derived from *T. aestivum* M chloroplasts (Pyke and Leech, 1987). This value is conservative, as it is on the lower end of the 0.4-0.5 moles 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. mays*, *S. officinarum* and *M.* x *giganteus*, typically show few or no stacked thylakoids in the BS. This arrangement leaves more space available for stroma, and therefore Rubisco, by comparison to bread wheat chloroplasts (Bellasio and Griffiths, 2014; Furbank, 2011; Voznesenskaya *et al.*, 2006; Voznesenskaya *et al.*, 2007).

Despite the use of conservative terms to calculate $A_{max,\,cp}$, this parameter was greater than published light-saturated photosynthetic rates (A_{sat}) for all four studied species (Fig. 4) (Friesen and Sage, 2016; Glowacka *et al.*, 2014; Glowacka *et al.*, 2016; Glowacka *et al.*, 2015; Long, 1983; Naidu and Long, 2004; Naidu *et al.*, 2003; Osborne *et al.*, 2008; Spitz, 2015). This was even true at low temperatures, where Rubisco has been predicted to be a strong limitation to C_4 photosynthesis (Kubien and Sage, 2004; Kubien *et al.*, 2003; Pearcy, 1977; Pittermann and Sage, 2000). 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.* x *giganteus* on chilling (Long and Spence, 2013). Additional evidence comes from a recent transgenic upregulation of Rubisco content by >30% above wild type in leaves of *Z. mays* (Salesse *et al.*, 2018).

Based on genetic diversity, the assumed origin of the C_4 grass tribe *Andropogoneae* is tropical South-east Asia (Arthan *et al.*, 2017; Hartley, 1958). Tropical origins are common across the C_4 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 C_4 cycle and associated anatomy. The literature has already addressed these additional restrictions and the evolution of chilling tolerant C_4 photosynthesis (Long, 1983; Long, 1999; Long and Spence, 2013).

Several C_4 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 CO_2 assimilation at chilling temperatures that equal or exceed rates achieved by temperate and even arctic/alpine C_3 grasses. Notably, the C_4 grass *M. x. 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. x 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: Long and Spence, 2013). Gene expression analyses suggest that

these increases are part of a syndrome of acclimative changes that allow efficient C_4 photosynthesis under chilling conditions (Spence *et al.*, 2014), and in turn the exceptional productivities achieved by *M. x. giganteus* in temperate climates (Dohleman and Long, 2009). Therefore, while Rubisco content clearly co-limits photosynthesis in many C_4 species under chilling conditions, the findings here show that this does not directly result from restricting Rubisco to the BS in C_4 grasses.

In conclusion, while the volume of the cells that can hold Rubisco in C_4 grass leaves is lower than in their C_3 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 C_4 grasses to cold environments.

Supplementary material

Supplementary Figure S1: Video of the full 3D image of leaf, bundle-sheath (BS) cells, mesophyll (M) cells, and chloroplasts seen in Fig. 2. The initial 3D image, collected by confocal microscopy, consists of raw fluorescence data emitted by stained cell walls (green) and chloroplastic photosystem II (red). The BS and M compartments are hand-delineated (blue). The chloroplasts within these compartments (bold red) are then identified from the photosystem II autofluorescence.

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Tables and Figures

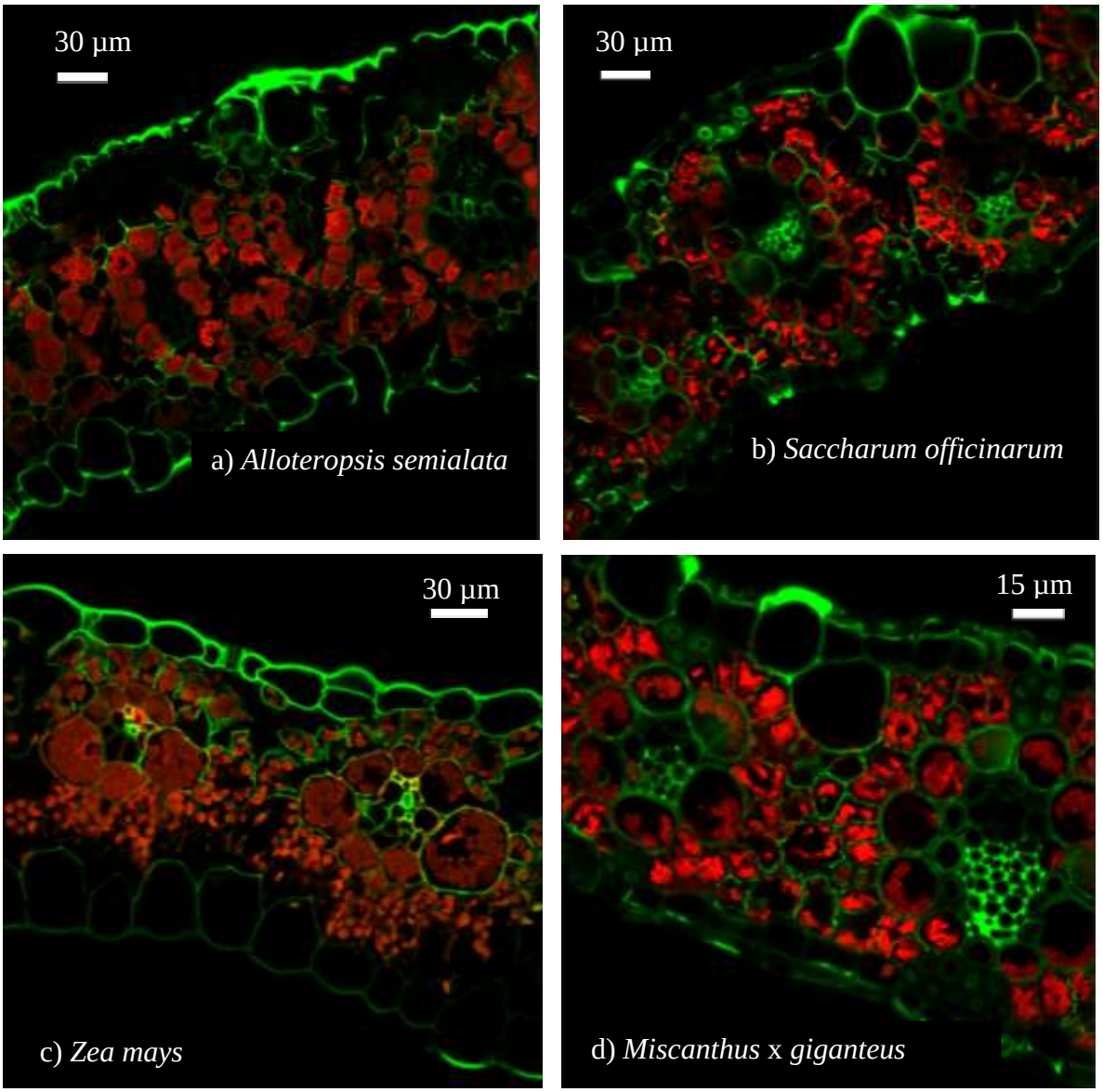
Figure 1. Individual single depth slices of representative leaf cross-sections. Cell walls labeled with FM 1-43FX are green. Chlorophyll fluorescence is red. The darker red bundle-sheath fluorescence of *Saccharum officinarum* L.*, Zea mays* L. and *Miscanthus* x *giganteus* Greef et Deu. reflects the lower photosystem II content in the chloroplasts, which is the primary emitter of chlorophyll fluorescence in the detection bandpass of 630-700 nm. The full 3D image of the *Z. mays* leaf is available as a video in Figure S1.

Figure 2. Fluorescent image of a representative *Zea mays* L. leaf. 2D compression of a 3D cross-section of *Z. mays*, 300 µm in length and 15 µm in depth. The full 3D image is available as a video in Figure S1. Cell walls labeled with FM 1-43FX are green. Chlorophyll fluorescence is red. Delineated volume reconstruction of the bundle-sheath and mesophyll compartments are shown in blue in panels a) and b), respectively. Chlorophyll fluorescence was used by the software to reconstruct chloroplast volumes within the bundle-sheath and mesophyll; these are shown in bold red in panels a) and b), respectively.

Figure 3. Leaf anatomical characteristics and differences between the study-species. Mean + SE of a) leaf thickness, b) inter-veinal distance (*IVD*), c) bundle-sheath volume per leaf area (vol_{BS}), d) mesophyll volume per leaf area (vol_{M}), e) bundle-sheath chloroplast volume per leaf area ($vol_{BS,\,cp}$), f) mesophyll chloroplast volume per leaf area ($vol_{M,\,cp}$), g) occupancy of the bundle-sheath by chloroplasts ($\%_{BS,\,cp}$), and h) occupancy of the mesophyll by chloroplasts ($\%_{M,\,cp}$) in *Zea mays* L. (n=7), *Saccharum officinarum* L. (n=5), *Alloteropsis semialata* J. Presl (n=6), and *Miscanthus* x *giganteus* Greef et Deu. (n=6). Letters indicate Tukey groups, with black letters indicating significant difference at $p<0.05$ and grey letters indicating significant difference at $p<0.1$.

Figure 4. Comparison of theoretical maximum vs. achieved leaf photosynthetic carboxylation rates at different temperatures. a) Symbols indicate published rates of net CO_2 uptake (A_{sat}) measured on leaves at different temperatures. Lines show estimated leaf maximal photosynthetic capacity ($A_{max,\,cp}$) calculated from bundle-sheath chloroplast volume per unit leaf area. b) Measurements of A_{sat} expressed as a percentage of $A_{max,\,cp}$. Measurements were obtained for *Zea mays* L. (Glowacka *et al.*, 2016; Long, 1983; Naidu and Long, 2004; Naidu *et al.*, 2003), *Saccharum officinarum* L. (Glowacka *et al.*, 2016; Spitz, 2015), *Alloteropsis semialata* J. Presl

(Osborne *et al.*, 2008), and *Miscanthus* x *giganteus* Greef et Deu. (Friesen and Sage, 2016; Glowacka *et al.*, 2014; Glowacka *et al.*, 2016; Glowacka *et al.*, 2015; Naidu and Long, 2004; Naidu *et al.*, 2003; Spitz, 2015) at different temperatures and at an incident photon flux ≥ 1000 μmol m^{-2} s^{-1}.



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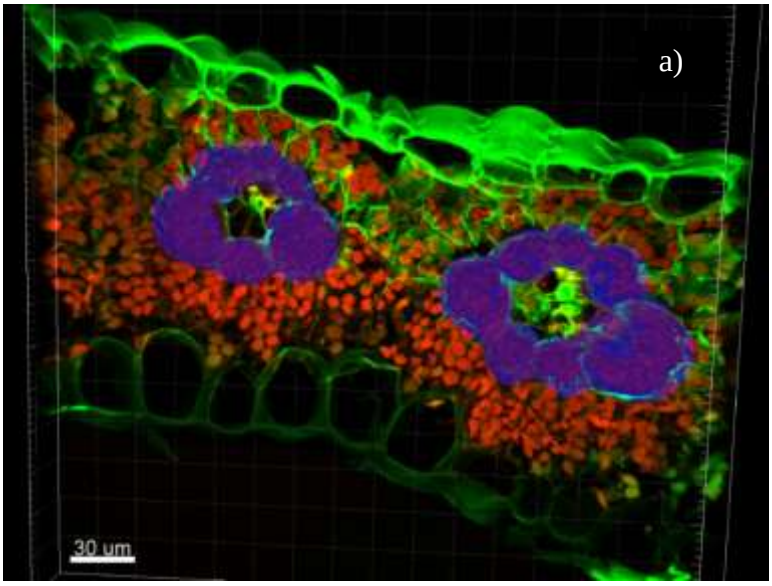

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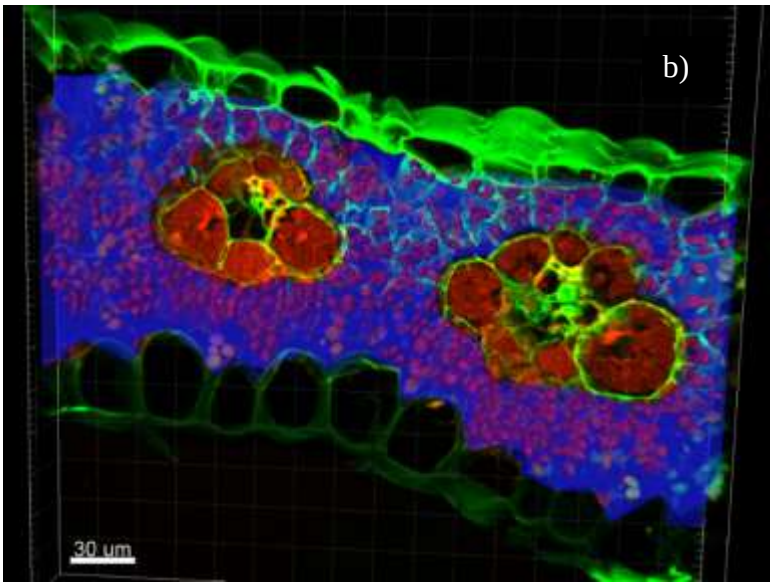

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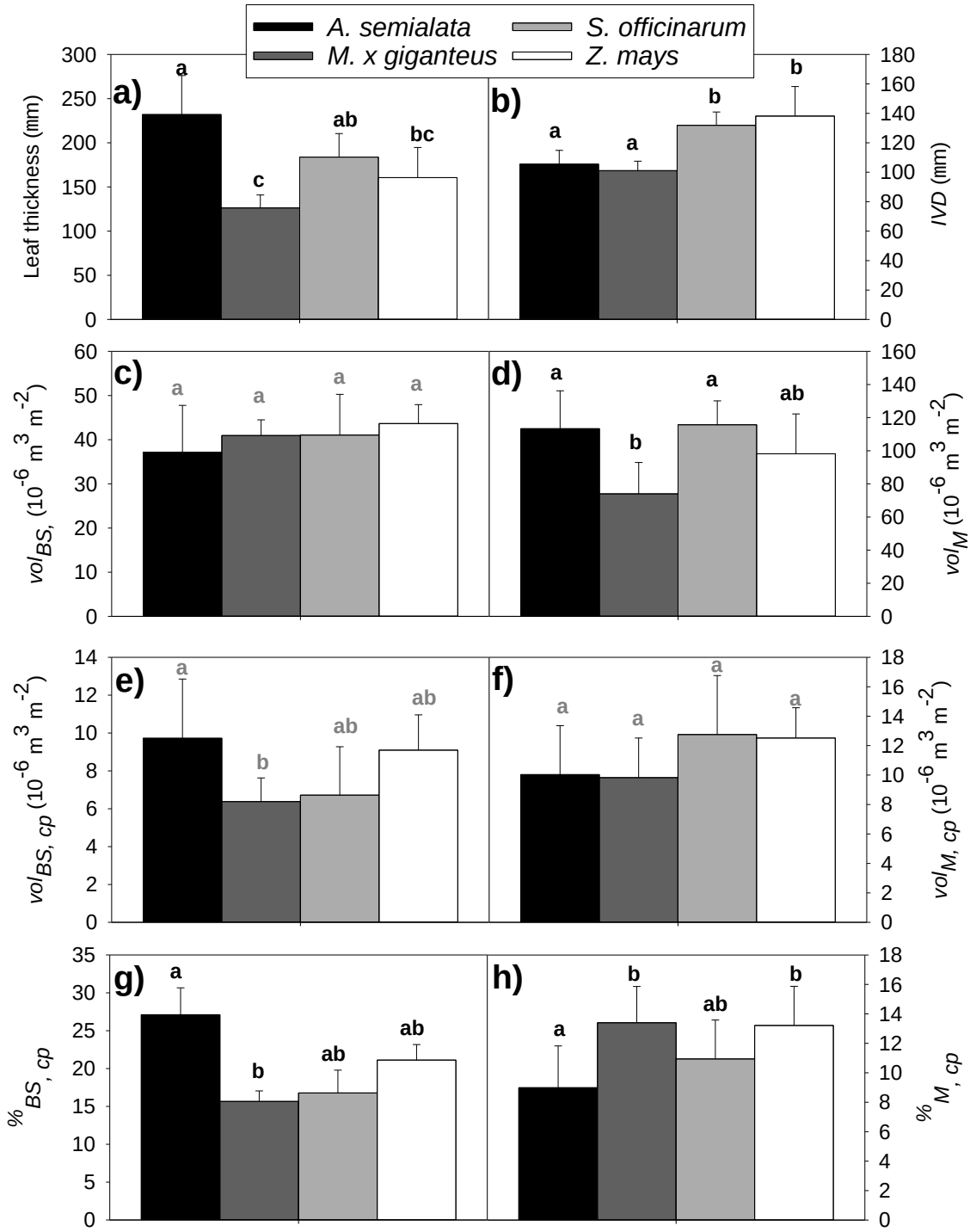

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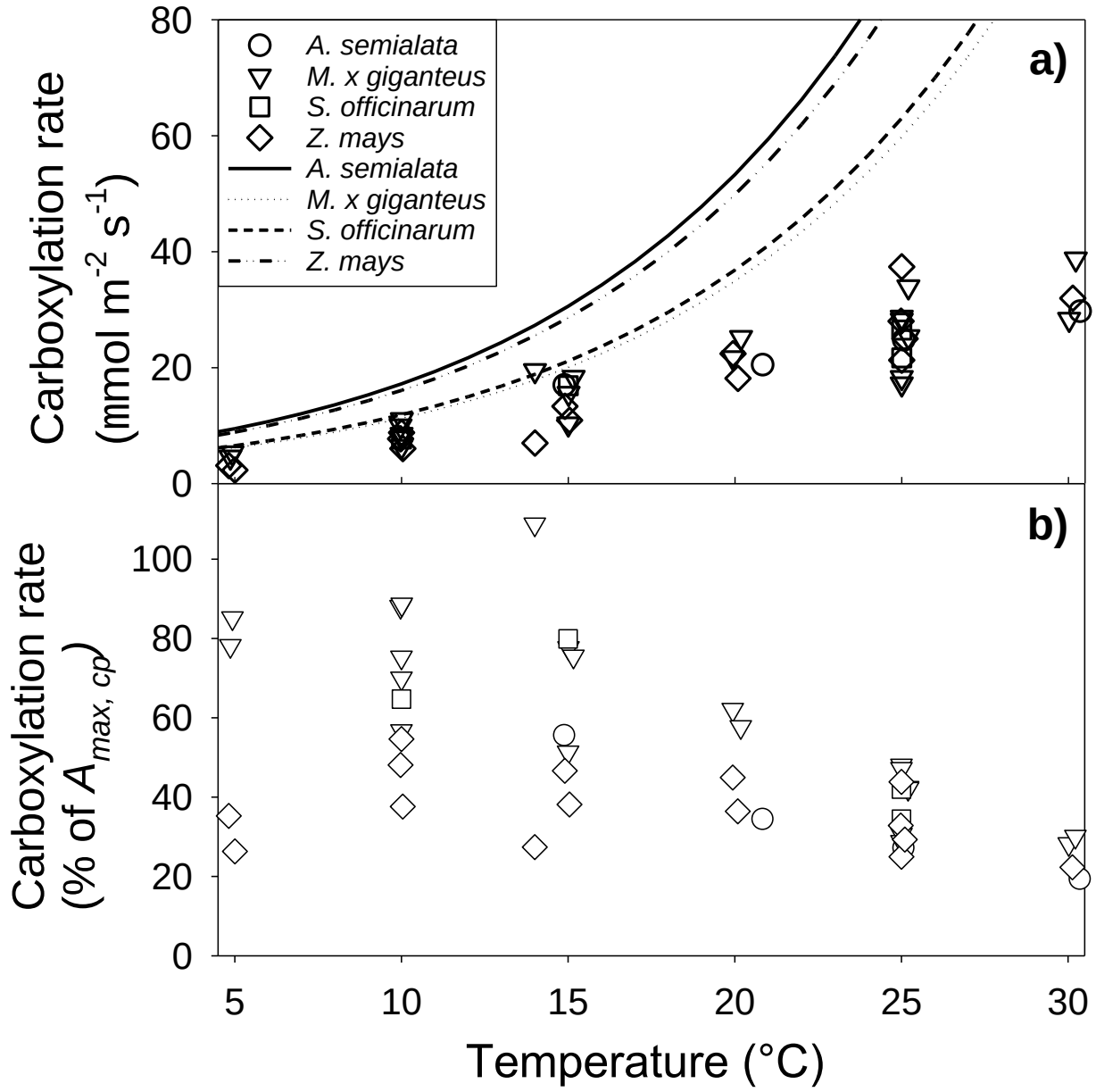

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625 Figure 4