Submission to *Plant, Cell & Environment* for consideration as an ‘Original Article’

Dark respiration rates are not determined by differences in mitochondrial capacity, abundance and ultrastructure in C4 leaves

Yuzhen Fan1,3, Andrew P. Scafaro1,3, Shinichi Asao3, Robert T. Furbank2,3, Antony Agostino3, David A. Day4, Susanne von Caemmerer2,3, Florence R. Danila2,3, Melanie Rug5, Daryl Webb5, Jiwon Lee5, Owen K. Atkin1,3\*

1ARC Centre of Excellence in Plant Energy Biology, Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia

2ARC Centre of Excellence for Translational Photosynthesis, Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia

3Division of Plant Sciences, Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia

4College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia

5Centre for Advanced Microscopy, The Australian National University, Canberra, ACT 2601, Australia

\***Author for correspondence**: Owen K. Atkin ([Owen.Atkin@anu.edu.au](mailto:Owen.Atkin@anu.edu.au); tel +61-2-61255046)

Number of Figures: 6 (+8 in Supporting Information)

Number of Tables: 3

Number of References: 48

Number of Pages (main text): 14

Word count (incl. in-text citations): Total (5,747), Abstract (186), Introduction (1,255), Materials and Methods (1,816), Results (1,169), Discussion (1,507)

Running title: Mitochondrial respiration in C4 leaves

## **Abstract**

Our understanding of the regulation of respiration in C4 plants, where mitochondria play different roles in the different types of C4 photosynthetic pathway, remains limited. We examined how leaf dark respiration rates (*R*dark), in the presence and absence of added malate, vary in monocots representing the three classical biochemical types of C4 photosynthesis (NADP-ME, NAD-ME and PCK) using intact leaves and extracted bundle sheath strands. In particular, we explored to what extent *R*dark are associated with mitochondrial number, volume and ultrastructure. We found that the respiratory response of NAD-ME and PCK type bundle sheath strands to added malate was associated with differences in mitochondrial number, volume, and/or ultrastructure, while NADP-ME type bundle sheath strands did not respond to malate addition. In general, mitochondrial traits reflected the contributions mitochondria make to photosynthesis in the three C4 types. However, despite the obvious differences in mitochondrial traits, no clear correlation was observed between these traits and *R*dark. We suggest that *R*dark is primarily driven by cellular maintenance demands and not mitochondrial composition *per se*, in a manner that is somewhat independent of mitochondrial organic acid cycling in the light.

## **Keywords**

Bundle sheath, C4 photosynthetic pathway, C4 plants, mitochondria, mitochondrial ultrastructure, respiration

## **Introduction**

Leaves of C4 plants feature a suite of biochemical and anatomic traits that increase the CO2 concentration around Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), thereby reducing photorespiration (the efflux of CO2 due to oxygenation reactions of Rubisco) and increasing photosynthetic efficiency (Evans & von Caemmerer, 1996; Ghannoum et al., 2005; Ghannoum et al., 2011; Leegood, 2002). In all C4 leaves, CO2 is initially fixed into C4 acids by phosphoenolpyruvate (PEP) carboxylase in mesophyll cells. The C4 acids then diffuse to adjoining bundle sheath cells where they are decarboxylated and the released CO2 is fixed by Rubisco. Importantly, the enzymatic steps used to decarboxylate the C4 acid differ between the three biochemically distinct types of C4 photosynthesis: NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME), and PEP-carboxykinase (PCK). Likewise, the contribution of bundle sheath mitochondria to photosynthesis differs between the three C4 types (Fig. S1) (Furbank, 2011; Hatch, 1987; von Caemmerer & Furbank, 2016). In the NADP-ME type, mitochondria are not directly involved in the C4 pathway. In the NAD-ME type, aspartate is the form of carbon transported from mesophyll to bundle sheath cells. Aspartate is transaminated to produce oxaloacetate, then reduced to malate, and decarboxylated to pyruvate, in bundle sheath mitochondria; the CO2 released by this process is fixed by Rubisco. In the PCK type, roughly one-third of total photosynthetic carbon flux enters bundle sheath cells as malate, which is decarboxylated to pyruvate in the mitochondria. The other two-thirds of fixed carbon diffuses to bundle sheath cells as aspartate, is transaminated to oxaloacetate and decarboxylated by PEP carboxykinase with consumption of ATP. It is believed that this ATP is generated in the mitochondria utilising the NADH produced by pyruvate decarboxylation (Hatch, 1987). The CO2 released from decarboxylation of malate and oxaloacetate is fixed by Rubisco for photosynthesis. Given these different carbon flux pathways, the rate of carbon processed by bundle sheath mitochondria in the light relative to that in the dark (i.e. *R*dark) is thought to be ten-fold, three-fold, and unchanged in NAD-ME, PCK, and NADP-ME types, respectively (Byrd et al., 1992; Furbank et al., 1990; Gardeström & Edwards, 1985).

Different C4 types exhibit distinct post-illumination CO2 bursts (PIB) when transitioning from light to dark, which is related to decarboxylation of remaining photosynthetic C4 acids by mitochondria (Downton, 1970; Laisk & Edwards, 1997; Ohsugi & Murata, 1980). However, after 30 minutes of dark adaptation, the three C4 types exhibit similar steady-state CO2-based *R*dark rates, suggesting all light-dependent C4 acid pools are exhausted within 30 minutes of darkness (Byrd et al., 1992; Siebke et al., 2003; Smith et al., 2019; Sonawane et al., 2017; Tjoelker et al., 2005; Wright et al., 2004). Interestingly, Agostino et al. (1996) showed that in extracted bundle sheath strands, the rate of respiratory O2 uptake in the dark when provided with saturating amount of malate in PCK type *Urochloa panicoides* was twice that of NAD-ME type *Panicum miliaceum*, and six times higher than in bundle sheath strands of NADP-ME type *Zea mays* (maize), which did not respond to added malate. Considered together, these results demonstrate that bundle sheath mitochondrial activity of PCK and NAD-ME types is distinct from that of the NADP-ME type, and these differences may influence rates of *R*dark. What is less clear, however, is whether the marked differences in malate-dependent bundle sheath O2 uptake between the three C4 types are associated with the abundance and ultrastructure of mitochondria.

In general, mitochondria in bundle sheath cells of C4 NAD-ME and PCK types are more abundant and larger compared to their mesophyll counterparts, and compared to those in both cell types in the C4 NADP-ME type (Gardeström & Edwards, 1985; Hatakeyama & Ueno, 2017; Khoshravesh et al., 2016; Muhaidat et al., 2011; Yoshimura et al., 2004). However, depending on what parameters are chosen to estimate the mitochondrial abundance, the results are not always consistent. For example, when measured as a percentage of mitochondrial area relative to chloroplasts per cell profile, bundle sheath mitochondria are more abundant in the NAD-ME than PCK type (Gardeström & Edwards, 1985; Hatch & Carnal, 1992). By contrast, when measured as counts of mitochondria per cell profile, bundle sheath mitochondria appear to be more abundant in the PCK than NAD-ME type (Yoshimura et al., 2004). Importantly, these studies of mitochondrial number were performed using transmission electron microscopy (TEM). Although TEM delivers a much higher magnification than confocal microscopy, these studies examined individual sections (~70-nm thick) that only provides information for each plane of sectioning at the time of fixation (Williams & Carter, 1996; Winey et al., 2014). Consequently, the numbers and areas of mitochondria measured by TEM are likely to be an under-estimation of the overall cell average. Mitochondrial numbers and size can be more accurately characterised using 3D confocal microscopy, providing that fluorescent tags can be linked to proteins of interest. With regard to this, Danila et al. (2016) successfully quantified the number of plasmodesmata in C3 rice, wheat, C4 *Setaria viridis* and maize using a method combining 3D immunolocalisation microscopy and the clearing technique, PEA-CLARITY (Palmer et al., 2015). This method was later applied to another 18 C3 and C4 monocots with environmental manipulation, demonstrating its successful application in C4 plants (Danila et al., 2018; Danila et al., 2019). However, to our best knowledge, no study has yet applied this approach to visualise mitochondria.

Given the different roles of bundle sheath mitochondria in the three C4 types, a further question is whether cristae (in-folds in the mitochondrial inner-membrane) develop to a greater extent in NAD-ME and PCK type bundle sheath mitochondria. One might expect so, considering more developed cristae are linked to higher relative number of mitochondrial electron transport chain (mETC) protein components and a greater ability to process substrates such as NADH (Armstrong et al., 2006). A previous non-quantitative comparison in C4 leaves suggested that NAD-ME bundle sheath mitochondria had the highest development of cristae, followed by PCK, and then NADP-ME type (Hatch & Carnal, 1992; Hatch et al., 1975); however, quantitative measurements of cristae to matrix area between the C4 types have yet to be reported.

In our study, we investigated the extent to which mitochondrial involvement in the C4 photosynthetic pathway of three monocot species is reflected in the capacity, abundance, and ultrastructure of mitochondria. We measured rates of *R*dark as CO2 efflux and O2 uptake in bundle sheath strands and whole leaves. We also applied a 3D immunolocalisation microscopy technique to quantify mitochondrial size and density, and used TEM to determine mitochondrial ultrastructure in mesophyll and bundle sheath cells. We hypothesised that the mitochondrial abundance and ultrastructure differ between the C4 types in a manner consistent with mitochondrial involvement in the C4 pathway: abundance would be the highest in the types where mitochondria play a central role in processing of organic acids (i.e. NAD-ME and PCK types), and cristae would be more developed in the types where the demand for ATP in the C4 pathway is the highest (i.e. PCK type). To explore how substrate-saturated rates of respiration differ between the species, O2 uptake by extracted bundle sheath strands was measured in the presence of a range of organic acids and adenylates. We hypothesised that malate would stimulate O2 uptake in darkness in NAD-ME and PCK bundle sheath cells, but not in bundle sheath cells of NADP-ME type plants. For some of the experiments (e.g. mitochondrial abundance and ultrastructure), we compared traits of the three C4 type monocots (NADP-ME type: *Zea mays*; NAD-ME type: *Panicum miliaceum*; PCK type: *Urochloa panicoides* or *Chloris gayana*; see Method) with that of a C3 monocot, wheat.

## **Materials and Methods**

**Plant materials and growth conditions**

For gas-exchange measurements, *Zea mays* (maize, cv. Delphine, NADP-ME type), *Panicum miliaceum* (NAD-ME type) and *Urochloa panicoides* (PCK type) were grown from seeds in 25 cm diameter pots in organic potting soil supplemented with slow-release fertiliser (Scotts Osmocote, Bella Vista, Australia). Plants were grown in a naturally illuminated glasshouse at The Australian National University, Canberra, Australia in September 2019. The most recent fully-expanded leaves from four-week-old plants were used in gas-exchange measurements. For microscopy, the same species were used with the addition of C3 *Triticum activum* (wheat, cv. Seri Rayon), except the use of *Chloris gayana* rather than *U. panicoides* for the C4 PCK type due to unsuccessful gemination of the latter. Plants were grown in Climatron growth cabinets (Thermoline Inc.) in a completely randomised order. The youngest fully expanded leaves from two to three-week-old plants were used in microscopic sample preparation. For both growth conditions, the temperatures were 30/25°C day/night, photoperiod was ~12 h day/night, CO2 concentration was ambient (~400 ppm) and plants were watered daily.

**Measurements of leaf *R*dark with CO2- and O2-based methods**

To explore whether patterns in post-illumination bursts (PIB) of CO2 efflux differed between the three C4 types in a manner consistent with past reports (Downton, 1970; Laisk & Edwards, 1997; Ohsugi & Murata, 1980), we measured CO2 efflux using a LI-COR 6400 XT infrared gas analyser (LI-COR BioSciences, Lincoln, NE, USA). All LI-COR measurements were made on the middle segment of a leaf using a 6-cm leaf chamber at 30°C with a flow rate of 500μmol s-1, and reference CO2 concentration of 400 μmol mol-1. PIB measurements were repeated on six leaves of six individual plants for each species. The leaf chamber was initially illuminated at 1,600 μmol quanta m-2 s-1 for 20 minutes to measure light-saturated photosynthesis. The light was then switched off and measurements were logged every one to three seconds for 160 seconds. For steady-state rates of O2 uptake in darkness, three leaves of three individual plants from each species were dark-adapted for 30 min in the glasshouse. All gas-exchange measurements in glasshouse were done in between 9:00 to 13:00 of the day. The leaves were subsequently cut, transported to the lab, and measured using a fluorophore based oxygen sensor (Astec Global, Maarssen, The Netherlands), as documented in O’Leary et al. (2017) and Scafaro et al. (2017). Leaf area was recorded and leaves then dried for at least two days at 60°C to determine dry mass.

**Determination of N content in leaves**

Dried leaves from gas-exchange measurements were ground and placed into tin capsules for combustion analysis using a system combining an elemental analyser (Heraeus CHN-O Rapid) for Dumas combustion of the samples, a Finnigan MAT Trapping box HT for automatic cryo-purification of the combustion products, and a Finnigan MAT mass spectrometer (delta D) with a dual inlet at a precision of 0.1‰ (Gebauer & Schulze, 1991).

**Measurements of *R*dark and chlorophyll concentration in bundle sheath strands**

Rates of O2-based *R*dark with addition of substrate and adenylates were measured in extracted bundle sheath strands using methods described in Agostino et al. (1996) and Ghannoum et al. (2005). Briefly, fully expanded leaves were harvested, and bundle sheath strands were extracted by mechanical blending (Omni International Inc, NW Kennesaw, GA) and membrane filtering. The extracted bundle sheath strands were placed in an O2 electrode with a temperature control unit (Hansatech Instruments Ltd., Norfolk, England) and rates of *R*dark were measured at 30°C. Once the basal rate of *R*dark stabilised, varying concentrations of malate, aspartate, and ADP were added and *R*dark was calculated from rates of O2 uptake after re-stabilisation. Selection of respiratory substrates is described in Method S1 and Figure S2. The bundle sheath strands in the electrode chambers were subsequently collected, resuspended in fresh medium and chlorophyll was extracted using methanol (Mackinney, 1941). Total chlorophyll concentration was calculated as a function of absorbance measured at 650 nm and 665 nm as described in Agostino et al. (1996).

To compare the *R*dark measurements on bundle sheath strands with those measured on intact leaf tissue, bundle sheath *R*dark on a chlorophyll basis was converted to a leaf area basis using the amount of chlorophyll in the bundle sheath compartment relative to the amount of chlorophyll of leaves with known area:

where *R*BS\_area is the converted bundle sheath *R*dark in area-based units (μmol O2 m-2 s-1), *R*BS\_chl is the measured bundle sheath *R*dark in chlorophyll-based units (nmol O2 mg-2 Chl s-1), BSChl is the bundle sheath chlorophyll concentration (mg mL-1), LeafChl is leaf chlorophyll concentration (mg mL-1), and Chlarea is chlorophyll concentration per leaf area (mg m-2). The ratio of bundle sheath and leaf chlorophyll concentration (i.e. BSchl/Leafchl) accounts for the proportion of bundle sheath cells within a certain amount of leaves.

**3D immunolocalisation confocal microscopy**

Quantification of mitochondrial size and abundance in mesophyll cells was performed on chemically fixed and cleared leaf tissue using the PEA-CLARITY technique as documented in Palmer et al. (2015) and Danila et al. (2016), with modifications. Due to thick bundle sheath cell walls, quantification of mitochondrial traits in bundle sheath cells was carried out on digested fixed strands without the clearing steps to prevent excessive tissue damage.

For leaf tissue preparation, middle sections of leaves (with mid-rib removed) were cut into squares (~0.25 cm2) and transferred into the initial fixative solution (16% paraformaldehyde with 0.01% Tween-20) followed by vacuum infiltration until tissue sank. The leaf tissue was transferred into ice-cold PEA-CLARITY fixative solution (PBS pH 7.4, 4% acrylamide, 4% paraformaldehyde, 0.05% bis acrylamide and 0.25% VA-44 initiator) and incubated at 4°C overnight, followed by a second overnight incubation at 37°C (120 rpm) to assist polymerisation. The polymerised leaf tissue was transferred into clearing solution (200 mM boric acid pH 8.5 and 4% SDS) for 6-8 weeks (~100 rpm, room temperature) to remove chlorophyll and pigments. The cleared leaf tissue was treated with cell wall digestion cocktail [recipe documented in Danila et al. (2016)] for 5-7 days (20-30 rpm, 37°C) to improve permeability of the antibodies. The digested leaf tissue was incubated with a monoclonal VDAC1 primary antibody (1:250 dilution; Harry Perkins Institute of Medical Research, WA, Australia) in TBST buffer (20 mM Tris, 154 mM NaCl and 0.1% Tween-20) at 4°C for 3-5 days, with 15-min vacuum infiltration each day. The reactivity and specificity of the VDAC1 antibody are documented in Method S2 and Figure S3, and the attempt to co-label the VDAC1 antibody with a known mitochondrial dye, MitoTracker, is documented in Method S3, Figure S4 and S5. The leaf tissue was rinsed with TBST buffer overnight then incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG H&L secondary antibody (1:500 dilution; catalog # ab150113, Abcam Inc.) in TBST buffer at 4°C for 3-5 days, with 15-min vacuum infiltration each day. On the last day of secondary antibody incubation, the leaf tissue was incubated for 2 h at 37°C to enhance antibody penetration and specific binding then stained with 0.05% Calcofluor White Stain (Sigma-Aldrich Co.) for 30 min to visualise cell walls. The leaf tissue was mounted onto glass slides with 87% glycerol to match the refractive index of the cleared leaf tissue.

Bundle sheath strands were extracted from leaf tissue, vacuum-infiltrated and fixed overnight at 4°C in fixative solution (25 mM sodium phosphate pH 7.2, 4% paraformaldehyde, 0.2% glutaraldehyde), followed by cell wall digestion (37°C, 2 h), as described above. The digested strands were hybridised with VDAC1 antibody (4°C, overnight) and Alexa Fluor 488 (37°C, 2 h) and mounted on glass slides with 50% glycerol. Bundle sheath extraction was not possible in C3 wheat due to C3 plants lacking suberin and lignin on the mesophyll-bundle sheath interface, thus bundle sheath strands are not structurally supported and are not retained after mechanical blending.

Leaf and bundle sheath samples were examined using Zeiss LSM 800 with Airyscan (Carl Zeiss AG) equipped with a water immersion objective lens, corrected for coverslip thickness (C-Apochromat 63x/NA 1.2W). Excitation at 488 nm allowed visualisation of the Alexa Fluor 488 signal (indicating location of the VDAC1 protein on mitochondria), with emission detected at 522-550 nm. Sequentially, fluorescence from Calcofluor White stained cell walls was detected at 434-445 nm following an excitation at 405 nm. Multiple 3D stacks were captured for each sample, and three leaf samples, each prepared from individual plants, were examined for each species.

**Transmission Electron Microscopy**

The middle sections of leaves free from midrib were cut into 1 x 3 mm2 pieces while submerged in 0.1 M sodium phosphate buffer (pH 7.2) and transferred into primary fixative solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer). Leaf tissue was vacuum infiltrated with the primary fixative solution in a Biowave microwave vacuum (Pelco Biowave, Ted Pella, Inc.) at 8 inHg of pressure for 2 h, then transferred into secondary fixative solution (1% osmium tetroxide in distilled H2O; room temperature, 1 h). Fixed leaf tissue was dehydrated in graded ethanol series, and the samples were then embedded in 100% LR White resin, at 60°C). Ultrathin leaf sections of 70 - 80 nm were obtained using a Leica Ultramicrotome (Leica EM UC7, Leica Microsystems) equipped with a diamond knife and examined using a Hitachi HA7100 transmission electron microscope (Hitachi High Technologies America) at 8 kV accelerating voltage. At least 10 images were taken from 3-4 cells of each cell type (mesophyll and bundle sheath cells) from each of three ultrathin leaf sections, and repeated on three individual plants.

**Image analysis**

Confocal images were analysed using the 3D Object Counter function (Bolte & Cordelières, 2006) in FIJI software (Schindelin et al., 2012) for volume and counts of mitochondria per cell volume. A cell was identified from a 3D stack, and a rectangular box was drawn and placed on the border of the cell to best approximate its volume. The volume of this rectangular box, together with the volume and count of mitochondria within the box were quantified. More details of the 3D image analysis are available in Method S4 and Figure S6. TEM images were also analysed using FIJI software for mitochondrial count, area, cristae and matrix areas in mesophyll and bundle sheath cells. White regions of a mitochondrion were considered as cristae [i.e. swollen cristae; see (Armstrong et al., 2006; Pridham, 1968)], while black region is heavily stained matrix due to its high protein component.

**Statistical analysis**

ANOVA and linear mixed-effect models were used to compare mitochondrial number and volume, cristae ratio, bundle sheath- and leaf-level *R*dark rates among the examined cell types and species. In addition, Tukey’s honest significant differences and least squares means tests were applied when comparing pairwise in a linear model. Statistical analyses were carried out using the lmer, emmeans, lm, aov and TukeyHSD functions in the R program (R Core Team, 2018). Comparisons were considered significant if *P* < 0.05. All data were checked with Bartlett’s test for linearity, normality, and heteroscedasticity.

## **Results**

**Post-illumination CO2 burst**

C4 NAD-ME type *P. miliaceum* and PCK type *U. panicoides* showed a marked PIB in CO2 release during light-to-dark transitions (Fig. 1). In *P. miliaceum*, the PIB was pronounced but short in duration, whereas in *U. panicoides*, the PIB was less pronounced but longer in duration. By contrast, the NADP-ME typemaize did not show a marked PIB, with net assimilation gradually declining, leading to slow and steady release of CO2 as assimilation transitioned into *R*dark. The size of PIB peaks in the three species did not relate to light-saturating net assimilation rates during the preceding light period (*A*sat, *P* > 0.1, Table 1).

**Rates of dark respiration in intact leaves**

After 30 min in the dark, CO2-based *R*dark rates declined from the peaks of the PIB to a stable rate. Expressed on a leaf area basis (Table 1), *P. miliaceum* (NAD-ME type) exhibited significantly higher *R*dark rates (*P* < 0.05) than *U. panicoides* (PCK type) and maize (NADP-ME type). The species differences in *R*dark on an area-basis were confirmed by measurements on a larger sample size for which only CO2 efflux in darkness was measured (n = 12 ~ 20; Fig. S7). Surprisingly, the species differences in CO2-based *R*dark (i.e. Table 1) were not consistent with O2-based estimates of *R*dark: maize showed the highest O2-based *R*dark, whereas *P. miliaceum* showed the lowest rates, regardless of units (Table 2). For O2-based *R*dark expressed on a leaf area basis, maize was significantly higher than the other two species (*P* < 0.01), and a similar pattern was observed for mass-based *R*dark (*P* < 0.01). For *R*dark per N, maize was also significantly higher than *U. panicoides* (*P* < 0.001), which in turn was significantly higher than *P. miliaceum* (*P* < 0.05).

**Rates of dark respiration in bundle sheath strands**

The difference between the three C4 types in O2-based *R*dark rates of intact leaves was also reflected in O2-based rates in extracted bundle sheath strands in the absence of exogenous substrates and adenylates: the O2-based *R*dark rates per unit chlorophyll were the highest in maize, followed by *P. miliaceum*, and then *U. panicoides* (*P* < 0.01; Fig. 2 and Table 2). The addition of malate increased O2-based *R*dark in *P. miliaceum* and *U. panicoides* (*P* < 0.001 for both) but not in maize (Fig. 2), indicating that C4 acids can stimulate the mETC in the dark in *P. miliaceum* and *U. panicoides* but not maize. With added malate, O2-based *R*dark was similar in *P. miliaceum* and *U. panicoides* and both were higher than in maize (*P* < 0.01). In *P. miliaceum* and *U. panicoides*, rates did not increase with further addition of malate nor of a wide range of other potential respiratory substrates (Fig. S8), indicating that the amount of malate added was enough to maximise respiration (i.e. respiratory capacity). Surprisingly, *R*dark did not respond to the addition of ADP in combination with malate (Fig. 2). This suggests that the measured bundle sheath mitochondria may have contained high endogenous concentrations of ADP or had high cytosolic ADP:ATP ratios, given high ATP/ADP/AMP concentration could inhibit NAD-ME activity (Furbank et al., 1991). While it is also possible that exogenous application of ADP may not have entered bundle sheath mitochondria, the fact that larger molecules did suggests that ADP is likely to have made its way to the site of phosphorylation (Weiner et al., 1988).

Rates of O2-based *R*dark measured in extracted bundle sheath strands were scaled up to the whole leaf level and expressed on an area basis (Fig. 3). The scaled rates without malate addition were similar to the measured leaf-level rates in all examined species (Table 2), suggesting that most of the respiratory activity in C4 leaves is in bundle sheath cells (Furbank et al., 1990), or that *R*dark of bundle sheath cells is equal to that of mesophyll cells. By contrast, with malate addition *R*dark was approximately 3-fold and 2-fold greater than without malate addition in *P. miliaceum* and *U. panicoides*, respectively. The scaled *R*dark rate of maize was not affected by malate addition.

**The number and volume of mitochondria**

To examine whether the respiratory rates were reflected in mitochondrial anatomic traits, we adopted a novel 3D approach examining mitochondrial number and volume on cleared leaf tissue and extracted bundle sheath strands. Figure 4 shows representative micrographs demonstrating the localisation of mitochondria in mesophyll cells in cleared whole leaves (Fig. 4a-d) and in extracted bundle sheath cells (Fig. 4e-g).

There were significantly more mitochondria in bundle sheath than mesophyll cells in leaves of C4 NAD-ME and PCK types, but not the NADP-ME type or C3 (Fig. 5). Mitochondrial number per bundle sheath cell volume was approximately six times greater in C4 NAD-ME type *P. miliaceum* and PCK type *C. gayana*, compared to their C4 mesophyll counterparts and C3 mesophyll cells (*P* < 0.0001, Fig. 5a). Such a pattern was not seen in NADP-ME type maize, where mitochondrial number was similar in mesophyll and bundle sheath cells (Fig. 5a). This suggests that mitochondria were in higher density in bundle sheath cells of *P. miliaceum* and *C. gayana* compared to maize. Individual and total mitochondrial volumes per cell volume (i.e. individual and total mitochondrial volume densities; Fig. 5b and c, respectively) were similar in mesophyll cells of all species, although *P. miliaceum* did have a two-fold higher individual volume density than that of wheat and maize in mesophyll cells (Fig 5b). The pattern of total volume density (Fig. 5c) largely agrees with that of mitochondrial number per cell volume (Fig. 5a), suggesting mitochondrial number played a bigger role than individual mitochondrial volume in determining the total mitochondrial volume density. In bundle sheath cells, the total volume densities of mitochondria (both in number and in volume) were four times higher in *P. miliaceum* and *C. gayana* compared to maize. *P. miliaceum* and *C. gayana* had much denser mitochondria packed in their bundle sheath than mesophyll cells (*P* < 0.0001). No differences in mitochondrial density (both in number and total volume) between mesophyll cells of maize and wheat, or between mesophyll and bundle sheath cells of maize, were observed (Fig. 5b, c). These comparisons between wheat and maize agree with published 2D mitochondrial number/area results in rice wildtype and transgenic rice containing maize GOLDEN2-LIKE genes (Wang et al., 2017).

**Ultrastructure of mitochondria**

Figure 6 shows representative light micrographs of the cell arrangement of leaf cross sections, with the TEM micrographs indicating the ultrastructure of mitochondria in mesophyll and bundle sheath cells. Comparison among TEM micrographs of C3 and C4 species revealed that mitochondria had a significantly higher cristae to matrix ratio in *C. gayana* bundle sheath cells, compared to its mesophyll counterparts and both cell types of wheat, maize and *P. miliaceum* (*P* < 0.0001, Table 3). By contrast, mitochondria showed similar cristae to matrix ratios in mesophyll and bundle sheath cells in wheat, maize, and *P. miliaceum* (Table 3). The high cristae to matrix ratio in *C. gayana* bundle sheath mitochondria suggests potentially more mETC protein components in this species.

## **Discussion**

Bundle sheath mitochondria exhibited distinct respiratory capacities in species representing the three C4 biochemical types, linked to the very high demand for decarboxylation during photosynthesis in NAD-ME and PCK type leaves (Agostino et al., 1996). Our study shows that the rates of *R*dark in these bundle sheath cells - when supplemented with malate - are close to three-fold higher than the rates of *R*dark for corresponding whole leaves, and bundle sheath cells without malate supplementation. By contrast, this pattern was not observed in NADP-ME type maize that does not rely on mitochondrial decarboxylation of malate in photosynthesis, where *R*dark rates were similar regardless of the addition of exogenous malate or measuring system (i.e. whole leaf vs. bundle sheath strands) (Fig. 3). Further, we demonstrate that malate stimulation of bundle sheath strands in NAD-ME and PCK types was associated with: (1) an increase in the number of mitochondria per cell volume; and (2) an increase in the (individual and total) volume of mitochondria per cell volume (Fig. 5). The dramatic increase in respiration in the presence of added malate was also associated with a high cristae ratio in the PCK type, but this was not evident in the NAD-ME type (Table 3). Interestingly, the observed differences in malate stimulation of respiration and mitochondrial anatomic traits did not align with whole leaf-level *R*dark measured in the three C4 species.

**Implications of the post-illumination CO2 burst**

The patterns of PIB (Fig. 1) support a previous finding in maize that a light-to-dark transition prevents the NADP-malic enzyme from decarboxylating malate to NADPH and CO2 (and pyruvate) (Fig. S1) (Downton, 1970; Laisk & Edwards, 1997; Ohsugi & Murata, 1980). Once in the dark, malate in maize bundle sheath cells may be only slowly decarboxylated, observed as a poor response of O2 uptake to added malate (Agostino et al., 1996) and a large inactive internal malate pool (Arrivault et al., 2017). We found that the rate of *R*dark did not change when malate was added to extracted maize bundle sheath strands (Fig. 2), suggesting that this species has very little ability to decarboxylate chloroplast or cytosolic malate in the dark. This may reflect lower levels of NAD-ME in the mitochondrial matrix (Hatch & Carnal, 1992).

**More and larger mitochondria are needed for NAD-ME and PCK type photosynthesis**

Three-dimensional confocal microscopy revealed that bundle sheath cells of NAD-ME type *P. miliaceum* and PCK type *C. gayana* had significantly higher total mitochondrial volume density compared to those of NADP-ME type maize (Fig. 5). The higher total mitochondrial volume density was a combination of more and larger mitochondria in the NAD-ME and PCK types (Fig. 5a, b). Further, mitochondrial volume density was similar in mesophyll cells and bundle sheath cells of maize (Fig. 5c). Our 3D results confirm previously published 2D TEM data, where the size of mitochondria – measured as mitochondrial area per cell area and the diameter of mitochondria – was reported to be significantly higher in bundle sheath cells of NAD-ME and PCK types compared to their mesophyll counterparts and both cell types of C3 and C4 NADP-ME type (Gardeström & Edwards, 1985; Hatakeyama & Ueno, 2017; Koteyeva et al., 2011; Yoshimura et al., 2004). Given that mitochondria in C3 and C4 mesophyll and NADP-ME type bundle sheath cells are not directly involved in the C4 metabolic pathway (Furbank, 2011; Hatch, 1987; von Caemmerer & Furbank, 2016), the increase in total volume density in the bundle sheath cells of the other two types is likely to reflect the role of mitochondria in the light – that is, to support processing of C4 acids as part of the C4 photosynthesis pathway. This conclusion is supported by the stimulation of *R*dark by malate only in NAD-ME and PCK type bundle sheath cells (Fig. 2).

**More developed cristae support PCK type mitochondrial ATP production**

Bundle sheath cells of PCK type *C. gayana* had more cristae relative to matrix when compared to all other cell types and species (Table 3). This is interesting in that C4 PCK type photosynthesis is the only pathway that requires mitochondrial ATP to convert OAA to phosphoenolpyruvate and liberate CO2 for fixation (Furbank, 2011; Hatch, 1987; von Caemmerer & Furbank, 2016). Does the increase in cristae relative to matrix relate to this need for ATP use in the PCK type C4 pathway? A previous study found PCK type *U. panicoides* to have double the cytochrome *c* oxidise (COX) pathway capacity when compared to NAD-ME type *P. miliaceum* (Agostino et al., 1996). COX is the terminal oxidase of the phosphorylating branch of the mETC located in the mitochondrial inner membranes which make up the cristae. Given this, we suggest that the bundle sheath mitochondria of PCK type species invest more heavily in cristae than matrix to maximise the mETC capacity and meet the extra ATP demands of photosynthesis. Interestingly, the extra investment in cristae of the PCK type did not enhance *R*dark of bundle sheath cells for this C4 type.

**Contrasting *R*dark between bundle sheath strands and intact leaves**

When rates of *R*dark measured in bundle sheath strands in the absence of exogenous malate were extrapolated to a leaf area basis, the rates matched those measured using intact leaf discs (Fig. 3). This suggests there is likely to be little difference in dark-adapted rates of O2 uptake between bundle sheath mitochondria and mitochondria in other cell types of the leaf. Alternatively, since mitochondrial abundance in bundle sheath cells of *P. miliaceum* and *U. panicoides* was six-fold higher than that in their mesophyll cells, bundle sheath mitochondria are likely to dominate leaf *R*dark regardless of mitochondrial activity in the rest of the leaf. This was supported by similar pyruvate production (i.e. a proxy of mitochondrial activity) of isolated mitochondria from whole leaves versus of mitochondria in extracted bundle sheath cells in *P. miliaceum* (Furbank et al., 1990). It is worth noting that mitochondria are also present in non-photosynthetic cells such as epidermal and vascular sheath cells (Logan, 2006), but little is known of the differences between mitochondria of these cell types.

We also demonstrated that addition of malate to bundle sheath strands in *P. miliaceum* and *U. panicoides*, tripled and doubled *R*dark, respectively (Fig. 3). This was expected and notably in *U. panicoides* the difference roughly matched the theoretical amount of carbon substrate flux passing through PCK type bundle sheath mitochondria (i.e. approximately three-fold higher than the dark respiratory flux). However, it is unclear why the difference was much less in the NAD-ME type where the theoretical estimation of carbon flux through bundle sheath mitochondria is 10-fold. Since it is unlikely that bundle sheath mitochondria are the rate limiting step in NAD-ME type photosynthesis, the difference in respiratory product demands in the light versus dark may have contributed to the expected flux differential. As mentioned above, in the light NAD-ME type bundle sheath mitochondria are required to generate large amounts of CO2 for photosynthetic carbon fixation and pyruvate for the C4 acid recycling (Fig. S1b). Such demands do not exist in the dark when mitochondria predominantly are involved in cellular maintenance and protein turnover (O'Leary et al., 2019). Thus, the carbon flux passing through NAD-ME type is expected to reflect the real-time demands for cellular maintenance in the dark.

***R*dark and C4 mitochondrial characteristics do not align**

The variations in mitochondrial number, volume, and ultrastructure we observed between the C4 types are consistent with the role of mitochondria in supporting C4 photosynthesis in the light, but they do not explain variations in rates of O2-based leaf *R*dark between the types (Fig. 3). Rather, the difference observed in leaf *R*dark are likely to reflect differences in cellular maintenance demands between the three C4 species. For example, the highest O2-based *R*dark was in maize (Table 2), which has high protein turnover costs; both Penning de Vries (1975) and Ghannoum et al. (2005) reported faster protein turnover in maize than in plants with other C4 pathways, mostly attributed to faster Rubisco turnover. Finally, we observed greater CO2-based *R*dark in *P. miliaceum* than maize despite maize having higher O2-based respiration (Table 1 & 2). That is, *P. miliaceum* had a greater respiratory quotient than maize, suggesting that it makes greater use of organic acids as respiratory substrates in the dark compared to maize. Interestingly, Siebke et al. (2003) reported similar averaged respiratory quotients closed to unity for NADP-ME and NAD-ME type species in the dark. What is less clear, however, is how changes in respiratory substrate pools are correlated with *R*dark.

**Concluding remarks**

Our results show that the respiratory response of NAD-ME and PCK type bundle sheath mitochondria to malate (in contrast to that of the NADP-ME type) is associated with differences in mitochondrial number, volume, and/or ultrastructure. These differences in mitochondrial traits match the relative contributions mitochondria make to photosynthesis in these two C4 types. However, no clear connection could be made between mitochondrial number and characteristics and rates of *R*dark. This indicates that *R*dark in C4 leaves is primarily driven by cellular maintenance demand in a manner that is independent of mitochondrial organic acid cycling in the light.

## **Acknowledgement**

This work was funded by grants from the Australian Research Council and was supported by the ARC Centre of Excellence in Plant Energy Biology (CE140100008), and the ARC Centre of Excellence for Translational Photosynthesis (CE1401000015). The authors acknowledge the facilities and the scientific and technical assistance of Microscopy Australia at the Centre for Advanced Microscopy, Australian National University, a facility that is funded by the University and the Federal Government. Y.F. was supported by the ARC Centre of Excellence for Plant Energy Biology Warwick Hillier Honours Scholarships, ANU International PhD Scholarship (737/2018) and HDR Fee Remission Merit Scholarship. We thank Associate Professor Oula Ghannoum for providing seeds. The authors have no conflict of interest to declare.

## **Author contribution**

Y.F., S.A., R.T.F and O.K.A. planned and designed the study. Y.F., A.A., F.D., M.R., D.W. and J.L. conducted the experiments. Y.F., A.P.S., S.A., R.T.F., A.A., D.A.D., S.v.C. and O.K.A interpreted the data. Y.F., A.P.S and O.K.A. wrote the first draft; all authors contributed significantly to subsequent versions.

## **ORCID**

Yuzhen Fan: 0000-0003-1857-9244

Andrew P. Scafaro: 0000-0003-3738-1145

Shinichi Asao: 0000-0002-0334-5464

Robert T. Furbank: 0000-0001-8700-6613

David A. Day: 0000-0001-7967-2173

Susanne von Caemmerer: 0000-0002-8366-2071

Florence Danila: 0000-0002-7352-3852

Melanie Rug: 0000-0002-4687-9677

Jiwon Lee: 0000-0003-2382-2106

Owen K. Atkin: 0000-0003-1041-5202

## **Supporting Information**

Additional supporting information may be found in the online version of this article.

**Method S1** -Selection of chemical substrates used in bundle sheath *R*dark measurements.

**Method S2** - Examining reactivity and specificity of a monoclonal VDAC1 antibody.

**Method S3** -Examining mitochondrial anatomic traits using MitoTracker dyes.

**Method S4** -3D confocal image analysis and outlier removal.

**Figure S1** - Three types of classic C4 photosynthetic pathways.

**Figure S2** - Comparisons of substrate effect on *R*dark rates measured in bundle sheath strands.

**Figure S3** - Reactivities of the monoclonal VDAC1 antibody.

**Figure S4** - Overview of C4 NAD-ME *Panicum miliaceum* leaf slices *in situ* fluorescent immunolocalisation with VDAC1 antibody and MitoTracker Red dye.

**Figure S5** – MitoTracker Red *in situ* localisation of mitochondria in C4 NAD-ME *Panicum miliaceum* vascular bundles.

**Figure S6** - Schematic illustration of methods used in 3D confocal image analysis and outlier removal.

**Figure S7** - A screening of leaf *R*dark of C4 species measured on intact whole leaves *in situ* using LI-COR 6400 XT.

**Figure S8** - Stimulation of O2 uptake by mitochondria in extracted bundle sheath strands in the presence of chemicals.

## **Reference**

Agostino, A., Heldt, H. W., & Hatch, M. D. (1996). Mitochondrial respiration in relation to photosynthetic C4 acid decarboxylation in C4 species. *Australian Journal of Plant Physiology, 23*(1), 1-7. doi:10.1071/PP9960001

Armstrong, A. F., Logan, D. C., Tobin, A. K., O'Toole, P., & Atkin, O. K. (2006). Heterogeneity of plant mitochondrial responses underpinning respiratory acclimation to the cold in *Arabidopsis thaliana* leaves. *Plant, Cell & Environment, 29*(5), 940-949. doi:10.1111/j.1365-3040.2005.01475.x

Arrivault, S., Obata, T., Szecówka, M., Mengin, V., Guenther, M., Hoehne, M., . . . Stitt, M. (2017). Metabolite pools and carbon flow during C4 photosynthesis in maize: 13CO2 labeling kinetics and cell type fractionation. *Journal of Experimental Botany, 68*(2), 283-298. doi:10.1093/jxb/erw414

Bolte, S., & Cordelières, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy, 224*(3), 213-232. doi:doi.org/10.1111/j.1365-2818.2006.01706.x

Byrd, G. T., Sage, R. F., & Brown, R. H. (1992). A comparison of dark respiration between C3 and C4 plants. *Plant Physiology, 100*(1), 191-198. doi:10.1104/pp.100.1.191

Danila, F. R., Quick, W. P., White, R. G., Furbank, R. T., & von Caemmerer, S. (2016). The metabolite pathway between bundle sheath and mesophyll: quantification of plasmodesmata in leaves of C3 and C4 monocots. *The Plant Cell, 28*(6), 1461. doi:10.1105/tpc.16.00155

Danila, F. R., Quick, W. P., White, R. G., Kelly, S., von Caemmerer, S., & Furbank, R. T. (2018). Multiple mechanisms for enhanced plasmodesmata density in disparate subtypes of C4 grasses. *Journal of Experimental Botany, 69*(5), 1135-1145. doi:10.1093/jxb/erx456

Danila, F. R., Quick, W. P., White, R. G., von Caemmerer, S., & Furbank, R. T. (2019). Response of plasmodesmata formation in leaves of C4 grasses to growth irradiance. *Plant, Cell & Environment, 42*(8), 2482– 2494. doi:10.1111/pce.13558

Downton, W. J. S. (1970). Preferential C4-dicarboxylic acid synthesis, the postillumination CO2 burst, carboxyl transfer step, and grana configurations in plants with C4-photosynthesis. *Canadian Journal of Botany, 48*(10), 1795-1800. doi:10.1139/b70-263

Evans, J. R., & von Caemmerer, S. (1996). Carbon dioxide diffusion inside leaves. *Plant Physiology, 110*(2), 339-346. doi:10.1104/pp.110.2.339

Furbank, R. T. (2011). Evolution of the C4 photosynthetic mechanism: are there really three C4 acid decarboxylation types? *Journal of Experimental Botany, 62*(9), 3103-3108. doi:10.1093/jxb/err080

Furbank, R. T., Agostino, A., & Hatch, M. D. (1990). C4 acid decarboxylation and photosynthesis in bundle sheath cells of NAD-malic enzyme-type C4 plants: Mechanism and the role of malate and orthophosphate. *Archives of Biochemistry and Biophysics, 276*(2), 374-381. doi:doi.org/10.1016/0003-9861(90)90735-H

Furbank, R. T., Agostino, A., & Hatch, M. D. (1991). Regulation of C4 photosynthesis: Modulation of mitochondrial NAD-malic enzyme by adenylates. *Archives of Biochemistry and Biophysics, 289*(2), 376-381. doi:doi.org/10.1016/0003-9861(91)90426-J

Gardeström, P., & Edwards, G. E. (1985). Leaf mitochondria (C3 + C4 + CAM). In R. Douce & D. A. Day (Eds.), *Higher plant cell respiration* (Vol. 18, pp. 314-346). Berlin Heidelberg: Springer-Verlag.

Gebauer, G., & Schulze, E.-D. (1991). Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. *Oecologia, 87*(2), 198-207. doi:10.1007/BF00325257

Ghannoum, O., Evans, J. R., Chow, W. S., Andrews, T. J., Conroy, J. P., & 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*(2), 638-650. doi:10.1104/pp.104.054759

Ghannoum, O., Evans, J. R., & von Caemmerer, S. (2011). Nitrogen and water use efficiency of C4 plants. In A. S. Raghavendra & R. F. Sage (Eds.), *C4 photosynthesis and related CO2 concentrating mechanisms* (pp. 129-146). Dordrecht: Springer.

Hatakeyama, Y., & Ueno, O. (2017). Intracellular position of mitochondria in mesophyll cells differs between C3 and C4 grasses. *Journal of Plant Research, 130*(5), 885-892. doi:10.1007/s10265-017-0947-z

Hatch, M. D. (1987). C4 photosynthesis: a unique elend of modified biochemistry, anatomy and ultrastructure. *Biochimica et Biophysica Acta, 895*(2), 81-106. doi:10.1016/S0304-4173(87)80009-5

Hatch, M. D., & Carnal, N. W. (1992). The role of mitochondria in C4 photosynthesis. In H. Lambers & L. H. W. van der Plas (Eds.), *Molecular, Biochemical and Physiological Aspects of Plant Respiration* (pp. 135-148): The Hague: SPB Academic Publishing.

Hatch, M. D., Kagawa, T., & Craig, S. (1975). Subdivision of C4-pathway species based on differing C4 acid decarboxylating systems and ultrastructural features. *Functional Plant Biology, 2*(2), 111-128. doi:doi.org/10.1071/PP9750111

Khoshravesh, R., Stinson, C. R., Stata, M., Busch, F. A., Sage, R. F., Ludwig, M., & Sage, T. L. (2016). C3-C4 intermediacy in grasses: organelle enrichment and distribution, glycine decarboxylase expression, and the rise of C2 photosynthesis. *Journal of Experimental Botany, 67*(10), 3065-3078. doi:10.1093/jxb/erw150

Koteyeva, N. K., Voznesenskaya, E. V., Roalson, E. H., & Edwards, G. E. (2011). Diversity in forms of C4 in the genus *Cleome* (Cleomaceae). *Annals of Botany, 107*(2), 269-283. doi:10.1093/aob/mcq239

Laisk, A., & Edwards, G. E. (1997). Post-illumination CO2 exchange and light-induced CO2 bursts during C4 photosynthesis. *Australian Journal of Plant Physiology, 24*(4), 517-528. doi:10.1071/PP97002

Leegood, R. C. (2002). C4 photosynthesis: principles of CO2 concentration and prospects for its introduction into C3 plants. *Journal of Experimental Botany, 53*(369), 581-590. doi:10.1093/jexbot/53.369.581

Logan, D. C. (2006). Plant mitochondrial dynamics. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1763*(5), 430-441. doi:10.1016/j.bbamcr.2006.01.003

Mackinney, G. (1941). Absorption of light by chlorophyll solutions. *The Journal of Biological Chemistry, 140*(2), 315-322. doi:10.1016/S0021-9258(18)51320-X

Muhaidat, R., Sage, T. L., Frohlich, M. W., Dengler, N. G., & Sage, R. F. (2011). Characterization of C3–C4 intermediate species in the genus *Heliotropium* L. (Boraginaceae): anatomy, ultrastructure and enzyme activity. *Plant, Cell & Environment, 34*(10), 1723-1736. doi:10.1111/j.1365-3040.2011.02367.x

O'Leary, B. M., Asao, S., Millar, A. H., & Atkin, O. K. (2019). Core principles which explain variation in respiration across biological scales. *New Phytologist, 222*(2), 670-686. doi:10.1111/nph.15576

O’Leary, B. M., Lee, C. P., Atkin, O. K., Cheng, R., Brown, T. B., & Millar, A. H. (2017). Variation in leaf respiration rates at night correlates with carbohydrate and amino acid supply. *Plant Physiology, 174*(4), 2261-2273. doi:10.1104/pp.17.00610

Ohsugi, R., & Murata, T. (1980). Leaf anatomy, post-illumination CO2 burst and NAD-malic enzyme activity of *Panicum dichotomiflorum*. *Plant & Cell Physiology, 21*(7), 1329-1333. doi:10.1093/oxfordjournals.pcp.a076131

Palmer, W. M., Martin, A. P., Flynn, J. R., Reed, S. L., White, R. G., Furbank, R. T., & Grof, C. P. L. (2015). PEA-CLARITY: 3D molecular imaging of whole plant organs. *Scientific Reports, 5*, 13492. doi:10.1038/srep13492

Penning de Vries, F. W. T. (1975). The cost of maintenance processes in plant cells. *Annals of Botany, 39*(1), 77-92. doi:10.1093/oxfordjournals.aob.a084919

Pridham, J. B. (1968). *Plant Cell Organelles*: Academic Press.

R Core Team. (2018). A language and environment for statistical computing. In *R foundation for statistical computing*. <https://www.r-project.org/>: R v.4.0.5.

Scafaro, A. P., Negrini, A. C. A., O'Leary, B. M., Rashid, F. A. A., Hayes, L., Fan, Y., . . . Atkin, O. K. (2017). The combination of gas-phase fluorophore technology and automation to enable high-throughput analysis of plant respiration. *Plant Methods, 13*(16), 1-13. doi:10.1186/s13007-017-0169-3

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019

Siebke, K., Ghannoum, O., Conroy, J. P., Badger, M. R., & Von Caemmerer, S. (2003). Photosynthetic oxygen exchange in C4 grasses: the role of oxygen as electron acceptor. *Plant, Cell & Environment, 26*(12), 1963-1972. doi:10.1046/j.1365-3040.2003.01112.x

Smith, N. G., Li, G., & Dukes, J. S. (2019). Short-term thermal acclimation of dark respiration is greater in non-photosynthetic than in photosynthetic tissues. *AoB PLANTS, 11*(6), plz064. doi:10.1093/aobpla/plz064

Sonawane, B. V., Sharwood, R. E., von Caemmerer, S., Whitney, S. M., & Ghannoum, O. (2017). Short-term thermal photosynthetic responses of C4 grasses are independent of the biochemical subtype. *Journal of Experimental Botany, 68*(20), 5583-5597. doi:10.1093/jxb/erx350

Tjoelker, M. G., Craine, J. M., Wedin, D., Reich, P. B., & Tilman, D. (2005). Linking leaf and root trait syndromes among 39 grassland and savannah species. *New Phytologist, 167*(2), 493-508.

von Caemmerer, S., & Furbank, R. T. (2016). Strategies for improving C4 photosynthesis. *Current Opinion in Plant Biology, 31*, 125-134. doi:10.1016/j.pbi.2016.04.003

Wang, P., Khoshravesh, R., Karki, S., Tapia, R., Balahadia, C. P., Bandyopadhyay, A., . . . Langdale, J. A. (2017). Re-creation of a key step in the evolutionary switch from C3 to C4 leaf anatomy. *Current Biology, 27*(21), 3278-3287. doi:10.1016/j.cub.2017.09.040

Weiner, H., Burnell, J. N., Woodrow, I. E., Heldt, H. W., & Hatch, M. D. (1988). Metabolite diffusion into bundle sheath cells from C4 plants: relation to C4 photosynthesis and plasmodesmatal function. *Plant Physiology, 88*(3), 815-822. doi:10.1104/pp.88.3.815

Williams, D. B., & Carter, C. B. (1996). The transmission electron microscope. In D. B. Williams & C. B. Carter (Eds.), *Transmission electron microscopy: A textbook for materials science* (pp. 3-17). Boston, MA: Springer US.

Winey, M., Meehl, J. B., O'Toole, E. T., & Giddings, T. H. (2014). Conventional transmission electron microscopy. *Molecular Biology of the Cell, 25*(3), 319-323. doi:10.1091/mbc.e12-12-0863

Wright, I. J., Reich, P. B., Westoby, M., & Ackerly, D. D. (2004). The worldwide leaf economics spectrum. *Nature, 428*(6985), 821-827. doi:10.1038/nature02403

Yoshimura, Y., Kubota, F., & Ueno, O. (2004). Structural and biochemical bases of photorespiration in C4 plants: quantification of organelles and glycine decarboxylase. *Planta, 220*(2), 307-317. doi:10.1007/s00425-004-1335-1

## **Tables**

**Table 1** – Physiological and carbon flux characterisation of leaves from the three classical C4 types.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Species | *A*sat | *R*dark | *A*sat:*R*dark | LMA | Leaf N |
| (mol ) | (mol ) |  | () | () |
| C4 NADP-ME *Z. mays* | 27.39 0.72 a | 0.98 0.05 a | 28.14 1.59 a | 20.73 0.28 a | 44.11 0.43 a |
| C4 NAD-ME *P. miliaceum* | 33.21 2.06 a | 1.28 0.09 b | 26.18 1.66 a | 17.89 0.98 b | 59.06 1.91 b |
| C4 PCK *U. panicoides* | 29.68 1.77 a | 0.84 0.11 a | 37.31 7.72 a | 17.60 0.80 b | 46.92 1.18 a |

Light-saturated photosynthesis (*A*sat), CO2-based leaf dark respiration (*R*dark), area-based *A*sat-*R*dark ratio (*A*sat:*R*dark), leaf dry mass per area (LMA) and leaf nitrogen (N) concentration. Values are the means SE, with a sample size of 3-6 plant replicates for each species. Linear one-way ANOVA is performed to compare every parameter (i.e. every column) between the three species. Values indicated by the same letter within a column are not statistically different at *P* < 0.05.

**Table 2** – Differences in O2-based leaf-level *R*dark between the C4 types.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Species | *R*dark | | | |
| Leaf | | | Bundle sheath strands |
| (mol O2 m-2 s-1) | (nmol O2 g-1 DM s-1) | (mol O2 g-1 N s-1) | (mol O2 mg-1 Chl s-1) |
| C4 NADP-ME *Z. mays* | 0.89 ± 0.02 a | 42.68 ± 1.52 a | 0.97 ± 0.04 a | 24.31 1.69 a |
| C4 NAD-ME *P. miliaceum* | 0.57 ± 0.05 b | 31.79 ± 0.87 b | 0.54 ± 0.02 b | 8.82 0.71 b |
| C4 PCK *U. panicoides* | 0.60 ± 0.08 b | 33.42 ± 3.03 b | 0.71 ± 0.05 b | 12.73 1.25 b |

O2-based leaf dark respiration (*R*dark) expressed on a leaf area, dry mass (DM), Nitrogen (N), and bundle sheath O2 update rate expressed on chlorophyll (Chl) basis. Values are the means SE, with a sample size of 3-6 plant replicates for each species. Linear one-way ANOVA is performed to compare every parameter (i.e. every column) between the three species. Values indicated by the same letter within a column are not statistically different at *P* < 0.05.

**Table 3** – Ultrastructural characteristics of mitochondria measured in mesophyll (Meso) and bundle sheath (BS) cells of C3 and C4 leaves.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Species | Cell type | # per cell area (m-2) | Area (m2) | Cristae (m2) | Matrix (m2) | Cristae to matrix ratio |
| C3  wheat | Meso | 0.07 0.01 a,A | 0.40 0.02 a,A | 0.07 0.00 a,A | 0.33 0.02 a,A | 0.17 0.01 a,A |
| BS | 0.10 0.01 a,A | 0.32 0.02 a,A | 0.09 0.01 a,A | 0.23 0.02 b,A | 0.24 0.02 a,A |
| C4 NADP-ME  *Z. mays* | Meso | 0.05 0.01 a,A | 0.17 0.01 a,B | 0.06 0.01 a,A | 0.11 0.01 a,B | 0.55 0.05 a,B |
| BS | 0.12 0.01 a,AB | 0.27 0.01 b,A | 0.10 0.00 b,A | 0.17 0.01 a,B | 0.61 0.02 a,B |
| C4 NAD-ME  *P. miliaceum* | Meso | 0.05 0.00 a,A | 0.29 0.02 a,C | 0.09 0.01 a,A | 0.19 0.01 a,C | 0.50 0.03 ab,B |
| BS | 0.16 0.01 b,AB | 0.63 0.02 b,B | 0.25 0.01 b,B | 0.38 0.01 b,C | 0.63 0.01 a,B |
| C4 PCK  *C. gayana* | Meso | 0.04 0.00 a,A | 0.23 0.02 a,BC | 0.06 0.00 a,A | 0.17 0.01 a,BC | 0.40 0.04 a,B |
| BS | 0.17 0.01 b,B | 0.33 0.01 b,A | 0.17 0.00 b,C | 0.15 0.00 a,B | 1.02 0.02 b,C |

Mitochondrial number per cell area (m2), mean cross-sectional area of individual mitochondria (m2), area of matrix and cristae in individual mitochondria (m2) and ratio of cristae area to matrix area (termed as ‘cristae to matrix ratio’) in individual mitochondria are shown. Values represent the mean of three replicate leaves ( SE), with each replicate value being made up of the average of 21-62 cells of the measured cell types of 51-566 sectioned mitochondria. Linear mixed-effect model was run to compare the traits within a species (between the three cell types) denoted with lower-case letters (i.e. a, b and c), and the traits within a cell type (between the four species) indicated with upper-case letters (i.e. A, B and C). Values indicated by the same letter and case within a column are not statistically different at *P* < 0.05.

## **Figure captions**

**Figure 1.** Post-illumination CO2 burst (PIB) of C4 NADP-ME *Z. mays*, C4 NAD-ME *P. miliaceum* and C4 PCK *U. panicoides* at 30°C and 400 ppm [CO2]. Prior to light being switched off at time = 0 s rates of light saturated net assimilation (*A*sat) were recorded under an irradiance of 1,600 μmol quanta m-2 s-1. Measurements were recorded every one to three seconds for 160 seconds and a running mean of two consecutive data points was computed when there is a gap in time. Faint points with the same colour are individual measurements of six independent replicates, while the solid lines show averaged trajectories for each species.

**Figure 2.** Rates of O2-based *R*dark on a chlorophyll basis in the extracted bundle sheath strands of the three C4 species. Malate (10 mM) is added as the C4 carbon acid to stimulate responses of mitochondria to photosynthesis-induced carbon flux, followed by addition of ADP (1 mM) to eliminate adenylate restriction. One outlier in the malate treatment of *U. panicoides* was more than four standard deviations away from the mean and thus was removed. Linear mixed-effect model was applied within each species and across the three species, with run and electrode number being the random effects. The number of biological replicates (i.e. individual plants) for *Z. mays*, *P. miliaceum* and *U. panicoides* was five, four and five, respectively. The number of technical replicates (i.e. electrode runs) was 15, 17 and 13 for the three species, respectively. Boxes indicated by the same letter are not statistically different at *P* < 0.05.

**Figure 3.** Comparison of O2-based *R*dark rates measured in extracted bundle sheath strands and the rates measured in intact whole leaves within each C4 species. One-way ANOVA and a Tukey HSD test examining leaf and bundle sheath *R*dark rates were applied in each species. Across-species comparisons of O2 uptake rates within the same system were reported in Fig. 2 and the main text. There were three to five biological replicates per species. Boxes indicated by the same letter are not statistically different at *P* < 0.05.

**Figure 4.** Three-dimensional (3D) confocal micrographs of VDAC1 localisation (as a proxy of mitochondria) in mesophyll and bundle sheath cells of C3 and C4 species. (a)-(d): 3D reconstructed image (from 146-356 single focal planes) of multiple mesophyll cells hybridised with primary antibody to VDAC1 proteins and secondary antibody tagged with Alexa Flour 488 (green) and stained with calcofluor white (grey) to show partial cell walls. (e)-(g): 3D reconstructed image (from 97-157 single focal planes) of bundle sheath cells, where VDAC1 proteins are shown in green and chlorophyll autofluorescence is indicated in red. One, one and three bundle sheath cells are shown in (e), (f), and (g), respectively. Meso = mesophyll, BS = bundle sheath. Scale bar = 20 m.

**Figure 5.** Three-dimensional (3D) mitochondrial traits in mesophyll and bundle sheath cells of C3 and C4 species expressed per cell volume. (a) mitochondrial number; (b) individual mitochondrial volume; (c) total mitochondrial volume. Values represent the mean of three replicate leaves ( SE), with each replicate being the average value of 7-18 cells of the measured cell types consisting of 8-215 mitochondria per cell. Total mitochondrial volume per cell volume (c) is calculated by multiplying mitochondrial number (a) and individual mitochondrial volume per cell volume (b). Linear mixed-effect models and least squares means test were applied on the averaged mitochondrial traits per cell between mesophyll and bundle sheath cells of the four species, with cell and plant numbers being the random effects. Bars indicated by the same letter are not statistically different at *P* < 0.05. Meso = mesophyll, BS = bundle sheath.

**Figure 6.** Micrographs of leaf cellular structure and mitochondrial ultrastructure in C3 and C4 species. (a)-(d): cellular structure of cross-sectioned leaves under light microscope. (e)-(h): ultrastructure of mitochondria in mesophyll cells under TEM. (i)-(l): ultrastructure of mitochondria in bundle sheath cells under TEM. For individual mitochondria, white regions are cristae (in-fold of inner membrane of a mitochondrion) and darken regions are matrix. Scale bars are indicated in the figure. Meso = mesophyll, BS = bundle sheath, VB = vascular bundle, M = mitochondrion and C = chloroplast.