C₄ anatomy can evolve via a single developmental change

Authors: Marjorie R. Lundgren¹ ² (m.lundgren@lancaster.ac.uk), Luke T. Dunning¹
(1.dunning@sheffield.ac.uk), Jill K. Olofsson¹ (j.k.olofsson@sheffield.ac.uk), Jose J. Moreno-Villena¹ (jjmorenovillena1@sheffield.ac.uk), Jacques W. Bouvier¹ (jbouvier1@sheffield.ac.uk), Tammy Sage³ (tammy.sage@utoronto.ca), Roxana Khoshravesh³ (r.khoshravesh@utoronto.ca), Stefanie Sultmanis³ (stefanie.sultmanis@mail.utoronto.ca), Matt Stata³ (matt.stata@mail.utoronto.ca), Brad Ripley⁴ (b.ripley@ru.ac.za), Maria S. Vorontsova⁵ (M.Vorontsova@kew.org), Guillaume Besnard⁶ (guillaume.besnard@univ-tlse3.fr), Claire Adams⁴ (claire3889@gmail.com), Nicholas Cuff⁷ (Nicholas.Cuff@nt.gov.au), Anthony Mapaura⁸ (mapaura@yahoo.com), Matheus Bianconi¹ (mebianconi1@sheffield.ac.uk), Christine M. Long⁹ (christine.long@nt.gov.au), Pascal-Antoine Christin¹ (p.christin@sheffield.ac.uk), Colin P. Osborne¹* (c.p.osborne@sheffield.ac.uk)
1Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK
2Current address: Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK
3Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks Street, Toronto, Ontario M5S 3B2, Canada
4Botany Department, Rhodes University, Grahamstown 6139, South Africa
5Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, UK
6Laboratoire Évolution & Diversité Biologique (EDB UMR5174), Université de Toulouse, CNRS, ENSFEA, UPS, IRD, 118 route de Narbonne, 31062 Toulouse, France
7Northern Territory Herbarium, Department of Environment and Natural Resources. PO Box 496, Palmerston, NT 0831, Australia
8National Herbarium and Botanic Garden, Harare, Zimbabwe
9Department of Primary Industry and Fisheries, Northern Territory Government, Darwin, NT 0801, Australia

*Author for correspondence: Colin P. Osborne; c.p.osborne@sheffield.ac.uk; Tel: +44-114-222-0146; Fax: +44-114-222-0002

Running title: One anatomical change key for C₄ emergence

Keywords: *Alloteropsis*, bundle sheath, C₃-C₄ intermediate, C₄ photosynthesis, evolution, grass, leaf anatomy, mesophyll, vein density

Article type: Letter
Statement of authorship: MRL, PAC and CPO designed the study. MRL produced and analyzed the data, with the help of LTD, JJMV, and JWB. TS and RK performed the immunolocalisations and TEM imaging. SS assisted with immunolocalisation sample preparation. MS assisted with tissue fixation. MRL, LTD, JKO, BR, MSV, GB, CA, NC, AM, MB, CML, PAC, and CPO contributed plant material. MRL, PAC, and CPO interpreted the results and wrote the paper, with the help of all the authors.

Data accessibility statement: Should this manuscript be accepted, the data supporting the results will be archived in the public repository Dryad and the data DOI will be included at the end of the article.

Abbreviations: BS, bundle sheath; CCP, CO₂ compensation point, IS, inner sheath; LDA, linear discriminant analysis; M, mesophyll; OS, outer sheath
Abstract

C₄ photosynthesis is a complex trait that boosts productivity in warm environments. Paradoxically, it evolved independently in numerous plant lineages, despite requiring specialized leaf anatomy. The anatomical modifications underlying C₄ evolution have previously been evaluated through interspecific comparisons, which capture numerous changes besides those needed for C₄ functionality. Here, we quantify the anatomical changes accompanying the transition between non-C₄ and C₄ phenotypes by sampling widely across the continuum of leaf anatomical traits in the grass Alloteropsis semialata. Within this species, the only trait that is shared among and specific to C₄ individuals is an increase in vein density, driven specifically by minor vein development that yields multiple secondary effects facilitating C₄ function. For species with the necessary anatomical preconditions, developmental proliferation of veins can therefore be sufficient to produce a functional C₄ leaf anatomy, creating an evolutionary entry point to complex C₄ syndromes that can become more specialized.
INTRODUCTION

The vast majority of plants use C₃ photosynthesis, but some lineages evolved the C₄ pathway to overcome environmentally induced limitations on carbon fixation (Ehleringer et al. 1991; Sage et al. 2011). Net carbon fixation by C₃ photosynthesis is decreased in warm, high light, arid, and saline environments that lower CO₂ concentrations within the leaf and increase photorespiration, the process initiated when O₂ instead of CO₂ is fixed by the enzyme Rubisco (Chollet & Ogren 1975). To circumvent the losses of carbon and energy caused by photorespiration, the C₄ pathway spatially separates the initial fixation of carbon and its assimilation by Rubisco across two leaf compartments, thereby concentrating CO₂ at the enzyme’s active site to promote CO₂- rather than O₂-fixation (Downton & Tregunna 1968; Hatch 1976). A number of anatomical and biochemical functions must work in concert to sustain the high fluxes of the C₄ cycle, and comparisons of average C₄ and C₃ plants suggest that the evolution of the C₄ phenotype required a large number and scale of changes (Hattersley 1984). Despite this apparent complexity, the C₄ trait evolved many times independently (Sage et al. 2011). Resolving this paradox requires the quantitative distinction of changes that were involved in the evolutionary transition from C₃ to C₄, from those that preceded or followed it.

In most C₄ plants, carbon fixation within leaf mesophyll tissue (M) is used to concentrate CO₂ and boost Rubisco activity within bundle sheath tissue (BS), whereas Rubisco in C₃ plants operates within the M where it depends on atmospheric CO₂ diffusion (Fig. 1; Brown 1975; Hattersley et al. 1977; Hatch 1987). Efficient C₄ leaves require large BS volumes to accommodate the necessary photosynthetic organelles, including chloroplasts containing abundant Rubisco, and a small distance between M and BS compartments to allow the rapid transfer of metabolites (Fig. 1; Hattersley & Watson 1975; Lundgren et al. 2014). These traits vary among C₃ plant lineages and, in grasses, C₄ photosynthesis evolved only within those groups with large fractions of BS (Christin et al. 2013; Lundgren et al. 2014). Comparisons of multiple C₄ lineages with their C₃ relatives indicate that the evolution of C₄ leaf anatomy involved ultrastructural rearrangements and further decreases to the relative volume of M compared to BS tissue (Hattersley 1984; Dengler et al. 1994; McKown &
Dengler 2007; Christin et al. 2013). These properties can be achieved via a variety of leaf structural modifications, allowing C₄ anatomy to be realized differently each time it evolved, in some cases involving the use of different tissue types for the C₄ BS function (Brown 1975; Soros and Dengler 2001; Christin et al. 2013; Freitag and Kadereit 2014; Lundgren et al. 2014). While the differences between a diverse range of C₃ and C₄ species are well known, the minimum set of leaf anatomical modifications required to carry out C₄ photosynthesis remains to be established.

The grass Alloteropsis semialata provides an outstanding system to capture the early events during C₄ evolution because it includes genetically divergent C₄ individuals, as well as a diversity of non-C₄ plants encompassing C₃ and C₃-C₄ intermediate phenotypes (Ellis 1974; Lundgren et al. 2016), which emerged in the paleotropics (Lundgren et al. 2015). The inner sheath (i.e., the mestome sheath), which is present in all C₃ grasses, has been co-opted for the C₄ BS function in A. semialata. Previous studies have compared leaf properties among C₄ and non-C₄ leaves of a few A. semialata accessions (Ellis 1974; Frean et al. 1983; Ueno & Sentoku 2006; Lundgren et al. 2016; Dunning et al. 2017), but a broader sampling is required to establish which properties are unique to each photosynthetic type.

The primary focus of this study is to compare leaf anatomy in accessions spanning the diversity of each photosynthetic type to distinguish the structural diversifications that occurred before, during, and after C₄ emergence in this species. We hypothesize that the properties that predate C₄ evolution will be shared by at least some of the non-C₄ individuals, while those that happened after C₄ evolution in a phase of subsequent adaptation will be restricted to a subset of the C₄ populations. Properties unique to, and common among all, C₄ accessions represent those that were involved in the initial transition to a C₄ physiology. We conducted a large scan of the diversity within the species using traits linked to the number and size of different cell types, and used controlled growth experiments to verify that anatomical differences are not environmentally induced. This evaluation of the gross leaf morphology was accompanied by a focused study in some individuals to identify ultrastructural changes that may also differ between C₄ and non-C₄ accessions. Overall, our work
shows that a complex trait of large ecological significance can evolve via a few key developmental changes.

**MATERIALS AND METHODS**

**Characterizing photosynthetic types**

Photosynthetic type was determined by a combination of stable isotope and CO$_2$ compensation point (CCP) data (Table S1; Dataset S1), as previously described (Lundgren et al. 2016). The carbon isotope composition of plant tissues ($\delta^{13}$C) distinguishes photosynthetic types (von Caemmerer et al. 2014), such that plants with $\delta^{13}$C values higher than -17‰ were considered to have a fully functioning C$_4$ system, while those with values lower than this threshold were considered either C$_3$ or C$_3$-C$_4$. CCPs were used to distinguish C$_3$-C$_4$ from C$_3$ plants, and to support the $\delta^{13}$C results. The CCP indicates the CO$_2$ concentration within the leaf at which CO$_2$ assimilation via photosynthesis equals CO$_2$ loss via photorespiration and respiration. Because less CO$_2$ is ultimately lost to photorespiration in C$_3$-C$_4$ plants, they have very low CCPs compared to C$_3$ plants. Thus, non-C$_4$ plants with CCPs greater than or equal to 35 μmol mol$^{-1}$ were classified as C$_3$, while those less than 35 μmol mol$^{-1}$ were classified as C$_3$-C$_4$. CCPs were calculated on 27 living accessions (6 C$_3$, 4 C$_3$-C$_4$, and 17 C$_4$), following published protocols (Bellasio et al. 2016a,b; Lundgren et al. 2016). Non-C$_4$ accessions for which live material was unavailable were assumed to have the same photosynthetic type as their closest relatives, as identified by phylogenetic relationships (Table S1).

**Leaf samples**

Fifty *Alloteropsis semialata* (R.Br.) Hitchc. accessions distributed across the species’ geographic range, including 17 C$_3$, 6 C$_3$-C$_4$, and 27 C$_4$, were used to assess intraspecific anatomical variation. Leaf samples from 44 of the 50 accessions were collected from their original field site and preserved until embedding was possible. For the remaining six accessions, leaf samples were taken from plants grown under controlled environment conditions as in Lundgren et al. (2016). For all samples, leaf
pieces 3-5 mm in length were embedded in methacrylate embedding resin (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim, Germany), sectioned 6-8 μm thick on a manual rotary microtome (Leica Biosystems, Newcastle, UK), and stained with Toluidine Blue O (Sigma-Aldrich, St. Louis, MO, USA). Stained leaf sections were imaged using microscopy-imaging software with a camera mounted on a microscope (Cell A, Olympus DP71, and Olympus BX51, respectively; Olympus, Hamburg, Germany) and the images were stitched together using DoubleTake (v2.2.9, Echo One, Frederikssund, Denmark).

**Leaf anatomy measurements**

Anatomical traits were measured using ImageJ (Fig. S1; Schneider *et al.* 2012) from the cross-section of a single leaf segment from the centre of the leaf blade, avoiding segments immediately adjacent to the midrib and lateral edges of the cross-section. Vein orders were distinguished following Renvoize (1987). A single segment was defined as the leaf area falling between two secondary veins, which are large veins with metaxylem. Tertiary and minor veins (*e.g.*, quaternary and quinary orders) lack metaxylem. In this species, the extraxylary fibres that flank both the adaxial and abaxial edges of tertiary veins distinguish them from higher order minor veins, which can be flanked by fibres on one side only (Fig. S1).

The cross-sectional area of the whole segment, combining M, BS, epidermis and bulliform cells, extraxylary fibres, and BS extensions, as well as any transverse veins or tear spaces was measured. For all accessions, the total BS (*i.e.*, the mestome sheath; the compartment used for the Calvin cycle in *C₄* *A. semialata*), outer sheath, and vein areas were measured separately for secondary, tertiary, and any minor veins. The area of M tissue was calculated as the total area remaining after accounting for all other tissue types. In addition, the cross-sectional area of individual M and BS cells (hereafter 'size') was measured (Fig. S1). Although the depth of individual cells can vary, it is their cross-sectional areas, and not their three-dimensional volumes, that primarily influence the proportion of each tissue in the leaf.
Linear discriminant analysis

We used a Linear Discriminant Analysis (LDA) to explain the variation between photosynthetic types (i.e., the test maximizes between-group variance while minimizing within-group variance). We performed the LDA on the 50 accessions with leave-one-out cross-validation and then bootstrapping over 100 runs, using the MASS package in R (Venables & Ripley 2002). Prior probabilities were based on the relative sample size of the categorical variable (i.e., 0.34, 0.12, and 0.54 for C3, C3-C4, and C4 groups, respectively). We chose predictor variables that were likely to influence the M:BS ratio, including the number of M cells between major veins, average size of individual M cells, number of minor veins per segment, leaf thickness, and the average size of BS cells on tertiary veins.

To test the generality of our findings from *A. semialata*, we carried out an equivalent LDA for a larger sample of 157 grasses including one C3 and one C4 *A. semialata* and representing 17 independent C4 lineages. Predictor variables in this analysis were based on the anatomical measurements of Christin *et al.* (2013) and chosen to best match the variables used in the *A. semialata* LDA described above, including the number of mesophyll cells between veins, mesophyll cell width, proportion of veins that are minor, leaf thickness, inner BS cell width, and outer BS cell width. The species were grouped as C3, C4 species using the inner BS, and C4 species using the outer BS.

Vein order analysis

To determine whether the pattern of vein density observed in the main dataset was maintained across a larger sample, we counted the total number of veins per segment and the presence or absence of minor veins in 91 additional accessions consisting of herbarium specimens that had been rehydrated in distilled water overnight at 4°C prior to embedding, sectioning, staining, and imaging as described above. Together with the 50 previous samples, this larger dataset included a total of 72 C4 (i.e., δ13C > -17‰) and 69 non-C4 (i.e., δ13C < -17‰) accessions distributed across the species’ geographic range.
Ultrastructure and immunohistochemistry

To investigate whether ultrastructural changes might also differ between photosynthetic types within this species, we analysed the spatial distributions of organelles and enzymes in one population representing each of the C₃, C₃-C₄, and C₄ types. Recently expanded mature leaf tissue was prepared for transmission electron microscopy and processed for immunodetection of the large subunit of Rubisco (RBCL) and glycine decarboxylase H subunit (GLDH) as previously described (see Supporting Information Materials 1; Khoshravesh et al. 2017).

Leaf anatomy in a common environment

To determine the degree to which the various leaf anatomical phenotypes arose from plastic development responses to their differing native growth environments, we compared field phenotypes to those obtained from live tillers of 17 A. semialata accessions (5 C₃, 4 C₃-C₄, and 8 C₄) after growing for a minimum of three months in a common growth chamber, with conditions as described in Lundgren et al. 2016. The environmental conditions (Fick & Hijmans 2017) at the field collection sites are detailed in Table S2. On both field and controlled environment samples, the number and order of veins, minimum number of M cells separating veins, area of inner BS cells, segment length and thickness, and IVD were determined on one segment per leaf.

Plasticity for leaf anatomy in response to low CO₂

To further test whether C₄-compatible phenotypes could emerge from plastic responses to the environment, as previously suggested (Li et al., 2014), we carried out a CO₂ manipulation experiment designed to promote photorespiration. One C₄ (MDG, South Africa) and one C₃ (GMT, South Africa) plant were initially grown from seed in a controlled environment chamber set as described in Lundgren et al. 2016, but with 400 μmol mol⁻¹ CO₂ concentration. Both plants were split into five replicate cuttings and kept in the same growth chamber conditions to re-establish for four months. From each replicated clone, one fully expanded, mature leaf was sampled and fixed in 4:1...
ethanol:acetic acid solution. The growth chamber was then set to 180 μmol mol$^{-1}$ CO$_2$ concentration for the next four months to promote photorespiration, while maintaining the other environmental conditions, and one new fully expanded leaf was again sampled and fixed. All leaf samples from the 400 (i.e., ambient) and 180 (i.e., low) CO$_2$ treatments were embedded, sectioned, and imaged as described above. To determine whether the plants used different photosynthetic pathways in the two CO$_2$ growth environments, we determined CCP and carboxylation efficiency, as described in Lundgren et al. (2016).

RESULTS

*Alloteropsis semialata* presents a continuum of leaf anatomy

The ratio of M to BS tissue, a trait known to differ among C$_3$ and C$_4$ species (Hattersley 1984), forms a continuum within *Alloteropsis semialata*, along which photosynthetic types are sorted (Fig. 2). Indeed, the smallest values are restricted to C$_4$ accessions and the largest are found in C$_3$ individuals. When considering the area in cross-section between two secondary veins (i.e., a leaf segment from here onward; Fig. S1), the M area is over ten times larger than the BS area in C$_3$ accessions, but less than five times larger in C$_4$ accessions (Dataset S1). As expected, C$_3$-C$_4$ accessions are intermediate in their overall leaf anatomy, with five to ten times more M than BS. These M:BS ranges are consistent with those measured in other C$_3$ and C$_4$ grasses (Christin et al. 2013).

C$_3$, C$_3$-C$_4$, and C$_4$ *Alloteropsis semialata* have distinct leaf anatomy

Variation in M:BS ratios can arise via changes to several underlying traits (Lundgren et al. 2014). Our modelling shows that M area is the product of leaf thickness and interveinal distance (IVD; Table 1). The latter is predicted by the number and size of M cells between veins (Table 1). BS area is explained by the number of BS units (i.e., the number of veins per segment) and the size of BS cells (Table 1). When these potential explanatory traits are incorporated within an LDA, all variance
between the three photosynthetic types is captured (Fig. 3a). In a bootstrapped sample, the mean overall predictive accuracy is 0.986, which is statistically indistinguishable from 1.0 (95% CI = 0.966 – 1.000). The mean predictive accuracy for C$_4$ (0.999, 95% CI = 0.995 – 1.000), C$_3$ (0.976, 95% CI = 0.918 – 1.000), and C$_3$-C$_4$ (0.926, 95% CI = 0.805 – 1.000) accessions are also statistically indistinguishable from 1.0. The analysis therefore confirms that leaf anatomy varies among photosynthetic types in a statistically predictable manner.

The first axis of the LDA explains 97.37% of the variance between photosynthetic types and clearly distinguishes C$_4$ from non-C$_4$ accessions (Fig. 3a). This axis is most strongly associated with the number of minor veins per segment, which were absent from all non-C$_4$ accessions in this analysis (Table 2). The second axis explains 2.63% of the variance between groups, clearly distinguishes C$_3$ from C$_3$-C$_4$ plants, and is most strongly associated with the number of M cells between major veins (i.e., secondary and tertiary order veins) and the number of minor veins (Fig. 3a; Table 2). Since minor veins are restricted to C$_4$ individuals, their contribution to LD2 is linked to diversity within the C$_4$ group. These results indicate that most of the variance in the dataset stems from the contrast between C$_4$ and non-C$_4$ individuals, and is driven entirely by a single underlying trait, the presence of minor veins. The phenotypic distance between C$_3$ and C$_3$-C$_4$ individuals is very small, being explained by the number of M cells between major veins. Conversely, leaf thickness and the cross-sectional areas of individual BS and M cells poorly distinguish photosynthetic types in this species.

The first two axes of an LDA of anatomical traits on the larger species dataset explain 89.62% and 10.38% of the variation, respectively. The first axis clearly distinguishes C$_4$ species that use the inner bundle sheath from C$_4$ species using the outer sheath and the C$_3$ species (Fig. 3b), and is mostly associated with the proportion of minor veins, while the remaining anatomical traits are weakly correlated with both axes (Table 2).

**Differences between C$_4$ and non-C$_4$ phenotypes arise from the development of minor veins**

In *A. semialata*, the presence of minor veins is the only variable consistently distinguishing C$_4$ and
non-C₄ accessions. When the M:BS ratio is calculated in the absence of minor veins, the clear
distinction between C₃-C₄ and C₄ accessions disappears, with nearly half the C₄ accessions
overlapping with C₃-C₄ plants (Fig. 2). This shows that the development of minor veins in C₄
accessions reduces the M:BS ratio by increasing BS area and displacing M area. To confirm the
restriction of minor veins to C₄ individuals, we screened vein architecture in a larger dataset (Fig.
4a,b; Dataset S2). Minor veins were present in all C₄ accessions and absent in all but five non-C₄
accessions. Four of these had only occasional and irregularly spaced minor veins, while the final
accession is an individual originating from a natural cross between C₃-C₄ and C₄ individuals
(Olofsson et al. 2016). Our data therefore show that the presence of frequent and regularly spaced
minor veins is universally and uniquely associated with the C₄ genomic background, captures nearly
all of the anatomical variation between C₄ and non-C₄ phenotypes, and explains overall differences
in relative M and BS areas.

The proliferation of minor veins explains a number of patterns associated with C₄ anatomy.
As expected, the number of M cells between consecutive veins differs among photosynthetic types,
being the smallest in C₄ accessions (1-3), compared to C₃-C₄ (3-6) and C₃ (5-11) plants (Fig. S2).
However, the number of M cells between major veins overlaps between the C₄ and non-C₄ groups
(Fig. 4c), which indicates that the reduced distance between any pair of M and BS cells in C₄
accessions is caused by the differentiation of ground meristem cells into minor veins rather than a
reduced proliferation of M cells. The high vein density of C₄ plants following the development of
minor veins is accompanied by more than a twofold increase in extraxylary fibres (i.e., tissue area per
segment length) than is found in non-C₄ accessions (Fig. S3). Because the area of extraxylary fibres
per vein does not differ between the photosynthetic types (Fig. S3), the increased fibre area in C₄
plants derives entirely from their greater vein density.

Other anatomical changes happened before or after the transition from C₃-C₄ to C₄ physiology
The development of minor veins explains the overall anatomical difference between C₄ and non-C₄
accessions, and is therefore linked to the emergence of a fully functioning C₄ physiology from a C₃-C₄ intermediate state. Evolutionary changes that happened once this C₄ physiology was in place would be restricted to some, but not all, C₄ individuals. In our dataset, such changes include further reductions to the M:BS ratio, potentially achieved via contractions to M airspace (Byott 1976) or increases in BS cell size. Indeed, although BS cell sizes of different photosynthetic types overlap, large increases to BS cell size characterize some African C₄ accessions (Figs S4-S5). The BS cell enlargement was therefore involved in the adaptation of C₄ physiology after it had emerged, possibly to accommodate more or larger organelles for a more efficient C₄ cycle, rather than being involved in its origin. Occasional hybridization between C₄ and non-C₄ individuals could affect the distribution of trait values, however non-C₄ A. semialata individuals are restricted to Africa, so that hybridization outside of Africa is unlikely. Yet, Asia and Australian accessions exhibit some of the smallest BS cells among C₄ accessions (Fig. S5).

Some characters observed in C₄ accessions are also present in C₃-C₄ individuals, but not C₃ ones, indicating that they are not associated with the transition to fully functional C₄ physiology, but might have facilitated it. These include a small increase in BS cell sizes in C₃-C₄ compared with C₃ plants and a decrease in outer sheath cell size, with C₃-C₄ accessions bridging the anatomical gap between C₃ and C₄ outer sheath cell sizes (Fig. 4c-d). This reduced outer sheath in C₃-C₄ and C₄ A. semialata likely facilitates metabolite exchanges between M and BS cells.

**Differences between C₄ and non-C₄ leaves are not environmentally induced**

Alloteropsis semialata plants grow naturally in diverse environments, depending on their photosynthetic background and evolutionary history (Lundgren et al. 2015). To verify that the differences we observe among photosynthetic types are not induced by environmental variations, we compared the leaves of field-collected plants after transplanting and growing them in a common controlled environment growth chamber for at least three months (Dataset S3; Fig. S6). Compared to field conditions, C₃ accessions produced more M cells between veins (p = 0.044) in the common
environment. Moreover, C3-C4 plants produced thicker leaves \((p = 0.040)\), such that leaf thickness of the three photosynthetic types converged in the common environment, which is likely a result of the non-limiting light, nutrients, and water available in these conditions. However, the other traits were not influenced by growth conditions and leaf anatomy of the three photosynthetic types remained distinct when grown in the common environment.

We further verified that historical changes in atmosphere composition did not influence the leaf phenotype by comparing C3 and C4 A. semialata under current ambient (400 ppm) and the Pleistocene minimum (180 ppm) CO2 concentrations. Plants grown under the low CO2 concentration experience elevated photorespiration rates, which might have induced a more C4-like anatomy. However, we found that plants did not shift photosynthetic state under the differing CO2 conditions \(i.e.,\) mean CCPs in ambient/low CO2 for C3 = 49.8/53.1 and C4 = 4.6/8.1 \(\mu\)mol mol\(^{-1}\); Dataset S4). Both C3 and C4 plants produced thinner leaves in the low CO2 environment \((p = 0.0049\) C3 / 0.0065 C4), and C4 plants developed smaller BS cells \((p = 0.011;\) Fig. S6), probably because the lower carbon supply restricted development (Ripley \textit{et al.} 2013). Importantly, the C3 plants did not produce more veins (or any minor veins), larger BS cells, or fewer M cells between veins when grown under this high photorespiration condition. These results show that, even when photorespiration is high, a C4-like phenotype is not plastically induced in C3 A. semialata.

**DISCUSSION**

Photosynthetic types form a continuum, along which multiple biochemical, anatomical, and ultrastructural alterations increase the proportion of CO2 fixed via the C4 cycle. The emerging model of C4 evolution involves gradual and overlapping phenotypic changes (Heckmann \textit{et al.}, 2013; Sage \textit{et al.}, 2014; Bräutigam & Gowik, 2016; Schlüter & Weber, 2016; Dunning \textit{et al.}, 2017), with traits acquired in differing orders among C4 lineages (Williams \textit{et al.}, 2013). Different traits may be involved in the initial transition to a C4 phenotype, and the subsequent adaptation and diversification
of that phenotype (Christin & Osborne, 2014; Watcharamongkol et al., 2018). Within the grass
Alloteropsis semialata, we have shown that the only gross leaf property distinguishing all C₄ from all
non-C₄ phenotypes is the development of frequent minor veins. The presence of these minor veins
has multiple consequences, including an overall increase of vein density, enlargement of the total
volumes of BS tissue, and a displacement of M tissue. These anatomical changes combine to facilitate
C₄ cycle activity, as demonstrated by a strong correlation between leaf vein frequency and carbon
isotope composition observed for this species (Lundgren et al., 2016). Our analyses of leaf ultrastructure indicate that the evolution of C₄ photosynthesis in A. semialata may have involved
additional changes in organelle distribution among cell types (Supporting Information Materials 1;
Figs S7-9), although the small sample of populations prevents us from differentiating ultrastructural
changes linked to the transition to C₄ from those that happened later.

The change in venation inferred during the evolution of C₄ photosynthesis in A. semialata
may have a number of physiological and ecological consequences. First, the increase in vein
frequency is accompanied by an enhancement of unpigmented extraxylary fibres, which improves
light transmission to the BS, and thus ATP production in these cells, facilitating photosynthetic
carbon reduction (Bellasio & Lundgren 2016). Enhanced fibre density may also increase leaf
toughness, reduce digestibility, and consequently deter herbivores (Caswell et al. 1973; Wilson et al.
1983). Secondly, the insertion of additional veins may influence leaf hydraulics. Model simulations
for other plant species demonstrate that an increase in minor vein density can lead to greater leaf
hydraulic conductance (McKown et al., 2010). However, empirical studies show that this is unlikely
to improve drought tolerance, since the decline in hydraulic conductance during drought arises
primarily outside veins (Scoffoni & Sack, 2017; Scoffoni et al., 2017a), while embolisms arise first
in the midrib, not minor veins (Scoffoni et al., 2017b).

Our results complement those from previous comparisons among species, which show that an
additional order of minor veins develops during the evolutionary transition from non-C₄ to C₄ forms
of Flaveria (McKown & Dengler, 2009), while BS cell size is large in both C₃ and C₄ Flaveria species
(Kümpers et al., 2017). Our further analysis of leaf gross anatomy across multiple grass species shows that the insertion of additional minor veins is a frequent developmental mechanism for decreasing the M:BS ratio in those C₄ grasses that primarily localise Rubisco within the mestome sheath. The insertion of minor veins could occur via relatively few developmental changes, likely underpinned by changes to auxin, brassinosteroids, SHORTROOT/SCARECROW, and/or INDETERMINATE DOMAIN transcription factors (Kumar & Kellogg 2018; Sedelnikova et al. 2018). In grasses, vein orders develop sequentially as leaves grow wider, such that minor veins are initiated considerably later than other vein orders, usually once the leaf ceases to widen (Nelson & Langdale 1989; Sedelnikova et al. 2018). Thus, the development of functional minor veins likely arises via the heterochronic regulation of the existing machinery for vein formation, sustaining vein differentiation beyond that of non-C₄ plants (Nelson 2011; Sedelnikova et al. 2018), probably through the prolonged production of auxin during later phases of leaf elongation (Scarpella et al. 2010). Alternatively, minor veins may also result from a heterotopic specialization of auxin maxima that permits them to form closer together (Kumar & Kellogg 2018).

The possibility that a transition from non-C₄ to C₄ states can be caused by a single developmental alteration is a plausible explanation for the recurrent origins of C₄ leaf anatomy, and helps to resolve the paradox of how this complex trait emerged so many times. We also show that organelle number and size differs among photosynthetic types of A. semialata, but, here too, recent work indicates that one gene can control multiple ultrastructural modifications (Wang et al. 2017). Finally, transcriptome comparisons show that few genes encoding enzymes are upregulated during the transition from non-C₄ to C₄ in A. semialata (Dunning et al. 2017). We therefore conclude that the overall transition from a non-C₄ state to the form of C₄ photosynthesis observed in A. semialata involved relatively few genetic mutations.

The limited number of changes involved in the emergence of C₄ anatomy in A. semialata is partially explained by the presence of relatively enlarged BS in the C₃-C₄ accessions, since C₃ A. semialata BS size is similar to C₃ species from other grass lineages (Lundgren et al., 2014; 2016;
Dunning et al. (2017)). The C₃ A. semialata phenotype might represent an evolutionary reversal from a C₃-C₄ state (Dunning et al. 2017), such that its leaf anatomy derives from an ancestral C₃-C₄ intermediate form. C₃-C₄ A. semialata are characterised by fewer M cell compared to C₃ accessions, and higher BS organelle abundance (Figs 3a, 4c and S7-9). These properties that had been selected for the C₃-C₄ physiology eased the subsequent transition to a full C₄ state, but it is important to note that the physiology and anatomy of C₃-C₄ A. semialata are typical for C₃-C₄ plants in general (Lundgren et al., 2016), and their anatomical characteristics can be found among C₃ grasses (Hattersley 1984; Christin et al. 2013; Lundgren et al. 2014). The background against which C₄ anatomy evolved in A. semialata is therefore not exceptional.

Our conclusion that C₄ leaf anatomy can arise from one key developmental modification is apparently incompatible with the great anatomical specialization of other C₄ lineages, as well as the large phenotypic gaps separating them from their closest C₃ relatives (Dengler et al. 1994; Christin et al. 2013). However, most C₃ and C₄ sister lineages are separated by long periods of evolution, and comparing these groups therefore captures all of the changes that happened after the origin of C₄ photosynthesis to improve the efficiency of C₄ physiology and adapt it to various organismal and ecological contexts (Christin & Osborne 2014). Indeed, photosynthetic efficiency may be significantly lower in C₄ A. semialata than in species from some older C₄ lineages (Lundgren et al., 2016; Bräutigam et al., 2018). This suggests that C₄ photosynthesis in A. semialata may represent a rudimentary version of the physiological trait (Ueno & Sentoku 2006). The biochemical characteristics of the C₄ cycle in A. semialata may be one reason for this (Bräutigam et al., 2018), and the presence of Rubisco protein in M could be another (Ueno & Sentoku, 2006). Anatomical diversity may also explain some of the variation in physiological efficiency among A. semialata populations (Lundgren et al. 2016). Indeed, in A. semialata, enlargements of the BS cells beyond those seen in non-C₄ individuals are restricted to a subset of C₄ populations (Fig. 2) and thus happened after the emergence of C₄ physiology. Over time, accumulated modifications will move C₄ leaf anatomy far beyond that realized via a single developmental change. However, the fact that an initial
C₄ phenotype and the associated physiology can be accessed via a single modification likely placed multiple groups on a selective highway to highly specialized and successful variants of the C₄ syndrome.

ACKNOWLEDGEMENTS

This work was funded by a University of Sheffield Prize Scholarship to MRL, an ERC grant (grant number ERC-2014-STG-638333) and a Royal Society Research Grant (grant number RG130448). LTD and JKO are supported by a NERC grant (grant number NE/M00208X/1) and PAC is supported by a Royal Society University Research Fellowship (grant number URF120119). JWB was supported by 301 and Think Ahead Sheffield Undergraduate Research Experience grants to MRL. The work on ultrastructure was supported by a Natural Sciences and Engineering Research Council of Canada grant (no 2015-04878) to TLS. The authors thank Peter Westhoff, Stefanie Schulze, and Udo Gowik for use of their GLDH antibody, Susanne von Caemmerer for advice about outer bundle sheath cell resistance, Paul Hattersley for leaf samples and ¹³C isotope data, John Thompson for field assistance and sample collection, Emma Jardine for discussion of linear discriminant analysis, Heather Walker for mass spectrometry assistance, Gareth Fraser for the use of his vibratome, and Emanuela Samaritani for histology assistance. Herbarium leaf samples were obtained from Kew Herbarium at the Royal Botanic Garden, the National Herbarium of South Africa in Pretoria, and National Museums of Kenya in Nairobi, and the National Botanic Garden of Belgium, Brussels, with the assistance of Martin Xanthos, Lyn Fish, Caroline Mashau, and Itambo Malombe.
REFERENCES


Ripley, B.S., Cunniff, J. & Osborne, C.P. (2013). Photosynthetic acclimation and resource use by the C\textsubscript{3} and C\textsubscript{4} subspecies of \textit{Alloteropsis semialata} in low CO\textsubscript{2} atmospheres. \textit{Glob. Change Biol.}, 19, 900-910.


Table 1. Results of linear regression analyses on leaf components underlying M:BS in *Allotropis semialata*.

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>DF</th>
<th>Adj R^2</th>
<th>p-value</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total M area / segment (μm²)</td>
<td>59.35</td>
<td>2, 47</td>
<td>0.704</td>
<td>1.38 x 10^{-13}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.98</td>
<td>0.00024</td>
</tr>
<tr>
<td>Interveinal distance (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.58</td>
<td>5.07 x 10^{-14}</td>
</tr>
<tr>
<td>Interveinal distance (μm)</td>
<td>343.4</td>
<td>2, 47</td>
<td>0.933</td>
<td>&lt; 2.2 x 10^{-16}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number M cells between veins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.48</td>
<td>&lt;2 x 10^{-16}</td>
</tr>
<tr>
<td>M cell size (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.97</td>
<td>9.22 x 10^{-9}</td>
</tr>
<tr>
<td>Total BS area / segment (μm²)</td>
<td>124.8</td>
<td>2, 47</td>
<td>0.835</td>
<td>&lt; 2.2 x 10^{-16}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS cell size (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.52</td>
<td>1.54 x 10^{-12}</td>
</tr>
<tr>
<td>Vein density (veins/segment)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.23</td>
<td>0.00011</td>
</tr>
</tbody>
</table>

aAverage distance between all veins; bNumber of mesophyll (M) cells between all veins; cCross-sectional area of inner bundle sheath (BS) cells on tertiary order veins.
Table 2. Coefficients of linear discriminants in a linear discriminant analysis on (top) five leaf anatomical traits expected to drive overall mesophyll to bundle sheath area ratios in *Alloteropsis semialata* and on (bottom) six leaf anatomical traits in 157 grass species, grouped as C₃ species, C₄ species using the inner sheath, and C₄ species using the outer sheath.

**LDA on *Alloteropsis semialata* accessions**

<table>
<thead>
<tr>
<th>Trait</th>
<th>LD1</th>
<th>LD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of minor veins per segment</td>
<td>1.3591</td>
<td>0.3663</td>
</tr>
<tr>
<td>Number of mesophyll cells between major veins</td>
<td>-0.315</td>
<td>0.4881</td>
</tr>
<tr>
<td>Average area inner bundle sheath cell on tertiary veins (μm²)</td>
<td>0.0123</td>
<td>-0.0104</td>
</tr>
<tr>
<td>Average area mesophyll cell (μm²)</td>
<td>-0.0012</td>
<td>-7.82E-05</td>
</tr>
<tr>
<td>Leaf thickness (μm)</td>
<td>-5.52E-04</td>
<td>0.0189</td>
</tr>
</tbody>
</table>

**LDA on 157 grass species + 1 C₃ and 1 C₄ *Alloteropsis semialata* accession**

<table>
<thead>
<tr>
<th>Trait</th>
<th>LD1</th>
<th>LD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>proportion of veins that are minor</td>
<td>4.145779</td>
<td>-0.34605</td>
</tr>
<tr>
<td>outer BS cell width (μm)</td>
<td>-0.06433</td>
<td>0.080823</td>
</tr>
<tr>
<td>inner BS cell width (μm)</td>
<td>0.301257</td>
<td>0.004749</td>
</tr>
<tr>
<td>Leaf thickness (μm)</td>
<td>-0.0083</td>
<td>-0.00729</td>
</tr>
<tr>
<td>Mesophyll cell width (μm)</td>
<td>0.01989</td>
<td>-0.01048</td>
</tr>
<tr>
<td>Number of mesophyll cells between veins</td>
<td>-0.08007</td>
<td>-0.22237</td>
</tr>
</tbody>
</table>
**FIGURE LEGENDS**

**Figure 1. Schematic of leaf anatomy and photosynthetic pathway in C₃, C₃-C₄, and C₄ grasses.** In C₃ plants, CO₂ assimilation via the Calvin-Benson cycle (solid black circle) and CO₂ release via photorespiration (dashed black circle) both occur in mesophyll cells (light green). C₃ leaves consequently have larger areas of mesophyll tissue than bundle sheath tissue, where no photosynthetic activity occurs. C₃-C₄ plants use an intermediate physiology called C₂ photosynthesis, where the Calvin cycle occurs in mesophyll cells, like in C₃ plants. However, because glycine decarboxylase (GDC) is specifically localized to bundle sheath cells, the photorespiratory cycle is split across these two cell types, creating a weak CO₂-concentrating mechanism, where CO₂ is released in the bundle sheath and can be reassimilated via the Calvin cycle. C₂ photosynthesis therefore requires large areas of mesophyll for photosynthesis via an initial Calvin cycle, but also close contact between mesophyll and bundle sheath cells for the photorespiratory CO₂ pump. C₄ plants have a strong CO₂ concentrating mechanism whereby CO₂ is biochemically shuttled from the mesophyll into the bundle sheath. The high CO₂ concentration in the bundle sheath largely avoids oxygenation and thus, photorespiration. Photosynthesis via the C₄ cycle therefore requires large areas of bundle sheath tissue, but less mesophyll, which can be achieved via the insertion of minor veins. Dark blue, bundle sheath lacking chloroplasts; dark green, bundle sheaths with chloroplasts; light green, mesophyll cells; yellow, extraxylary fibers/bundle sheath extensions; grey, epidermal cells; light blue, veins; white, metaxylem.

**Figure 2. Continuous variation in Alloteropsis semialata leaf anatomy, but distinct division among C₃, C₃-C₄, and C₄ types.** Ratios of mesophyll (M) to bundle sheath (BS) area of individual accessions of C₃ (blue circles), C₃-C₄ (green circles), and C₄ (solid red circles) plants, ranked by M:BS value. n = 50. Lines delineating M:BS ratios that distinguish C₃ from C₃-C₄ (green) and C₃-C₄ from C₄ (red) are shown. For C₄ individuals, M:BS ratios are also calculated in the absence of minor veins (open red circles).

**Figure 3. Linear discriminant analysis of leaf anatomical traits.** The first (LD1) and second (LD2) dimensions of the LDA are plotted against each other with histograms of each dimension shown on the opposing axis for (A) the LDA on C₃, C₃-C₄, and C₄ Alloteropsis semialata accessions and (B) the LDA on 157 additional C₃, C₄ inner sheath, and C₄ outer sheath grass species. One C₃ and one C₄ A. semialata accession...
were included this larger LDA and denoted by solid blue and red circles, respectively. Loading plots are overlaid via black arrows. M, mesophyll, IS, inner sheath; OS, outer sheath, nb.M, number of mesophyll cells between veins.

**Figure 4. Diversity of intraspecific anatomical components.** Histograms of (A) vein density (i.e., the total number of veins per segment) and (B) the number of major veins per segment in C₄ (red; n=72) and non-C₄ (grey, n= 69) accessions. Scatter plots show (C) the average number of mesophyll (M) cells between major veins versus the average area of individual bundle sheath (BS) cells, with dot size scaled to the M:BS ratio, and (D) BS cell area versus outer sheath cell area, with dot size scaled to the number of veins per segment. Colors indicate photosynthetic type with C₃ (blue; n=17), C₃-C₄ (green; n=6), and C₄ (red; n=27).
FIGURES

C₃ anatomy

C₃-C₄ anatomy

C₄ anatomy

CO₂ → Rubisco → CO₂

O₂ → Rubisco → CO₂

CO₂ → GDC → CO₂

mesophyll

bundle sheath

C₃ photosynthesis

C₄ photosynthesis

FIGURE 1
FIGURE 2
FIGURE 3
**FIGURE 4**
SUPPORTING INFORMATION

Supporting Information Materials 1. Ultrastructural characterization of C₃, C₃-C₄ and C₄ accessions

Dataset S1. Comprehensive anatomical measurements for all accessions

Dataset S2. Larger vein density dataset

Dataset S3. Field v controlled environment plasticity dataset

Dataset S4. Growth CO₂ concentration plasticity dataset

Figure S1. Example leaf anatomy measurement methods

Figure S2. Relationships between anatomical traits

Figure S3. Comparison of extraxylary fibre area in C₃, C₃-C₄ and C₄ accessions

Figure S4. Relationships between inner BS cell size and overall BS areas

Figure S5. Comparison of bundle sheath size in African and non-African C₄ accessions.

Figure S6. Plasticity for leaf anatomical components by photosynthetic type

Figure S7. Organelle abundance differs between photosynthetic types.

Figure S8. Immunodetection of GLDH

Figure S9. Immunodetection of Rubisco large subunit

Table S1. Details used to determine photosynthetic pathway for accessions

Table S2. Details of the accessions used in the plasticity dataset