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REVIEW PAPER

Deconstructing Kranz anatomy to understand C_4 evolution

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Abstract

C_4 photosynthesis is a complex physiological adaptation that confers greater productivity than the ancestral C_3 photosynthetic type in environments where photorespiration is high. It evolved in multiple lineages through the coordination of anatomical and biochemical components, which concentrate CO_2 at the active site of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In most C_4 plants, the CO_2-concentrating mechanism is achieved via the confinement of Rubisco to bundle-sheath cells, into which CO_2 is biochemically pumped from surrounding mesophyll cells. The C_4 biochemical pathway relies on a specific suite of leaf functional properties, often referred to as Kranz anatomy. These include the existence of discrete compartments differentially connected to the atmosphere, a close contact between these compartments, and a relatively large compartment to host the Calvin cycle. In this review, we use a quantitative dataset for grasses (Poaceae) and examples from other groups to isolate the changes in anatomical characteristics that generate these functional properties, including changes in the size, number, and distribution of different cell types. These underlying anatomical characteristics vary among C_4 origins, as similar functions emerged via different modifications of anatomical characteristics. In addition, the quantitative characteristics of leaves all vary continuously across C_3 and C_4 taxa, resulting in C_4-like values in some C_3 taxa. These observations suggest that the evolution of C_4-suitable anatomy might require relatively few changes in plant lineages with anatomical predispositions. Furthermore, the distribution of anatomical traits across C_3 and C_4 taxa has important implications for the functional diversity observed among C_4 lineages and for the approaches used to identify genetic determinants of C_4 anatomy.

Key words: C_4 photosynthesis, complex trait, convergent evolution, co-option, Kranz anatomy, leaf.

Introduction

During the diversification of flowering plants, C_4 photosynthesis evolved from C_3 ancestors more than 62 times independently in several distantly related groups (Sage *et al.*, 2011). C_4 photosynthesis is characterized by a biochemical CO_2 pump formed by the coordination of several evolutionary novelties, which increase the relative concentration of CO_2 around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) to nearly eliminate photorespiration (Ludwig and Canvin, 1971; Hatch, 1987; von Caemmerer and Furbank, 2003; Skillman, 2008; Sage *et al.*, 2012). The CO_2-concentrating mechanism relies on the primary fixation of atmospheric carbon by phosphoenolpyurvate carboxylase (PEPC) coupled with carbonic anhydrase. These reactions are spatially separated from the secondary refixation of CO_2 by Rubisco (Hatch, 1987; von Caemmerer and Furbank, 2003). An efficient segregation of these C_4 biochemical reactions requires specific leaf functions (Hattersley, 1984; Dengler *et al.*, 1994; Muhaidat *et al.*, 2007).

As a result of its multiple origins, C_4 photosynthesis does not present a consistent and discrete phenotype, so is better considered a functional trait involving a suite of coordinated leaf anatomical and biochemical characteristics (Brown and Smith, 1972; Laetsch, 1974). These components can assemble differently during each origin of C_4 photosynthesis, and these divergent evolutionary histories result in high anatomical and biochemical diversity among, and sometimes within, C_4 lineages (Hattersley and Watson,1992; Sinha and Kellogg,

Abbreviations: PEPC, phosphoenolpyurvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

1996; Kadereit *et al.*, 2003; Muhaidat *et al.*, 2007; Edwards and Voznesenskaya, 2011; Freitag and Kadereit, 2014). An understanding of the evolutionary transitions leading to the recurrent assembly of C_4 photosynthesis requires investigation of the individual characteristics that together generate C_4 function, not only in C_4 species but also in C_3 species variously related to C_4 taxa (Christin and Osborne, 2013). It is particularly important to differentiate the present function of each component from its identity and developmental origin. In this work, we focus on the variation observed in both C_3 and C_4 plants in each of the anatomical traits that together generate leaf functions compatible with C_4 photosynthesis. We combine a review of the literature with analyses of a quantitative leaf anatomy dataset compiled from 155 C_3 and C_4 grass species (Christin *et al.*, 2013). The C_4 grasses in this dataset encompass eight of the nine structural C_4 forms described for this family (Edwards and Voznesenskaya, 2011).

What is C_4 leaf anatomy?

Differential arrangements of cells and organelles within the leaves of taxa that we now recognize as C_3 and C_4 were first observed and published more than 80 years before the C_4 pathway itself was discovered (Duval-Jouve, 1875; Haberlandt, 1884). The association between specific cell and organelle arrangements and the C_4 pathway was then identified soon after the discovery of C_4 photosynthesis (El-Sharkawy and Hesketh, 1965; Downton and Tregunna, 1968; Berry *et al.*, 1970; Welkie and Caldwell, 1970). Since then, C_4 photosynthesis has usually been affiliated closely with a suite of leaf properties referred to as 'Kranz' anatomy (after Haberlandt's description in German of a wreath-like arrangement of cells). Kranz anatomy can be described as two distinct concentric layers of chlorenchyma cells, formed by a bundle sheath containing most of the chloroplasts, surrounded by an outer layer consisting of a small number of mesophyll cells. The visual identification of such arrangements in transverse section has been used in numerous anatomical surveys of leaves to identify the photosynthetic pathway for hundreds of species (Welkie and Caldwell, 1970; Carolin *et al.*, 1973, 1975, 1977; Brown, 1977; Hattersley *et al.*, 1982; Renvoize, 1987*a*).

Surveys of numerous C_3 and C_4 species over the past five decades have shown that leaf anatomies cannot be easily and consistently grouped into discrete categories corresponding to the two photosynthetic types but come in many flavours (Brown, 1975; Edwards and Voznesenskaya, 2011). It is true that the leaf anatomy of a randomly selected C_3 plant is highly likely to deviate significantly from that of a randomly selected C_4 plant. For example, *Viburnum punctatum*, like most C_3 eudicots, has distinct horizontal layers of mesophyll cells in its leaves (Fig. 1A), arranged such that it does not conform to the general anatomical pattern generally present in C_4 plants, whereby the bundle-sheath and mesophyll cells form concentric circles around the vasculature (Fig. 1C). This concentric arrangement of cells can be found in many C_3 grasses though (Figs 1B and 2) (Hattersley *et al.*, 1982; Dengler *et al.*, 1994; Besnard *et al.*, 2013) and, as detailed below, individual leaf characteristics that are usually associated with a C_4 function can be found in at least some C_3 plants. Furthermore, some plants achieve C_4 photosynthesis without the segregation of photosynthetic reactions into different types of cells (Bowes and Salvucci, 1984; Bowes and Salvucci, 1989; Freitag and Stichler, 2000; Edwards *et al.*, 2004). Despite this variation, C_4 physiology is still associated with a suite

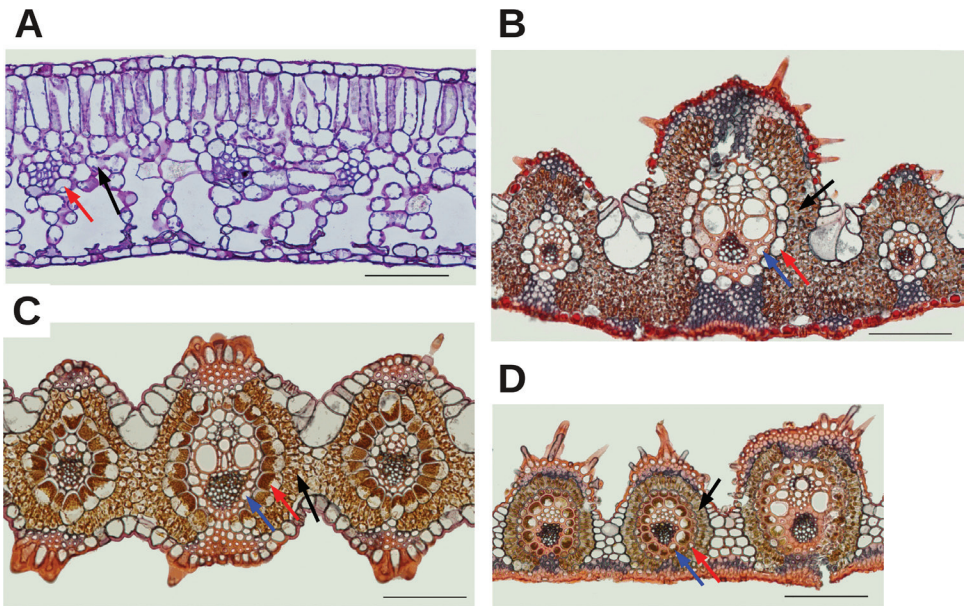

Fig. 1. Examples of C_3 and C_4 leaf cross-sections. The C_3/C_4 pair on the left (A, C) are unrelated, belonging to different major groups of flowering plants. By contrast, the C_3/C_4 pair on the right (B, D) is composed of closely related species, belonging to the same subfamily of grasses. (A) *Viburnum punctatum* (C_3, Adoxaceae), (B) *Sartidia angolensis* (C_3, Poaceae), (C) *Centropodia mossamedensis* (C_4, Poaceae), and (D) *Aristida mollissima* (C_4, Poaceae). Black arrows indicate the mesophyll, red arrows the outer bundle sheath, and blue arrows the inner sheath of grasses (=mestome sheath). The four cross-sections are shown at the same scale. Bars, 100 μm. Picture (A) was kindly provided by Dr David Chatelet from Brown University and pictures (B), (C) and (D) come from the collections of Professor J. Travis Columbus from Rancho Santa Ana Botanic Garden, CA, USA, with permission.

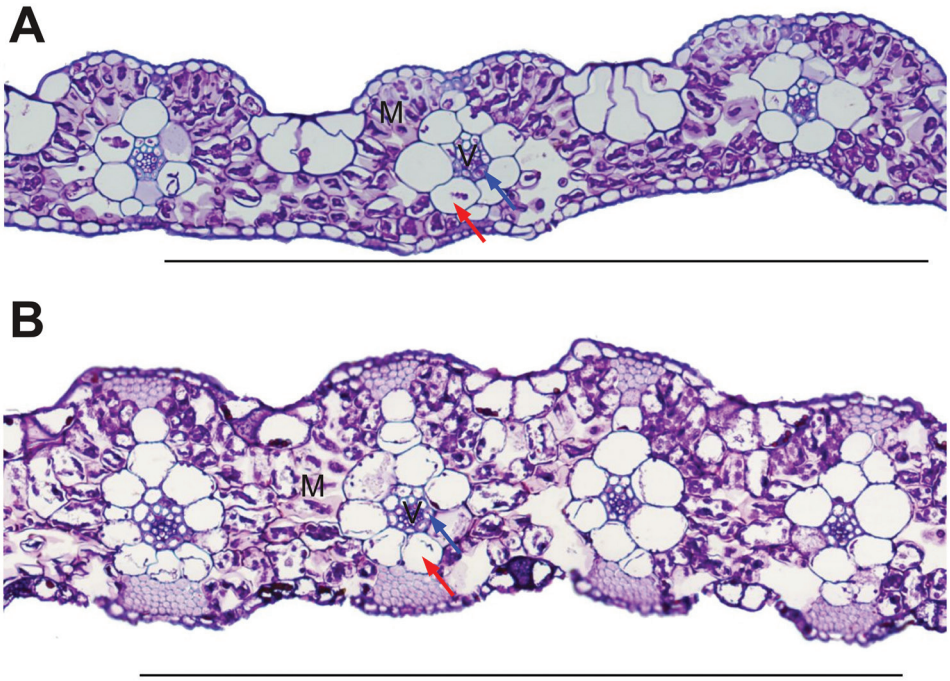

Fig. 2. Examples of C_3 grasses with leaf anatomy close to the C_4 requirements. (A) *Panicum pygmaeum* (C_3), (B) *Panicum malacotrichum* (C_3). The mesophyll (M) and vascular tissue (V) are indicated on the sections. Red arrows indicate the outer bundle sheath while blue arrows indicate the inner sheath (=mestome sheath). Bars, 500 μm.

of functional properties (Brown and Smith, 1972; Edwards and Voznesenskaya, 2011), which must first be considered before analysing diversity in the identity and developmental origins of the characteristics that generate them. Based on the literature, the following functional properties of leaves are considered essential requirements for C_4 photosynthesis (Hattersley *et al.*, 1977; Leegood, 2002; von Caemmerer and Furbank, 2003; Edwards and Voznesenskaya, 2011; Nelson, 2011). Note that these apply equally to all C_4 plants, whether or not they use distinct types of cells.

1. There must be two distinct compartments arranged so that atmospheric gases reach the first compartment more easily than the second. The first compartment houses the PEPC reactions, while the second, with characteristics that restrict CO_2 efflux, houses the Calvin cycle.
2. The two compartments must be in close contact to allow the rapid exchange of metabolites.
3. The compartment where the Calvin cycle occurs must occupy a large enough fraction of the leaf to accommodate a significant number of chloroplasts.
4. Chloroplasts must be abundant in the Calvin cycle compartment.

These functional properties are extremely important for C_4 physiology and biochemistry. However, to understand the gradual evolutionary changes leading to the recurrent assembly of C_4 photosynthesis, it is important to account for exact changes in cellular characteristics and the genetic determinants of these characteristics. In the following sections, we therefore discuss how each of the four functional properties listed above is generated from underlying characteristics. We look at how these characteristics vary qualitatively and quantitatively among C_3 and C_4 lineages, and show how there is an overlap between the values observed in C_3 and C_4 species.

Two compartments differentially connected to the atmosphere

In C_3 plants, the Calvin cycle occurs in most of the leaf, while it is restricted to specific locations in C_4 plants. It is well known that the identity of the compartments co-opted for the segregation of the atmospheric CO_2 fixation by PEPC and its refixation by the Calvin cycle differs among C_4 origins (e.g. Brown, 1975; Dengler *et al.*, 1985). For instance, some single-celled C_4 species have evolved separate compartments for the PEPC and Calvin cycle reactions through the rearrangements of organelles or vacuoles within individual photosynthetic cells (Edwards *et al.*, 2004). In the majority of C_4 plants, however, the PEPC and Calvin cycle reactions are segregated in different types of cells. In C_3 species, the mesophyll and bundle sheath represent two physiologically distinct types of cells, and the central position of bundle-sheath cells within the leaf gives the opportunity for minimal contact with the atmosphere (Figs 1A, B and 3, and Supplementary Fig. S1 available at *JXB* online). The bundle sheaths have consequently been co-opted for Calvin cycle reactions across most C_4 origins, while the mesophyll cells, which are better connected to the atmosphere, are used for the PEPC reactions. Despite this convergence in function, the bundle-sheath cells recruited for C_4 photosynthesis are not homologous among all C_4 origins.

In some C_4 species within the grass genera *Arundinella*, *Garnotia*, *Arthropogon*, *Achlaena*, *Dissochondrus*, *Anrthraxon*,

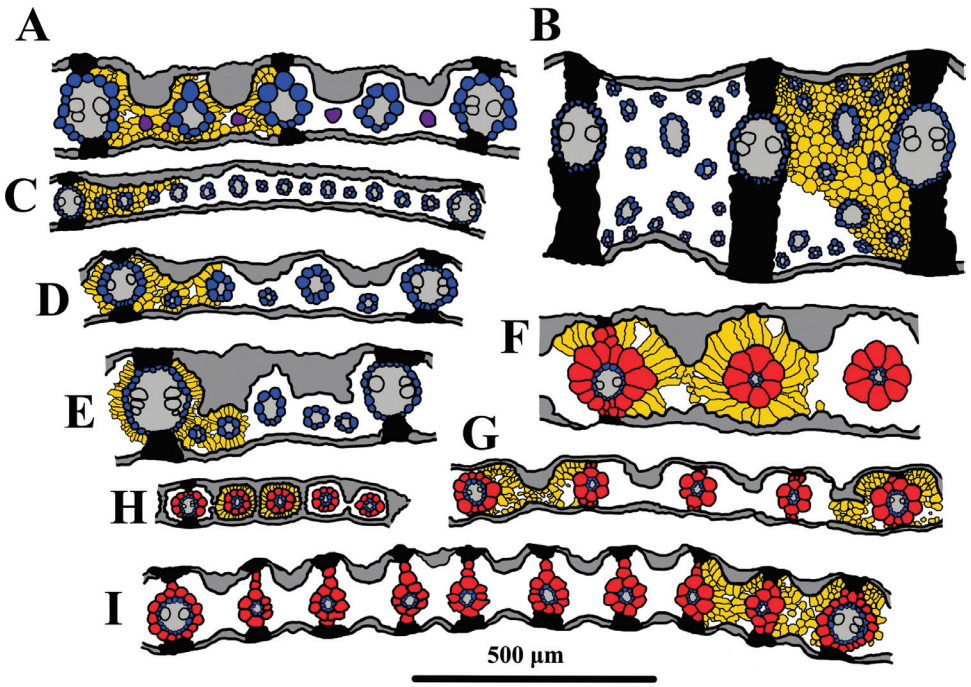

Fig. 3. Leaf anatomy for selected cross-sections of grasses. (A) *Arundinella nepalensis* (C_4), (B) *Anthaenantia lanata* (C_4), (C) *Axonopus compressus* (C_4), (D) *Ischaemum afrum* (C_4), (E) *Chrysopogon pallidus* (C_4), (F) *Alloteropsis cimicina* (C_4), (G) *Panicum pygmaemum* (C_3), (H) *Bouteloua stolonifera* (C_4) and (I) *Panicum malacotrichum* (C_3). The diagrams highlight the mesophyll cells (yellow), outer bundle sheaths (red), inner bundle sheaths (blue), and distinctive cells (purple). Uncoloured central areas are composed of mesophyll cells and intercellular airspace. Vein (light grey), epidermis (dark grey), and sclerenchymatous girders (solid black) are also shown. Where only one bundle sheath is present, it is assumed that the outer bundle sheath has been lost and the inner bundle sheath remains. All cross-sections are drawn at the same scale, indicated at the bottom. The corresponding pictures can be found in Supplementary Fig. S1 available at JXB online.

and *Microstegium*, the Calvin cycle also occurs in distinctive cells, which are atypical bundle-sheath-like cells, differentiated within the mesophyll but not associated with vascular bundles (Fig. 3A) (Tateoka, 1958; Hattersley and Watson, 1992; Ueno, 1995; Dengler *et al.*, 1996; Wakayama *et al.*, 2003). In addition, grasses and sedges possess multiple layers of sheath cells, with inner layers derived from procambium (often referred to as the 'mestome sheath') and outer layers from ground meristem (Dengler *et al.*, 1985; Soros and Dengler, 2001; Martins and Scatena, 2011). In studies of C_4 photosynthesis, consideration of the different cells is often based on their function. However, for evolutionary studies, the ontogenic origin of each type of cell needs to be established independently of its function. The C_4 lineages within grasses and sedges have alternatively co-opted one or both of these cell types, while the second cell layer is often lost, for example in the numerous C_4 grasses with a single sheath layer (Fig. 3A–E) (Brown, 1975; Dengler *et al.*, 1996; Soros and Dengler, 2001; Martins and Scatena, 2011). This diversity in the identity of the two compartments co-opted for the segregation of C_4 reactions, together with phylogenetic analyses, has been used previously to argue for multiple independent C_4 origins, rather than fewer origins followed by reversals in closely related C_3 species (Kellogg, 1999; Christin *et al.*, 2010).

The limited connection of the Calvin cycle compartment to the atmosphere is also achieved via different mechanisms in the different C_4 lineages. First, tightly packing mesophyll cells around the bundle sheath reduces the fraction of cells from the latter that are in contact with the atmosphere (Dengler *et al.*, 1994; Muhaidat *et al.*, 2007), although similar packing also occurs in some C_3 grasses (Fig. 1B) (Dengler *et al.*, 1994) and some C_3 eudicots (Muhaidat *et al.*, 2007). In addition, the bundle-sheath cell walls can also be covered with a layer of suberin, which limits gas diffusion. This is the case in C_4 monocots that have co-opted the inner sheath layer for a C_4 function (Hattersley and Browning, 1981; Ueno *et al.*, 1988*b*). However, the presence of suberin layers on the inner sheath cell walls can also be found in most C_3 grasses (Hattersley and Browning, 1981). Neither of the characteristics reducing contact of the Calvin cycle with the atmosphere is therefore found exclusively in C_4 plants.

Distance between the two compartments

Close contact between the PEPC and Calvin cycle compartments is guaranteed in plants with a single-celled C_4 system. In plants with a dual-celled C_4 system, the presence of mesophyll cells not directly adjacent to the bundle sheaths will increase the average distance between the compartments containing PEPC and Rubisco. This problem is usually solved in C_4 plants by limiting the number of cells separating consecutive Calvin cycle compartments, and by organizing mesophyll cells into one or two layers around the bundle sheath (Fig. 1C, D), which produces the classical pattern of Kranz anatomy. In some species, this configuration is achieved through the development of a

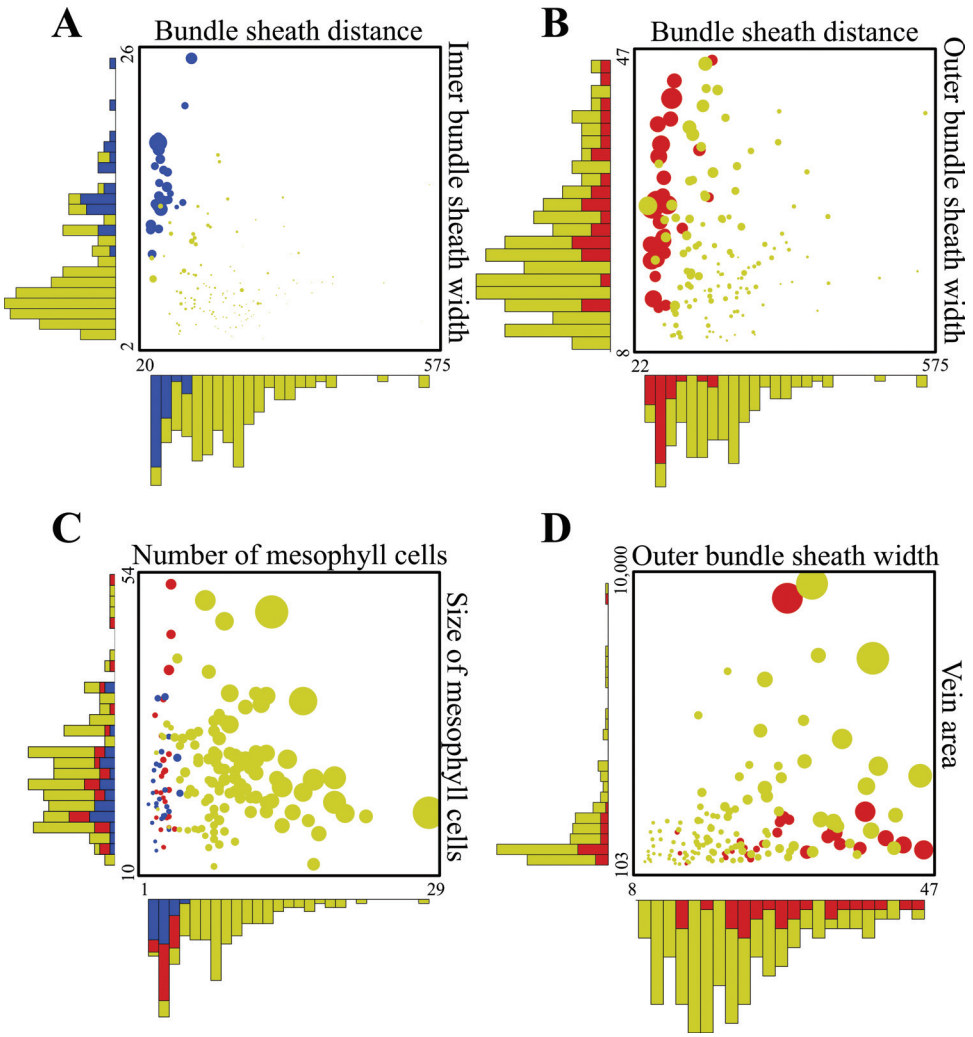

Fig. 4. Multidimensionality of C_4 anatomy in grasses. Scatter plots for anatomical variables associated with the C_4 syndrome are shown, along with frequency distributions for each trait, arranged along the axes. For each pair of variables, dot size is proportional to a third variable. C_3 grass species are shown in yellow, C_4 grass species using the outer sheath for the Calvin cycle in red, and C_4 grass species using the inner sheath for the Calvin cycle in blue. Relationships are shown between means of: (A) distance between consecutive bundle sheaths (μm) and inner bundle-sheath cell width (μm), with dot size proportional to the percentage of inner bundle-sheath area; (B) distance between consecutive bundle sheaths (μm) and outer bundle-sheath cell width (μm), with dot size proportional to the percentage of outer bundle-sheath area; (C) number of mesophyll cells between consecutive bundles and mesophyll cell length (μm), with dot size proportional to the distance between consecutive bundle sheaths (μm); and (D) outer bundle sheath cell width (μm) and area of vasculature (μm^2), with dot size proportional to the outer bundle sheath area (μm^2) per vein number. The data for 170 grasses (representing 155 species) come from Christin *et al.* (2013).

single bundle-sheath layer that encompasses all the vasculature within the leaf and often water-storage cells as well, and a single layer of mesophyll that surrounds the bundle sheath. Variations on this anatomical theme are common among C_4 eudicots and have been found in the Asteraceae, Amaranthaceae, and Cleomaceae families (Carolin *et al.*, 1975; Das and Raghavendra, 1976; Kadereit *et al.*, 2003; Peter and Katinas, 2003; Edwards and Voznesenskaya, 2011; Koteyeva *et al.*, 2011). Some C_4 grasses have similar bundle sheaths that extend horizontally from the vascular tissue and join together, such that the mesophyll becomes isolated in small patches (Renvoize, 1983).

For C_4 lineages with multiple photosynthetic units formed by concentric cell layers of mesophyll, bundle sheath, and vascular tissue, the presence of fewer mesophyll cells between consecutive veins can be achieved via two different developmental mechanisms. First, the number of cells that develop between consecutive bundle sheaths can be directly reduced during ontogeny. Second, extra Calvin cycle compartments, such as distinctive cells or minor veins, can be added to decrease the average distance between compartments, as has been documented in both monocots (e.g. Poaceae; Fig. 3A–E; Renvoize, 1987*a*; Dengler *et al.*, 1994; Ueno *et al.*, 2006; Christin *et al.*, 2013) and eudicots (e.g. Asteraceae; McKown and Dengler, 2007; McKown and Dengler, 2009; Cleomaceae; Marshall *et al.*, 2007).

Interveinal distance (or vein density) is often considered a proxy for the number of mesophyll cells between consecutive bundles, and largely overlaps between C_3 and C_4 grasses (Christin *et al.*, 2013) and eudicots (Muhaidat *et al.*, 2007). However, the relationship between interveinal distance and the number of mesophyll cells is only partial. First, because

interveinal distance is influenced both by the diameter of the veins and the size of the bundle sheaths, measuring the actual distance between bundle sheaths is more relevant. This distance is influenced by the size of individual mesophyll cells, their orientation, and finally their number (Fig. 4C). Some C_4 species, such as *Alloteropsis cimicina*, have relatively large interveinal distances but with only a few large mesophyll cells between consecutive bundles (Fig. 3F, 4C). In addition, the number of mesophyll cells containing PEPC below and above veins can influence the average distance between the PEPC and Calvin cycle reactions independently of the distance between consecutive bundles. Some thick C_4 leaves, such as those of *Anthaenathia lanata* (Fig. 3B) or some *Portulaca* (Ocampo *et al.*, 2013), consequently require a three-dimensional venation system. Finally, leaf thickness is often reduced between veins so that there are few mesophyll cells in positions most distant from the bundle sheaths, and interveinal distance can greatly exceed the average distance between photosynthetically active mesophyll cells and bundle-sheath cells (Figs 1 and 3). For instance, in leaves of the C_3 grass *Panicum pygmaemum*, the average number of mesophyll cells between bundles greatly exceeds four. However, because its leaf thickness decreases between veins, the number of mesophyll cells separated from the bundle sheath by more than one cell is smaller than the number of mesophyll cells separated from the bundle sheath by zero or one cell (38 versus 73 cells between the three veins in Fig. 2). Finally, the distance between consecutive bundles can be increased by the presence of achlorophyllous cells that do not influence the average path length from PEPC to Calvin cycle cells (e.g. Fig. 1D).

The number of mesophyll cells between consecutive bundles will distinguish C_3 from C_4 taxa with a high success rate and has consequently been proposed as a criterion to recognize C_4 plants (Hattersley and Watson, 1975; Renvoize, 1987*a*; Sinha and Kellogg, 1996). However, the C_3 and C_4 distributions for this trait also overlap (Fig. 4C). For instance, *Panicum malacotrichum* is a C_3 grass with less than four mesophyll cells between veins (Fig. 2). The variation observed in both C_3 and C_4 taxa is probably due to the importance of vascular architecture for both photosynthetic types. While the distance between consecutive bundles affects the efficiency of C_4 photosynthesis (Ogle, 2003), vein density also influences the transport of metabolites, leaf hydraulics and other physiological characteristics in C_3 plants (Sack and Scoffoni, 2013; Sack *et al.*, 2013). In summary, both interveinal distance and the number of mesophyll cells between consecutive bundles overlap in C_3 and C_4 taxa, so that C_4 values represent only a subset of those observed among all photosynthetic types (Fig. 4A–C) (Muhaidat *et al.*, 2007; Christin *et al.*, 2013).

The transport of metabolites between the PEPC and Calvin cycle compartments in C_4 plants is also facilitated by a number of plasmodesmata connecting mesophyll and bundle-sheath cells that exceeds the number found in C_3 plants (Olesen, 1975; Weiner *et al.*, 1988; Botha, 1992). However, plasmodesmata frequency is known in only a few C_3 species, so the overall variation in this trait cannot be established with confidence.

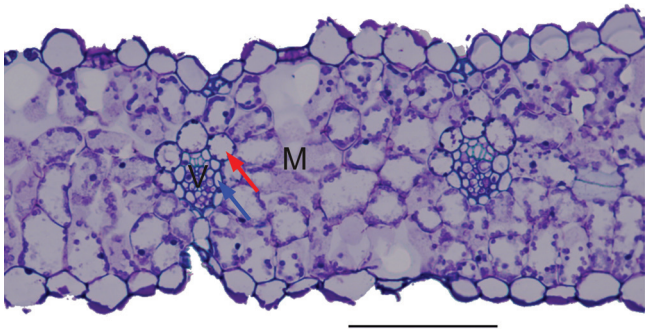

Fig. 5. Detail of a cross-section for *Dactylis glomerata*. The mesophyll (M) and vascular tissue (V) are indicated on the section of this C_3 species. The red arrow Indicates the outer bundle sheath, while the blue arrow indicates the inner sheath (=mestome sheath). Bar, 100 μm. Note the incomplete outer sheath.

Large Calvin cycle compartment

The amount of CO_2 that can be re-fixed by Rubisco in the Calvin cycle will depend on the number of chloroplasts within the compartment co-opted for this function. The size of this compartment, not including the volume occupied by the vacuole, will influence the number of chloroplasts that can be accommodated. Thus, C_4 plants tend to have enlarged bundle-sheath cells able to accommodate numerous chloroplasts. More than the size of individual bundle sheath cells, the cumulative volume of bundle sheath relative to the PEPC compartment (mesophyll) is relevant, and seems to be constrained within a given range in C_4 plants (Hattersley, 1984; Dengler *et al.*, 1994; Muhaidat *et al.*, 2007). This might represent a trade-off between having sufficient chloroplasts in the Calvin cycle compartment and still conserving enough mesophyll volume for PEPC.

Similar bundle sheath:mesophyll ratios can be achieved through different combinations of the numerator (volume of bundle sheath) and denominator (volume of mesophyll). For instance, similar proportions of bundle sheath can be achieved through alternative developmental mechanisms, involving the production of either larger or more numerous bundle-sheath cells (the latter is generally achieved through a proliferation of veins; Fig. 3) (Hattersley, 1984; McKown and Dengler, 2009). The cross-sectional area of mesophyll per vein is mainly a function of the distance between veins, the thickness of the leaf (including the thickness between veins in comparison to that at the veins) and the presence of achlorophyllous cells (Christin *et al.*, 2013). On the other hand, when viewed in transverse section, the total area of a given type of bundle sheath per vein is a function of the size of the bundle-sheath cells, the diameter of the veins, and, in some cases, the completeness of the bundle sheath (Fig. 4) (Christin *et al.*, 2013). For instance, the external bundle sheath of many grasses is not developed on the abaxial side of the leaf, which reduces the total volume of this tissue (Fig. 5) (e.g. Renvoize, 1985, 1987*b*). Thus, the relative amount of bundle-sheath tissue is a function of at least five distinct traits, which may all vary independently. Functionally similar characteristics can consequently arise through different developmental modifications, as highlighted by the diversity of C_4 leaf anatomy (Fig. 4).

The five components that dictate the relative amount of bundle-sheath tissue are important determinants of the gross leaf anatomy associated with C_4 photosynthesis. However, each component shows an essentially continuous distribution across C_3 and C_4 values, such that C_4-compatible ranges merely represent a subset of the distribution found in C_3 taxa (Fig. 4; Marshall *et al.*, 2007; McKown and Dengler, 2007). The C_4-suitability of one parameter depends on the values of the other parameters. For instance, large volumes of bundle-sheath tissue can arise in the presence of significant distances between consecutive bundles if the bundle-sheath cells are enlarged (Fig. 4A, B). This is highlighted by a comparison of *Alloteropsis cimicina* and *Axonopus compressus* (Fig. 3F and C, respectively), which achieved similar ratios of bundle sheath per mesophyll area [BS/(BS+M) of 0.26 and 0.21, respectively] through different means. *Alloteropsis cimicina* has very large outer bundle sheaths that are separated by long distances of mesophyll, while *Axonopus compressus* has small inner sheaths that are separated by very short mesophyll distances in particularly thin leaves (Fig. 3F and 3C, respectively).

During the course of evolution, numerous alterations in the characteristics that generate each leaf function occur either stochastically or in response to selective pressures. For instance, leaf thickness often represents an adaptation to the amount of light received by plants (Boardman, 1977; Terashima *et al.*, 2001). The number and size of veins alters the hydraulics of a plant, which, in turn, affects the sorting of plants across environments (McKown *et al.*, 2010; Sack *et al.*, 2012). Finally, the bundle sheath controls water flux between the mesophyll and vascular tissue such that an increase in bundle-sheath size might provide better protection against cavitation in arid environments (Sage, 2001; Leegood, 2008; Griffiths *et al.*, 2013). Recurrent and independent changes in different leaf properties repeatedly led to the emergence of tissues suitable for C_4 photosynthesis, which characterize numerous extant C_3 plants (Muhaidat *et al.*, 2007; Edwards and Voznesenskaya, 2011; Muhaidat *et al.*, 2011; Kadereit *et al.*, 2012; Christin *et al.*, 2013; Griffiths *et al.*, 2013).

Distribution of organelles

One of the most important requirements for C_4 photosynthesis probably lies in the distribution of chloroplasts. Although they are present in all photosynthetic cells of C_3 plants, chloroplasts are especially abundant in mesophyll cells and can vary from equally abundant to completely absent in bundle-sheath cells (Figs 1, 2 and 5) (Crookston and Moss, 1970). In C_4 plants, the light-dependent and light-independent functions of chloroplasts are often decoupled, and chloroplasts of the PEPC and Calvin cycle compartments can become morphologically and functionally differentiated (Woo *et al.*, 1970; Laetsch, 1974; Hattersley *et al.*, 1977; Bowman *et al.*, 2013). Although the characteristics and distribution of organelles vary among C_4 lineages (Ueno *et al.*, 1988*b*; Voznesenskaya *et al.*, 2006; Edwards and Voznesenskaya, 2011), the Calvin cycle compartment of C_4 plants consistently has a high concentration of chloroplasts, where the enzymes of the Calvin cycle are preferentially expressed.

No quantitative census of chloroplast distribution is available for randomly selected plants; however, the organelle distribution has been investigated in species closely related to C_4 lineages, which shows that some plants maintain significant numbers of chloroplasts in bundle-sheath cells, despite lacking a functional C_4 pathway (Hattersly *et al.*, 1982; Ueno and Sentoku, 2006; Christin *et al.*, 2013). This is particularly common in plants using C_2 photosynthesis, a weak CO_2-concentrating mechanism based on a glycine shuttle from mesophyll to bundle-sheath cells (Edwards and Ku, 1987; Sage *et al.*, 2012). When chloroplast abundance in bundle-sheath cells is compared among taxa, there is a gradient from closely related C_3 to C_2, and then from C_2 to C_4 species (Muhaidat *et al.*, 2011; Sage *et al.*, 2013). The C_2 trait is consequently often considered an evolutionary intermediate between C_3 and C_4 types (Hylton *et al.*, 1988; Sage *et al.*, 2012; Williams *et al.*, 2013). Therefore, as for other anatomical traits, the number of chloroplasts in bundle-sheath cells varies and may form a continuum between C_3 and C_4 species. Despite this, a high concentration of chloroplasts in bundle-sheath cells might be the only trait that occurs systematically within dual-celled C_4 photosynthesis that is never present in non-C_4 plants. The tight association between C_4 physiology and chloroplast distribution is explained by the fact that C_4 physiology results from a differential distribution of the Calvin cycle (among other biochemical reactions), which is usually linked to the distribution of chloroplasts.

Other ultrastructural properties associated with some C_4 plants include the distribution of mitochondria and peroxisomes among compartments, the distribution of organelles within compartments and the ultrastructure and photochemical properties of the chloroplasts (Bruhl and Perry, 1995; Edwards and Voznesenskaya, 2011). Some of these properties are also observed in non-C_4 species closely related to C_2 and C_4 taxa (Sage *et al.*, 2012)

Plasticity for C_4-suitable anatomy

Phenotypic plasticity to environmental cues creates an additional layer of variation and further blurs the dichotomy between C_4 and non-C_4 anatomy. Specifically, plasticity for the anatomical traits relevant to photosynthesis (e.g. compartmentalization, interveinal distance, mesophyll cell size and number, bundle-sheath cell size, and organelle distribution) could partially explain the variation found in these anatomical characteristics or, more importantly, the shift of C_3 plants into the C_4-suitable space. Plasticity for these traits has been documented in the literature. For example, the C_3 grass *Phragmites australis* acquires C_4-like traits when it grows at low soil water potentials (Gong *et al.*, 2011). Specifically, interveinal distance decreases, chlorophyll content within bundle-sheath cells increases, and the activity of C_4-related enzymes increases as soil water potential becomes more negative across a natural precipitation gradient (Gong *et al.*, 2011). The C_4-like *Flaveria brownii* lacks the complete suite of anatomical characteristics required for a fully functioning C_4 system (Araus *et al.*, 1990). However, this species can plastically increase its degree of C_4 photosynthesis by nearly doubling its investment in

bundle-sheath tissue relative to mesophyll in response to high irradiance compared with when it is grown at low irradiance (1200 vs 80 μmol m^{-2} s^{-1} photosynthetic photon flux density, respectively; Araus *et al.*, 1991). Furthermore, interveinal distances decreased in the C_3 grasses, *Festuca arundinaceae* (43% decrease), and the C_3/C_4 intermediate grass *Panicum miliodies* (34% decrease), when grown in high versus low nitrogen levels (Bolton and Brown, 1980).

In addition to the plasticity of individual anatomical components, two different modes of environmentally induced C_4 photosynthesis exist. First, several aquatic species of Hydrocharitaceae, and possibly some Alismataceae and Cyperaceae, are able to switch from C_3 to single-cell C_4 photosynthesis (Bowes *et al.*, 2002). The environmental cue for this plasticity may be exposure to low-CO_2 conditions as they become submerged under water, or seasonal variation in temperature (Bowes *et al.*, 1979; Bowes, 2011). In contrast, some aquatic *Eleocharis* species use C_3 or C_3/C_4 intermediate photosynthesis when submerged but induce C_3/C_4 or C_4 photosynthesis by developing C_4-compatible leaf anatomy and expressing C_4 enzymes in the emergent leaves (Ueno *et al.*, 1988*a*; Ueno, 2001; Murphy *et al.*, 2007). Finally, some amphibious C_4 grasses seem to switch from a C_4 system that functions without C_4-associated leaf anatomy in aquatic leaves to a classical dual-cell C_4 cycle in aerial leaves (Keeley, 1998; Boykin *et al.*, 2008).

Phenotypic plasticity for C_4-associated traits might have important implications for the evolution of C_4 photosynthesis (Sultan, 1987; West-Eberhard *et al.*, 2011). First, the direction and degree of phenotypic change in response to an environmental gradient is heritable (Schlichting and Levin, 1986; Schlichting and Pigliucci, 1993), and the reaction norm for a trait is genetically distinct from the trait itself. Selection can therefore act independently on both a trait and on the plasticity for that trait. Plasticity may thus deter the evolutionary transition from C_3 to C_4 photosynthesis by diluting the effects of natural selection. However, adaptive phenotypic plasticity may promote C_4 evolution if the plastic expression of C_4-suitable anatomical traits in C_3 plants allows the colonization of new niches, leading to selective pressures for the gradual acquisition of C_4 biochemistry (Heckmann *et al.*, 2013). Indeed, Sage and McKown (2006) reviewed the literature to find that C_3 plants seem to be inherently more plastic than C_4 plants overall. Thus, this capacity for phenotypic plasticity might affect the probability of evolving C_4 photosynthesis. For instance, differential capacity in the phenotypic plasticity for important C_4 anatomical traits among plant lineages may explain the differential propensity for C_4 evolution. However, the plasticity of anatomical traits associated with C_4 photosynthesis remains mostly unknown in C_3 species, and more comparative work is required.

Consequences for the evolution of C_4-associated anatomy

When comparing the anatomy of a randomly selected C_3 taxon with that of a highly efficient C_4 species, the evolutionary transition from C_3 to C_4 anatomy can seem extraordinary (Fig. 1A, C). However, it is important to note that C_4 photosynthesis did not emerge from the average C_3 taxon but from C_3 ancestors with leaf anatomical properties much closer to the C_4 requirements (Figs 1B and 5) (Muhaidat *et al.*, 2011; Christin *et al.*, 2013; Sage *et al.*, 2013). In the Poaceae, some species apparently using the C_3 photosynthetic type have gross leaf anatomies that closely resemble those of C_4 plants. For instance, *Panicum malacotrichum* and *Panicum pygmaeum* (Fig. 2) are two C_3 grasses ($\delta^{13}C$ values of –27.4 and –29.7, respectively), which are closely related to several C_4 lineages (namely *Alloteropsis* and *Echinochloa*; Grass Phylogeny Working Group II, 2012). These species possess large proportions of bundle-sheath tissue that are firmly in the C_4 range [BS/(BS+M) of 0.26 and 0.23, respectively; Christin *et al.*, 2013], and most mesophyll cells are directly adjacent to the bundle sheath or separated by only one mesophyll cell (Fig. 2). Chloroplasts are still almost completely restricted to the mesophyll in these species. However, because the gross leaf anatomy is in place, fewer anatomical changes are necessary for the evolution of C_2 or C_4 photosynthesis. In other cases, such as the grass tribe Neurachninae, C_3 species that are closely related to C_4 species have both C_4-like gross anatomy [BS/(BS+M) of 0.14–0.16; Christin *et al.*, 2013] and the presence of conspicuous chloroplasts in the inner sheath, which was co-opted for C_4 photosynthesis in this group (Hattersley *et al.*, 1982). These examples show that the evolution of C_4-suitable anatomy might not always require drastic modifications, as C_3 lineages may possess C_4-like values for individual traits that can generate C_4 leaf functions.

Each component of C_4-compatible leaf anatomy may vary independently within C_3 ancestors, such that any combination of mesophyll cell size, bundle-sheath cell size, leaf thickness and interveinal distance could theoretically occur. However, the observed range is obviously more limited (Fig. 4), for a number of reasons. First, multiple traits might be influenced by the same gene (pleiotropy). For instance, genome size theoretically affects the size of all cells (Grime and Mowforth, 1982; Masterson, 1994; Beaulieu *et al.*, 2008; Šímová and

Table 1. *Degrees of co-variation among anatomical variables*

Co-variation in grasses between the mean distance between consecutive bundle sheaths (μm), outer bundle-sheath (OBS) cell width (μm), inner bundle-sheath (IBS) cell width (μm), number of mesophyll (M) cells between consecutive bundles, and leaf thickness (μm). R^2 values are provided for pairs of variables with significant correlations. Regressions with P values less than 0.05 are considered significant, while those with P values greater than 0.05 are indicated by NS. Phylogenetically controlled analyses were performed with the pgls function of the caper R package (Orme et al., 2012), using the data for 155 grass species from Christin et al. (2013).

BS distance				
0.02	OBS cell width			
NS	0.23	IBS cell width		
0.58	NS	0.05	No. M cells	
NS	0.28	0.43	0.12	Leaf thickness

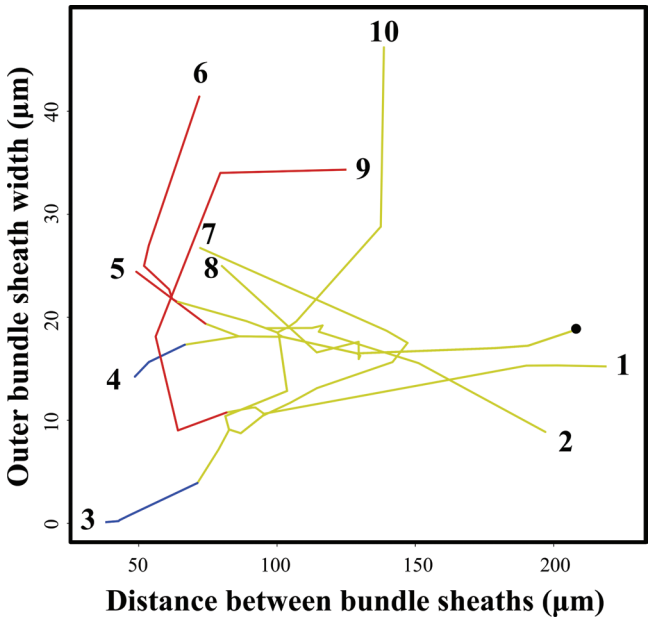

Fig. 6. Evolutionary trajectories toward C_4-compatible anatomical traits. Phylogenetic relationships are plotted in anatomical space for grass species selected to represent a diversity of anatomical traits. Values are the distance between consecutive bundle sheaths, and the width of outer bundle-sheath cells, which are observed for the tips and inferred for the internal nodes. The black point represents the root of the tree (see Christin *et al.*, 2013, for details). Yellow branches indicate a C_3 state, red branches a C_4 state using the outer sheath for the Calvin cycle, and blue branches a C_4 state using the inner sheath for the Calvin cycle. Numbers refer to the extant species: 1, *Dichanthelium acuminatum* (C_3); 2, *Danthonia spicata* (C_3); 3, *Heteropogon contortus* (C_4); 4, *Aristida congesta* (C_4); 5, *Stipagrostis obtusa* (C_4); 6, *Eleusine indica* (C_4); 7, *Panicum malacotrichum* (C_3); 8, *Oryza coarctata* (C_3); 9, *Panicum miliaceum* (C_4), 10, *Arundo donax* (C_3). Data from Christin *et al.* (2013).

Herben, 2012), so that an increase in bundle-sheath cell size might co-occur with increases in the sizes of mesophyll cells. Plants often escape this constraint via cell-specific endoreduplication, which allows an increase of one type of cell relative to others (Sugimoto-Shirasu and Roberts, 2003), and comparative analyses show that variation in the cell sizes of different components of C_4 anatomy is only partially correlated (Table 1). However, endoreduplication is not involved in the increase of bundle-sheath cell size, at least in the C_4 *Cleome gynandra* (Aubry *et al.*, 2013). It is also likely that some combinations of traits are not viable, as the whole-leaf structure influences plant fitness (Noblin *et al.*, 2008), not its individual components.

The multidimensionality of leaf characteristics associated with C_4 photosynthesis, as highlighted for the grass family, means that different combinations of underlying traits will generate C_4-compatible leaf anatomies (Fig. 4). For instance, both a proliferation of veins with small bundle-sheath cells and an increase of bundle-sheath cell size without additional veins would increase the relative amount of bundle-sheath cells (Fig. 6). This potential for alternative anatomical combinations to achieve the same functional outcome means that C_3 ancestors will repeatedly reach C_4-compatible areas of the multidimensional trait space (Fig. 6), and increases the likelihood of C_4 anatomy evolving (Williams *et al.*, 2013).

A sample of evolutionary trajectories in the Poaceae shows lineages for which repeated and independent alterations of the distance between bundle sheaths and bundle-sheath size led into different C_4-compatible regions of the anatomical space (Fig. 6). Obviously, not all C_3 lineages that acquired C_4-suitable leaf anatomical characteristics have evolved C_4 biochemistry. For example, *Panicum malacotrichum* and *Oryza coarctata* have C_4-suitable mesophyll distances between consecutive bundle sheaths and proportions of bundle-sheath tissue but have not developed the C_4 syndrome (Figs 2 and 6) (Christin *et al.*, 2013). Furthermore, *Cleome violacea*, *C. africana*, and *C. paradoxa* have small interveinal distances, and *C. africana* and *C. paradoxa* also display enlarged bundle-sheath cells similar to their C_4 congener *C. gynandra*, yet these three species do not employ the C_4 photosynthetic system (Marshall *et al.*, 2007). However, the presence of these characteristics probably enables C_4 evolution (pre-adaptation or exaptation *sensu* Gould and Vrba, 1982; Christin *et al.*, 2013; Griffiths *et al.*, 2013; Sage *et al.*, 2013). Once a C_4-compatible anatomy is in place, the C_4 biochemical pathway can evolve from a C_3 background in a stepwise sequence, where each step incrementally increases the efficiency of photosynthesis (Heckmann *et al.*, 2013). However, the multiple anatomical requirements for C_4 photosynthesis do not usually co-occur in C_3 plants. Interesting exceptions include plants with a C_2 physiology, which were probably co-opted for the evolution of C_4 photosynthesis (Christin *et al.*, 2011; Muhaidat *et al.*, 2011; Sage *et al.*, 2012).

Functional C_4 diversity as a consequence of evolutionary diversity

Because C_4-compatible leaf anatomy engages multiple components, each C_4 origin may involve different modifications and co-opt different compartments for the Calvin cycle (Brown, 1975; Dengler *et al.*, 1994; Edwards and Voznesenskaya, 2011; Christin *et al.*, 2013). The anatomy present in the C_3 ancestor might affect which C_4 phenotypes are possible. For instance, C_3 ancestors with enhanced water storage tissue are likely to give rise to C_4 leaves that maintain the same capacity to store water, with the PEPC and Calvin cycle compartments occupying other parts of the leaves (Voznesenskaya *et al.*, 1999; Kadereit *et al.*, 2003; Freitag and Kadereit, 2014). Similarly, C_4 species that use the inner bundle sheath for the Calvin cycle must evolve from C_3 ancestors that possessed two differentiated sheaths, as is the case with grasses and sedges (Dengler *et al.*, 1994; Soros and Dengler, 2001). Furthermore, C_4 phenotypes that are functionally similar can be achieved through different modifications, even when starting with similar C_3 ancestors.

Different modifications to fulfil the same C_4 requirements might have functional consequences. Indeed, the adaptation of C_4 photosynthesis through the evolution of thick leaves with large bundle-sheath cells (Fig. 3F) is likely to have different consequences from the evolution of thin leaves with small cells but very short interveinal distance (Fig. 3C). An increase in vein density will affect not only the hydraulics but

also the distribution of stomata, which tend to be located in between veins (Taylor *et al.*, 2012). Leaf thickness will have consequences for light-capture efficiency as well as ecologically meaningful traits such as specific leaf area (Wilson *et al.*, 2002). Similarly, light capture will also be affected by the different distribution of chloroplasts in mesophyll and bundle-sheath cells, and the relative abundance of each cell type, together with the orientation of mesophyll cells (Vogelmann *et al.*, 1996). The path length from stomata to the photosynthetically active cells will also be influenced by leaf thickness, interveinal distance, and amount of intercellular airspace (Noblin *et al.*, 2008). Finally, co-opting some areas of the leaf for C_4 photosynthesis while maintaining water storage cells will probably allow the C_4 descendants to thrive in more arid conditions (Voznesenskaya *et al.*, 1999; Kadereit *et al.*, 2012). All of these characteristics, which can be directly affected by the evolutionary path a species took to achieve C_4 function, will determine the physiology of a plant and thus its ecological preferences. Therefore, the diversity of evolutionary trajectories toward C_4-compatible leaf anatomy might partially explain the ecological diversity associated with distinct C_4 lineages (e.g. Taub, 2000; Kadereit *et al.*, 2012; Liu *et al.*, 2012).

Consequences for putative genetic determinism

A detailed discussion of genetic determinants is beyond the scope of this paper. However, it is worth pointing out that, despite recent important developments (e.g. Slewinski *et al.*, 2013; Wang *et al.*, 2013; Lundquist *et al.*, 2014), the genetic mechanisms necessary to introduce C_4-compatible anatomy into C_3 species remain largely unknown. This has particular implications for the bioengineering of C_4 photosynthesis into major C_3 crops, such as rice and wheat, which has the potential to greatly enhance yield (Covshoff and Hibberd, 2012; von Caemmerer *et al.*, 2012). First, the multiplicity of traits means that there are probably multiple genes involved. For instance, a phylogenetic analysis shows that the distance between consecutive bundle sheaths and the size of these bundle sheaths vary independently in grasses (Table 1), suggesting different underlying genetic changes. Second, as the variation in most traits presents a continuum from C_3 to C_4 plants, the determinism is likely to involve multiple genes with small effects and no master switch. Third, the diversity of strategies used to achieve leaf functions that are compatible with C_4 photosynthesis means that genetic determinism is likely to differ among C_4 lineages. Finally, the genetic changes that occur during the evolution of C_4 photosynthesis are likely to vary as a function of the condition in the C_3 ancestor.

Interestingly, similar variation in some of the underlying traits exists in C_3 and C_4 species, which suggests that useful genetic variants may be identified from the analysis of C_3 taxa that vary in only some of the traits, even if these C_3 taxa do not present C_4-like anatomies. For instance, a C_3 taxon with variation in the number of mesophyll cells between consecutive veins would be a good study system, even if the bundle sheath and distribution of chloroplasts were not C_4-compatible. Considering variation within C_3 taxa that are unrelated to C_4 lineages might therefore expose new ways to identify the adaptive significance of individual C_4 components, as well as their genetic determinism.

Conclusions

Overall, C_4 leaves can be defined by a set of important functions that characterize all C_4 plants. However, the underlying developmental characteristics that generate these functional properties are extremely variable, as a consequence of the taxonomic diversity of C_4 plants. The same functionally important traits are not homologous among all C_4 plants, and this has important implications for the evolution and underlying genetics of C_4-specific leaf anatomy. In addition, the developmental modifications that generate each of the essential requirements of C_4 leaf anatomy can happen independently. Thus, distantly related C_4 groups might arrive at the same phenotype for one of these requirements (e.g. both groups co-opt the same compartment for the Calvin cycle) but not another (e.g. they achieve small distances between the two compartments through either a reduction in the number of cells between veins or the development of additional veins).

Most of the anatomical characteristics that can generate functional properties of C_4 leaves exist in at least some C_3 plants. The only well-characterized exception is chloroplast concentration in the compartment co-opted for the segregation of the Calvin cycle, which seems to be specific to C_4 plants, and to some extent C_2 plants. Without considering the distribution of chloroplasts and hence C_4 physiology, leaves of C_3 and C_4 plants cannot be placed into mutually exclusive categories (see Fig. 3, for example), and there is continuous variation of the underlying traits among C_4 and C_3 species (Fig. 4). Hard categorization is meaningful from a functional perspective, but it wrongly suggests that the recurrent emergence of C_4 photosynthesis represents the same number of drastic transitions between two distinct and homogeneous characteristic states. Acknowledging the diversity present within both C_3 and C_4 taxa, and the continuum that exists between these two physiological states, is paramount to understanding the evolutionary processes that led to C_4 plants, as well as the genetic mechanisms responsible for C_4-compatible leaf anatomy.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Cross-sections corresponding to the diagrams shown in Fig. 3.

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