From proto-Kranz to C\textsubscript{4} Kranz: building the bridge to C\textsubscript{4} photosynthesis

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Received 2 January 2014; Revised 14 March 2014; Accepted 24 March 2014

Abstract

In this review, we examine how the specialized “Kranz” anatomy of C\textsubscript{4} photosynthesis evolved from C\textsubscript{3} ancestors. Kranz anatomy refers to the wreath-like structural traits that compartmentalize the biochemistry of C\textsubscript{4} photosynthesis and enables the concentration of CO\textsubscript{2} around Rubisco. A simplified version of Kranz anatomy is also present in the species that utilize C\textsubscript{2} photosynthesis, where a photorespiratory glycine shuttle concentrates CO\textsubscript{2} into an inner bundle-sheath-like compartment surrounding the vascular tissue. C\textsubscript{2} Kranz is considered to be an intermediate stage in the evolutionary development of C\textsubscript{4} Kranz, based on the intermediate branching position of C\textsubscript{2} species in 14 evolutionary lineages of C\textsubscript{4} photosynthesis. In the best-supported model of C\textsubscript{4} evolution, Kranz anatomy in C\textsubscript{2} species evolved from C\textsubscript{3} ancestors with enlarged bundle sheath cells and high vein density. Four independent lineages have been identified where C\textsubscript{3} sister species of C\textsubscript{2} plants exhibit an increase in organelle numbers in the bundle sheath and enlarged bundle sheath cells. Notably, in all of these species, there is a pronounced shift of mitochondria to the inner bundle sheath wall, forming an incipient version of the C\textsubscript{2} type of Kranz anatomy. This incipient version of C\textsubscript{2} Kranz anatomy is termed proto-Kranz, and is proposed to scavenge photorespiratory CO\textsubscript{2}. By doing so, it may provide fitness benefits in hot environments, and thus represent a critical first stage of the evolution of both the C\textsubscript{2} and C\textsubscript{4} forms of Kranz anatomy.

Key words: C\textsubscript{4} evolution, C\textsubscript{2} photosynthesis, C\textsubscript{4} photosynthesis, C\textsubscript{3}–C\textsubscript{4} intermediate, glycine shuttle, Kranz anatomy, photorespiration, proto-Kranz anatomy.

Introduction

A major feature of C\textsubscript{4} photosynthesis is the specialization of leaf structure to form Kranz anatomy, wherein CO\textsubscript{2} is first assimilated by PEPcase in a layer of mesophyll (M) cells that surround an inner layer of bundle-sheath-like Kranz (K) cells, where CO\textsubscript{2} is concentrated and refixed by Rubisco (Fig. 1; Brown, 1975). Of the 65 to 70 known lineages of C\textsubscript{4} plants (Sage et al., 2011a, 2012), only a few lack Kranz anatomy. These are the single-celled C\textsubscript{4} plants occurring in two terrestrial C\textsubscript{4} lineages of the Chenopodiaceae, a C\textsubscript{4} lineage of diatoms, and two lineages in the Hydrocharitaceae, a family of aquatic angiosperms (Bowes, 2011; Edwards and Voznesenskaya, 2011; Sage et al. 2011a). In terrestrial plants, variants of C\textsubscript{4}-Kranz anatomy have independently evolved at least 60 times, making the Kranz syndrome one of the most convergent structural types in the living world (Sage et al. 2011a; Edwards and Voznesenskaya, 2011).

Kranz anatomy encompasses many distinct forms (Hattersley and Watson, 1992; Edwards and Voznesenskaya, 2011; Kadereit et al., 2012; Freitag and Kadereit, 2013). The inner layer of K cells can be derived from parenchymatous bundle sheath (eudicots and many monocots), a mestome sheath around the vascular bundle (monocots only), or a sheath of parenchymatous cells around layers of water storage cells (eudicots only; Brown, 1975; Dengler and Nelson, 1999; Edwards and Voznesenskaya, 2011; see Supplementary Figs S1, S2 for examples). Kranz cells are generally more
conspicuous than the homologous cells of their C₃ relatives, owing to the presence of larger and more numerous chloroplasts, thick outer walls, tight packing around the vascular bundles or water-storage cells, and limited exposure to intercellular air spaces (Fig. 1, Supplementary Figs S1, S2; Brown, 1977; Hattersley and Watson 1992; Dengler and Nelson, 1999; Pyankov et al., 2000; Edwards and Voznesenskaya, 2011). Additionally, K cells in C₄ plants are commonly larger than the homologous cells of their more distant C₃ relatives; however, size differences between K cells and the equivalent layer in closely related C₃ relatives are often lacking (Hattersley et al., 1982; Muhaidat et al. 2007, 2011; Christin et al. 2013). Chlorenchymatous M cells between veins are reduced in number in C₄ relative to C₃ leaves, such that only one layer of photosynthetic M cells surrounds the K cells, and there is extensive wall-to-wall contact between the K and M cells (Fig. 1; Supplementary Figs S1, S2; Brown, 1977; Hattersley and Watson, 1992; Edwards and Voznesenskaya, 2011). This reduction in M cell layers minimizes the resistance to metabolite flux between the M and K cells (Bräutigam and Weber, 2011). In some Kranz types, an extra layer of cells lies between the M and K cells (Edwards and Voznesenskaya, 2011). The most common example of this occurs in grasses and sedges where an additional layer of cells with few chloroplasts separates M cells from mesophyte sheath cells where CO₂ is concentrated (Supplementary Fig. S2C; Hattersley and Watson, 1992; Soros and Dengler, 2001).

The many versions of the C₄ Kranz syndrome are also associated with distinct ultrastructural changes that are essential for C₄ function (Voznesenskaya and Gamaley, 1986; Hatch 1987; Hattersley and Watson, 1992; Dengler and Nelson, 1999; Edwards and Voznesenskaya, 2011). These include variation in organelle size, number, and position within the K cells (Dengler and Nelson, 1999; Voznesenskaya et al., 1999, 2007, 2010; 2013; Koteyava et al., 2011; Muhaidat et al., 2011; Sage et al., 2011b; Bissinger et al., 2014). Additionally, many C₄ monocots contain suberin in the wall of the K cells, a feature that is absent from the eudicots (Hattersley and Browning, 1981; Edwards and Voznesenskaya, 2011; Mertz and Brutnell, 2014). Suberin slows diffusive efflux, thus helping to trap CO₂ in the K cells (Laetsch, 1974; Mertz and Brutnell, 2014). Kranz cells with suberized walls often have centrifugally positioned organelles, whereas in leaves lacking suberized walls, the chloroplasts typically occur along the inner, centripetal region of the K cell (Supplementary Figs S1B–D; Hattersley and Watson 1992; Dengler and Nelson, 1999). The organelles of K cells are also altered to meet the different requirements of the three biochemical subtypes of C₄ photosynthesis. For example, photosystem II is depleted in the K cell chloroplasts of the NADP-ME subtypes, but not in the K-cell chloroplasts of the NAD-ME subtypes (Hattersley and Watson 1992; Dengler and Nelson, 1999; Edwards and Voznesenskaya, 2011; Furbank, 2011). In the NAD-ME subtype, the decarboxylating enzyme occurs in the mitochondria, whereas in the NADP-ME subtype, it is in the chloroplast (Hatch, 1987). As a result, K-cell mitochondria in NAD-ME species tend to be larger, more numerous, and closely associated with chloroplasts compared with NADP-ME species (Dengler and Nelson, 1999; Voznesenskaya et al., 2010; Koteyava et al., 2011; Khoshravesh et al., 2012; Oakley et al., 2014). In the M cells, C₄ plants produce about half the number of chloroplasts as their C₃ relatives, and the C₄ chloroplasts cover much less of the M cell periphery than in C₃ plants (Stata et al., 2014). This reduction in chloroplast number enhances CO₂ access to the PEP carboxylation sites in the cytosol of the C₄ M cell. Also, the cell walls between M and K cells are enriched in plasmodesmata, which increases the rate of metabolite exchange between the two cell types (Hattersley 1984; Evert et al. 1977; Botha, 1992; Bräutigam and Weber, 2011). In summary, when all features associated with Kranz anatomy are considered, it is apparent that it represents a highly sophisticated suite of structural adaptations that not only establish the necessary compartmentalization required by the C₄ carbon concentrating mechanism (CCM), but also produces the subcellular intricacy needed for efficient C₄ photosynthesis.

How Kranz anatomy evolved remains one of the great mysteries of plant biology, and has recently become a hot topic because of ongoing efforts to engineer C₄ photosynthesis into C₃ crops, and the recognition that C₄ evolution is a major
event in the formation of the modern biosphere (Edwards et al., 2010; Covshoff and Hibberd, 2012; Christin et al., 2013; Slewnski, 2013). Biologists are now in a much better position to resolve the Kranz enigma, as new developmental models and genomic tools facilitate the linkage of traits with the underlying genetic control (Covshoff et al., 2012, 2014; Williams et al., 2013; Fouracre et al., 2014). Long-standing concepts of C₄ evolution can also be examined using phylogenetically informed comparisons that include C₃-C₄ intermediate species from multiple lineages (McKown and Dengler, 2007; Muhaidat et al., 2011; Christin et al., 2013; Khoshravesh et al., 2012; Ocampo et al., 2013; Sage et al., 2013). Of particular value have been phylogenies with enough species coverage to identify close, sister taxa of C₃ and C₄ plants and numerous species with traits that are intermediate between the C₃ and C₄ conditions (McKown et al., 2005; Sage et al., 2007; Fedorova et al., 2010; Christin et al., 2011; Kaderet and Freitag, 2011; Roalson, 2011; Grass Phylogeny Working Group II, 2012; Khoshravesh et al., 2012; Freitag and Kaderet, 2013; Ocampo et al., 2013; Bissinger et al., 2014). As a result, it has been possible to propose models of C₄ evolution that postulate, and then test, the importance of intermediate steps (Monson and Rawsthorne, 2000; Sage 2004; Sage et al., 2012; Heckmann et al., 2013; Williams et al., 2013). A major aspect of these models has been the origin of Kranz anatomy.

In this review, we present a structure-function analysis addressing how Kranz anatomy may have evolved (see Fouracre et al., 2014 for developmental perspectives of the issue). We begin by describing the conceptual models of C₄ evolution proposed by Monson and Rawsthorne (Monson et al., 1984; Rawsthorne, 1992; Rawsthorne and Bauwe, 1998; Monson, 1999; Monson and Rawsthorne, 2000) as modified by Sage et al., (Sage, 2001, 2004; Sage et al., 2012). These models postulate a central role for glycine shunting in the evolution of the C₄ pathway. We also discuss the importance of photorespiration and present a case that Kranz anatomy originated as a structure to enable the trapping and recycling of photorespired CO₂. Photorespiration has been termed the “bridge to C₄ photosynthesis” because in dealing with its consequences, many of the structural features essential to C₄ photosynthesis first evolved (Bauwe, 2011). As part of this discussion, we examine recent papers evaluating the critical initial phases of C₄ evolution that occur in the C3 relatives of C₄ clades (for example, Muhaidat et al., 2011; Christin et al., 2013; Sage et al., 2013; Williams et al., 2013). These close C₃ sisters exhibit changes in organelle size, number and location that are associated with changes in the size and shape of the bundle sheath cells, such that an incipient version of Kranz anatomy is apparent. The term “proto-Kranz” has been coined to describe this condition, which may represent the initial phase of C₄ evolution (Muhaidat et al., 2011; Sage et al., 2012; 2013).

Before proceeding, we define and justify our use of certain key terms to avoid confusion with earlier uses and to have a system that allows us to delineate evolutionary transitions in anatomical forms and photosynthetic physiologies. The term C₂ photosynthesis refers to a CCM that uses a photorespiratory glycine shuttle to concentrate CO₂ into an inner, BS-like compartment from the M tissue. This physiology has commonly been called C₁-C₄ intermediacy, but “C₁-C₄ intermediate” is inappropriate as it equates one specific trait with an evolutionary process that is comprised of many transitional characteristics, not just the glycine shuttle. In addition, the glycine shuttle is found in many species having no relationship to C₄ clades, and thus are not technically C₁-C₄ intermediates (Sage et al., 2011a). Furthermore, “C₂” refers to the number of carbon atoms in the glycine molecule that transports CO₂ into the inner compartment, in the same manner that “C₄” refers to the four-carbon compound that transports CO₂ into the K cells. With respect to anatomical terminology, we restrict our definition of Kranz anatomy to those anatomical features where a wreath-like arrangement of M and BS-like cells enables a functioning CCM. This follows Haberlandt’s (1914) suggestion that the Kranz-type has a distinct functional adaptation. By this definition, C₁ taxa with enlarged BS cells that have been listed as having Kranz anatomy (see for example, Metcalfe and Chalk, 1979, pages 214–215) would not have it, whereas the anatomical specializations associated with the C₄ and C₂ CCMs would represent two versions of Kranz anatomy. For clarity, we refer to the pronounced wreath-like anatomy of C₄ species as “C₄-Kranz”, and the simplified wreath-like anatomy of C₂ species as “C₂-Kranz” (see Supplementary Fig. S2 for examples of each). "Sub-Kranz” might be a logical alternative to C₂-Kranz, but we feel this is inadequate as it implies the anatomy of C₂ species is an incomplete version of C₄-Kranz, rather than a structural adaptation in its own right that enables efficient function of the C₂ CCM. Sub-Kranz also does not connect the anatomy to the specific physiological adaptation, and can be confused with proto-Kranz, the term we use for anatomical changes in C₁ species that precede C₂-Kranz. Finally, we use the term BS (bundle sheath) cells in place of K cells because we repeatedly refer to the evolutionary transition from BS-like cells lacking a CCM to Kranz cells with a CCM, and the distinction between the two may not always be clear.

The Monson family of models for C₄ evolution

The high number of C₄ origins allow for the testing of evolutionary hypotheses using the methods of comparative biology, where each lineage represents an independent observation of one evolutionary transition (Ackerly, 1999; Christin et al., 2013). Using such approaches, the evidence from the many C₄ lineages consistently supports gradual models of C₄ origin, with a critical intermediate role for a glycine shuttle that concentrates photorespired CO₂ into a BS-like compartment (Fig. 2; Monson and Rawsthorne, 2000; Sage et al., 2012; Williams et al., 2013). The glycine shuttle CCM was first proposed by Monson et al. (1984) to explain the photosynthetic physiology of the C₁-C₄ intermediate species known at that time (Rawsthorne et al., 1988, Rawsthorne, 1992 and Monson 1999 for follow-ups). Glycine shuttling has since been identified in over 50 C₂ species from 20 or so evolutionary lineages
Fig. 2. A diagram illustrating the evolutionary progression from C₃ to C₄ photosynthesis via three distinct phases termed proto-Kranz, C₂ photosynthesis (C₂), and C₄-like photosynthesis. Arrows indicate the major changes between the phases. Note that bundle-sheath (BS) activation occurs within the C₃ group as indicated by the transition to grey shading; this refers to the increase in photosynthetic activity in the BS following an increase in organelle numbers and BS cell size. Immediately below are false-colour transmission electron micrographs showing chloroplasts (green) and mitochondria (red) for five Flaveria species that are classified as C₃, proto-Kranz, C₂, C₄-like, and C₄ plants. The bottom row illustrates carbon flow in the mesophyll (M) and BS tissues of the C₃ and proto-Kranz types, and between the M and BS in the C₂, C₄-like, and C₄ types. 2C indicates the two-carbon photorespiratory metabolite glycine; (CH₂)n refers to leaf carbohydrate. Type I refers to C₂ photosynthesis and little associated C₄ cycle; Type II refers to C₂ photosynthesis with a modest C₄ cycle. Developed from Edwards and Ku, 1987; Moore et al., 1987b; Ku et al., 1991; Monson and Rawsthorne, 2000; Muhaidat et al., 2011; and Sage et al., 2012. Bars=5 μm. Explanation: Flaveria cronquistii, F. robusta, and F. linearis demonstrate the development of BS from an expanded, activated C3 condition to the mitochondria-enriched BS of the proto-Kranz and C₂ conditions. The main difference between these proto-Kranz and C₂ species is the high expression of glycine decarboxylase (GDC) in the M cells of F. robusta, compared with low expression of GDC in the M cells of F. linearis (not shown). The shift from the C₂-Kranz to C₄-Kranz forms is accompanied by an enlargement of BS chloroplasts and a reduction in BS mitochondria in this C₄ NADP-ME lineage. With respect to carbon flow, the M and BS cells in C₃ plants operate independently, with each assimilating O₂ and CO₂ and processing the fixation products to either CO₂ (via glycine decarboxylase, GDC, in the mitochondria) or carbohydrate (via photosynthesis). The CO₂ produced by GDC can then either escape the cell or be refixed. In proto-Kranz species, the movement of mitochondria and a few chloroplasts to the inner wall of the BS cells forces the glycine formed by outer chloroplasts to migrate to the inner BS for decarboxylation, with the released CO₂ accumulating and increasing Rubisco efficiency. This represents a single-celled, BS-specific glycine shuttle. In Type I C₂ species, GDC is largely restricted to BS cells, so that photorespiratory glycine diffuses to GDC located in centripetal mitochondria. The released CO₂ accumulates and enhances Rubisco activity in the numerous chloroplasts in the inner BS. This is the two-celled photorespiratory glycine shuttle that boosts BS CO₂ levels. In the C₄-like pattern, a strong C₄ biochemical cycle moves 4C organic acids into the BS, whereas a weak two-celled glycine shuttle remains to process any glycine produced by the residual Rubisco in the M cells. In most C₄ leaves, the only way to move carbon from the M to BS cells is via the C₄ metabolic cycle involving PEP carboxylation.
Table 1. The classification of the known C$_4$-C$_3$ intermediate species into proto-Kranz, C$_3$ photosynthesis, and C$_4$-like photosynthesis

A clade of closely related C$_4$ species is also listed, if relevant. A listing of C$_3$ alone indicates the strength of C$_4$ metabolism is unknown. Compiled from Table 2 of Sage et al., 2011a; Table V in Sage et al., 1999 and references listed.

<table>
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<tr>
<th>C$_4$ clade</th>
<th>Species</th>
<th>Photosynthetic category</th>
<th>Closely related C$_4$ clade</th>
<th>Reference</th>
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<td>Flaveria A</td>
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Table 1. Continued

<table>
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<th>Closely related C₄ clade</th>
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<td>C₂ anatomy</td>
<td>Mesosetum and/or Arthropogon</td>
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Monson, Rawsthorne, and co-workers originally proposed a series of conceptual models for the evolutionary progression from C₃ to C₄ species, based on the variation observed in C₂ species of *Alternanthera*, *Flaveria*, *Mollugo*, *Moricandia*, *Neurachne*, and *Panicum/Steinchisma* (Monson and Moore, 1989; Monson, 1989, 1999; Rawsthorne, 1992; Rawsthorne and Bauwe, 1998; Monson and Rawsthorne, 2000). In recent years, numerous groups have built upon these conceptual models as data has become available from newly described C₃ to C₄ lineages (Sage, 2004; McCown and Dengler, 2007; Bauwe, 2011; Sage et al., 2012). All of these models propose the glycine shuttle-type CCM as the key intermediate step between the C₃ and C₄ conditions. For this reason, we classify these as the Monson family of C₄ evolutionary models.

Figure 2 presents a schematic of C₄ evolution that follows from Monson and Rawsthorne (2000) and a recent iteration in Sage et al. (2012). For simplicity, we present the model as a flow scheme that documents the transition from C₃ to C₄ as moving through a series of intermediate phases; these correspond to known physiological states in existing lineages, and their order is consistent with phylogenetic patterns observed in those lineages. Three distinct intermediate phases are delineated, which we term (i) proto-Kranz, (ii) C₃ photosynthesis or the photorespiratory glycolate shuttle, and (iii) C₄-like photosynthesis (Fig. 2). Four key transitions are noted. The first is BS activation, which occurs within the C₃ condition. Activation of the BS occurs when the BS cells engage in substantial photosynthetic activity owing to increases in their size and chloroplast number (Gowik and Westhoff, 2011; Sage et al., 2013). *Flaveria cronquistii* is representative of a C₃ plant with an activated BS (Fig. 2). The key transitions following BS activation are the shift in the location of mitochondria from the outer to the inner BS, the localization of glycine decarboxylase (GDC) to the BS cells, and the activation of the C₄ metabolic pump (Fig. 2). Figure 2 also indicates the transition from a Type I to Type II condition within the C₃ phase. In the Type I subphase, the glycine shuttle alone concentrates CO₂ in the BS, whereas in the Type II subphase, the glycine shuttle is accompanied by modest C₄ metabolism.

This follows the delineation of Type I and II C₃–C₄ intermediates by Edwards and Ku (1987).

The scheme in Figure 2 is conceptual and qualitative in nature. In the past year, two independent efforts have developed more complex, quantifiable models that use an adaptive landscape approach to analyse the details of the C₄ evolutionary process (Heckmann et al. 2013; Williams et al., 2013). Heckmann et al. (2013) use a theoretical photosynthesis model (von Caemmerer, 2000) to quantify a fitness landscape across which evolutionary trajectories are modelled for the following six parameters: (i) fraction of Rubisco in the M tissue, (ii) Rubisco turnover capacity, (iii) fraction of GDC activity in the BS, (iv) C₄ cycle activity, (v) the Kₘ of PEP carboxylase for bicarbonate, and (vi) the conductance of the BS for gases (Heckmann et al., 2013). In the model, these six traits were randomly altered between C₃ and C₄ values. If fitness increased following the single trait change, the trait could be fixed, and then built upon if a subsequent trait change increased fitness. This iterative process continued until the modelled phenotypes arrived at the C₄ condition for all traits. With respect to Kranz evolution, Heckmann et al. (2013) present two important findings. First, the formation of a glycine shuttle (and the C₄-Kranz anatomy that enables glycine shuttling) is the critical early phase in the biochemical evolution of C₄ photosynthesis. This theoretical result independently supports the empirical findings summarized in Fig. 2. Second, an extensive series of changes representing the biochemical evolution of the C₄ pathway largely corresponds to the “C₄ cycle activation” and “optimization” steps in Fig. 2 and thus would also correspond to the transition from C₃-Kranz to C₄-Kranz. Heckmann et al. (2013) did not directly model any specific anatomical change, but did include a number of parameters whose trait values would encompass anatomical changes. Of these, reduction in the BS conductance to CO₂ efflux is modelled to occur late, after activation of the C₄ biochemical cycle. Reduced BS conductance would largely reflect structural evolution, for example through thickening of the outer BS wall (von Caemmerer and Furban, 2003).

In Williams et al. (2013), a meta-analysis of 43 studies of C₃–C₄ intermediates was used to quantify 16 biochemical,
anatomical, and cellular traits to parameterize a phenotypic landscape. The evolution of C₄ photosynthesis across this landscape was then modelled as a transition network, and a time-ordered acquisition of traits was predicted based on the series of networks that were most compatible with the meta-analysis. For eudicots, the trait appearance predicted by the model was consistent with the qualitative scheme depicted in Fig. 2, in that changes in vein density, BS cell size, and GDC specificity occur early in C₄ evolution to establish the proto-Kranz and C₂-like conditions. Most of the biochemical changes were modelled to occur later, in what would correspond to the C₄ cycle activation and optimization phases of Fig. 2. The order of trait appearance in monocots differed, but Williams et al. (2013) had a limited set of C₃-C₄ intermediate grasses to parameterize the model, and so these predictions are tentative.

At this point, we evaluate the empirical and theoretical evidence for the structural changes that are thought to have occurred during C₄ evolution. We begin by discussing the anatomical traits in C₃ plants that may have enabled the initiation of C₄ evolution.

Setting the stage—the rise of anatomical enablers in the C₃ flora

Although C₄ photosynthesis evolved in taxonomic groups scattered throughout the angiosperm phylogeny, it tends to cluster in three major clades: the grasses (22–24 independent origins; GWPGII, 2012), the Caryophyllales (23 independent origins; Sage et al., 2011a), and the sedges (six independent origins; Besnard et al., 2009). As striking as this clustering is, the complete absence of C₄ photosynthesis in diverse and adaptable lineages such as the large orders containing the legumes, roses, lilies, and orchids is also noteworthy. Many of these groups are common in the same habits as C₄ species and numerous genera within these orders have evolved CAM, indicating photosynthetic flexibility (Smith and Winter, 1996; Sage, 2002). These patterns suggest there may be a series of predisposing traits that facilitate C₄ evolution in some taxa, whereas the lack of such traits may preclude C₄ evolution in other taxa. Preconditioning traits in C₃ species that may promote diversification of Kranz tissues include high vein density and enlarged BS cells (Sage, 2004; Muhaïdat et al., 2011; Sage et al., 2012; Christin et al., 2013; Griffiths et al., 2013).

The identification of most lineages of C₃ photosynthesis has allowed for an evaluation of the environmental conditions where the C₄ pathway arose. In the eudicots, centres of origin have been postulated for 35 of the 36 known clades and it is possible to identify centres of origin for a handful of grass lineages (Sage et al., 2011a; Christin et al., 2012). The centres of origin generally correspond to hot, monsoon-affected regions, or non-monsoon areas where there is sufficient soil moisture to support summer photosynthesis. Most areas also correspond to where periodic aridity and low atmospheric humidity promote recurring episodes of water and/or salinity stress. Salinity seems to be a particularly important driver for C₄ evolution in the Chenopodiaceae lines of central Asia (Djamali et al., 2012; Kadereit et al., 2012; Sage et al., 2011a). These conditions maximize the potential for photorespiration, but also create high evapotranspiration potentials that could lead to hydraulic crisis in leaves should the stomata remain open, or restrict carbon gain should stomata close to conserve water (Osborne and Sack, 2012; Sage, 2013). The key role of the monsoons is that they supply moisture during the summer season, creating moist growing conditions in what is often a hot, low humidity setting (Sage et al., 2012). In these conditions, high vein density in leaves is proposed to be adaptive because it can deliver water fast enough to maintain tissue water status and prevent premature stomatal closure (Sage, 2001, 2004; Osborne and Sack, 2012). Consistently, C₃ species from hot, semi-arid regions often have greater vein density (Roth-Nebelsick et al., 2001). High vein density seems to be particularly common in the C₃ sister clades of many C₄ lineages (Osborne and Sack, 2012; Sage et al., 2012; Griffiths et al., 2013). For example, vein density approaches or equals C₄ values in C₃ sister groups of Anticharis (Khoshravesh et al., 2012), Cleome (Marshall et al., 2007; Voznesenskaya et al., 2007), Heliotropium (Muhaïdat et al., 2011), Euphorbia (Sage et al., 2011b), Mollugo (Christin et al., 2011), and Salsola (Voznesenskaya et al., 2013). In a survey of species from 54 related C₃ and C₄ species from 13 eudicot families, no statistical difference was observed in vein density between the means of the C₃ and C₄ groups (Muhaïdat et al., 2007). Many C₃ grasses that are sister to the C₄ grass clades also have reduced interveinal distance, reflecting elevated vein density (Christin et al., 2013).

Large BS size is also considered an important anatomical feature that may be more common in dry environments. Larger BS cells are posited to improve hydraulic capacitance in leaves and thus buffer surges in transpiration caused by wind gusts (Sage, 2004; Griffiths et al., 2013). As with increased vein density, increased BS size is commonly found in the C₃ relatives of C₄ clades. Most of the C₃ sister groups of C₄ lineages in the PACMAD clade of grasses have increased BS volumes relative to M volumes (Brown et al., 1983; Christin et al., 2012, 2013; Griffiths et al., 2013). In Flaveria and related genera, enlarged BS cells are present in the C₃ species F. cronquistii (Fig. 2) and its sister genus Sartwellia (McKown and Dengler, 2007; Sage et al., 2013). Large BS cells are also present in a C₃ Euphorbia that is sister to the C₂ clade in Euphorbia section acutae (Sage et al., 2011b). In Heliotropium, the closest C₃ relatives to the C₄ clades have enlarged BS cells relative to less-related C₃ species (Muhaïdat et al., 2011). In Atriplex, the C₃ and C₄ species that are related enough to form fertile hybrids also have similar BS cell size (Oakley et al., 2014). In the Muhaïdat et al. (2007) comparison of 54 C₃ and C₄ species from 13 dicot families, C₃ species had on average smaller BS cell size than C₄ relatives. Understanding how increased vein density and BS size facilitates Kranz evolution requires close examination of BS ultrastructure and morphology. In C₃ species that branch in sister positions to C₂ or C₄ species in the Anticharis, Cleome, Euphorbia, Flaveria, and Heliotropium phylogenies, enlarged BS cells protrude into the mesophyll, forming...
spongy-parenchyma-like cells (Fig. 2; Marshall et al., 2007 for Cleome; Muhaidat et al., 2011 for Heliotropium; Sage et al., 2011b for Euphorbia; Sage et al., 2013 for Flaveria). The BS cell protrusions enhance exposure to the intercellular air space (IAS), thereby allowing many chloroplasts to be positioned along the perimeter of the BS cell that faces the IAS, as shown for F. cronquistii (Fig. 2). Notably, as observed in C₃ species of Euphorbia, Flaveria and Heliotropium, the BS chloroplasts facing the IAS occur near mitochondria, in an identical arrangement as that in M cells (Muhaidat et al., 2011; Sage et al., 2011b; Sage et al., 2013). This close arrangement of mitochondria and chloroplasts facilitates rapid flux of metabolites between the two organelles during photosynthetic metabolism (Busch et al., 2013). Enhancement of chloroplast numbers against the IAS wall of the BS cells is strong evidence that the BS has become heavily engaged in photosynthetic carbon assimilation, or following the terminology of Gowik and Westhoff (2011), the BS has become “activated”. Photosynthetic activation of the BS is suggested to compensate for the loss of M cell volume as veins become more abundant in the leaf and BS cells expand (Sage et al., 2012; 2013). Its significance for C₄ evolution is that the BS cells begin to generate large amounts of photosynthetic CO₂ in hot climates of low atmospheric CO₂. This formation of photorespired CO₂ represents an opportunity for improved carbon gain if the CO₂ production can be localized to a BS region where it can be trapped and refixed. As discussed next, the evolution of CO₂ trapping and refixation is proposed to give rise to the proto-Kranz condition and the first definitive step in C₄ evolution.

The proto-Kranz condition

As first described for two Heliotropium species (H. procumbens and H. karwinskyi), the proto-Kranz condition consists of enlarged BS cells relative to a typical C₃ Heliotropium species, and the BS tissue is more circular in outline than in the uneven edge produced by enlarged, spongy-like BS cells of the sister C₃ species H. tenellum (Muhaidat et al., 2011). The more uniform BS outline in the proto-Kranz species is associated with a reduction in the exposure of the BS cells to the IAS. Mitochondria in the BS are larger in the two proto-Kranz Heliotropium species, and in H. procumbens, there are double the number of mitochondria relative to the C₃ sister species. Of particular note is the localization of 82–97% of the mitochondria to the inner wall of the BS in H. karwinskyi and H. procumbens, respectively (Muhaidat et al., 2011). In H. tenellum, by contrast, mitochondria are spread around the cell periphery. The localization of mitochondria to the centripetal pole of the BS cells produced a distinct band of immunolocalization stain for glycine decarboxylase (GDC) along the inner edge of the BS, in a pattern that is similar to, though less intense, than that of C₂ species (Fig. 3A–C). Unlike the C₂ species, GDC is still common in the M cells of proto-Kranz species.

Muhaidat et al. (2011) drew attention to proto-Kranz anatomy described by Brown et al., (1983) in the Steinchisma clade of grasses. Steinchisma laxa (formerly Panicum laxum) is sister to a C₂ clade of Steinchisma, whereas Panicum hylaeicum is close to the Steinchisma clade (Aliscioni et al., 2003). Both species have C₃ gas exchange characteristics and BS cells that are of similar size as the C₂ species of Steinchisma; however, the BS cells exhibit over 3-fold more BS chloroplasts, and S. laxa has 8-fold more mitochondria than a typical C₃, Panicum species (Morgan and Brown, 1980; Brown et al., 1983). Nearly all of the BS mitochondria in S. laxa are arrayed along the inner BS wall where it contacts the vascular tissue. In S. laxa, there is also a layer of chloroplasts adjacent to the layer of mitochondria as is widely observed in C₃ species (Brown et al., 1983). Curiously, in both S. laxa and P. hylaeicum, many of the chloroplasts encapsulate the mitochondria (Brown et al. 1983), a feature that has been linked to increased refixation of photorespired CO₂ (Busch et al., 2013).

In addition to Heliotropium and Panicum/Steinchisma, the proto-Kranz condition has recently been recognized in Salsola montana of the Chenopodiaceae (Voznesenskaya et al., 2013) and two C₃ Flaveria species in the sunflower family, F. pringlei and F. robusta (Sage et al., 2013). In S. montana, the leaf has a “symplemoid” anatomy formed by concentric layers of mesophyll cells surrounding a central core of succulent water storage tissue and veins (Voznesenskaya et al., 2013). Bundle-sheath cells occur beside the veins at the edge of the water storage tissue. Mitochondria in S. montana are localized to the wall against the vascular tissue in these BS cells, leading to its designation as a proto-Kranz species (Voznesenskaya et al., 2013).

In Flaveria, detailed examination of the species branching at the basal nodes of the phylogeny has identified two species previously categorized as C₃ (F. pringlei and F. robusta) as having proto-Kranz features similar to those observed in Heliotropium (Sage et al., 2013). In the Flaveria phylogeny, F. pringlei and F. robusta branch at nodes between the C₃ F. cronquistii node and the nodes for the C₃ species F. sonorensis and F. angustifolia (McKown et al., 2005). In the basal branching F. cronquistii and its sister species Sartwellia flaveriinae, BS cells are enlarged with elongated spongy-like protrusions, whereas in F. pringlei and F. robusta, the BS cells have coalesced into a more even edged-sheath with low exposure to the IAS (Sage et al., 2013). Bundle-sheath cells in F. pringlei and F. robusta are enlarged compared with a typical C₃ species such as sunflower but are actually smaller than their immediate C₃ sister species that branch lower in the phylogeny (McKown and Dengler, 2007; Sage et al., 2013). This is due to the loss of the lobing observed in the expansive BS cells of F. cronquistii. In F. pringlei and F. robusta, organelle numbers are enhanced, and 75% or more of the BS mitochondria are centripetally located, which approximates the mitochondria distribution in the BS of C₃ species (Fig. 2; Sage et al., 2013). Both F. pringlei and F. robusta have a distinct band of GDC stain along the inner BS wall, yet both also express GDC in the M mitochondria (Fig. 3D–F). This GDC distribution is similar to what has been reported for the proto-Kranz species in the genus Heliotropium (Fig. 3; Muhaidat et al., 2011). In F. robusta, numerous chloroplasts also occur beside the
centripetal BS mitochondria in a pattern similar to that of many C₃ species (Figs 2, 4; Sage et al., 2013).

The observation that C₃ proto-Kranz species are closely related to C₂ species in four independent lineages supports a hypothesis that the proto-Kranz condition is an early phase of C₂ evolution, and in turn, C₄ evolution. If this is the case, it is logical to assume there is an adaptive benefit conferred by the proto-Kranz traits. Photosynthetic gas exchange responses demonstrate a subtle reduction of the CO₂ compensation point of photosynthesis (Γ), which could provide slight yet biologically meaningful enhancements in carbon gain at low intercellular CO₂ levels. In S. laxa, the gas exchange benefits are a 5–7 μmol CO₂ mol⁻¹ air reduction in Γ across a range of O₂ levels, and increased sensitivity of Γ to variation in light intensity (Morgan and Brown, 1980; Sage et al., 2013). In H. procumbens and H. karwinskyi, the benefit may be a slight reduction in the slope of the Γ versus O₂ response (Vogan et al., 2007). In Flaveria pringlei and F. robusta, Γ is also reduced by 5–10 μmol mol⁻¹ relative to the C₃ F. cronquistii and Sartwellia flaveriae (Sage et al., 2013); however, no consistent gas exchange differences were observed in Salsola montana relative to its C₃ relatives (Voznesenskaya et al., 2013). In F. robusta, Γ also exhibited greater light dependency than C₃ species (Sage et al., 2013). Large reductions in Γ with an increase in light intensity indicate a C₂-type of CCM may be active in higher plants (Sage et al., 2013). The greater light dependency in F. robusta was associated with a shift to lower intercellular CO₂ values (Cᵢ) of the relationship between net CO₂ assimilation rate (A) and Cᵢ, which is not observed in the C₃ species, but is pronounced.

**Fig. 3.** In situ immunolocalization of GDC p-protein subunit in the leaves of C₃ (A, D), proto-Kranz (B, E), and C₂ (C, F) species in the genus Heliotropium (A–C) and Flaveria (D, E). Hete, H. tenellum; Hepr, H. procumbens; Heco, H. convolvulaceum; Flcr, F. cronquistii; Flro, F. robusta; Flra, F. ramosissima. Brown stain indicates the presence of GDC. Asterisks indicate bundle sheath cells and arrows point out bundle sheath mitochondria. V indicates vascular tissue. Bars=50 μm.
in C$_2$ species. This was interpreted by Sage et al., (2013) to indicate that F. robusta operates has a weak glycine shuttle which allows it to refix proportionally more photorespired CO$_2$ than C$_3$ species, although much less than in C$_2$ species. This refixation mechanism may represent the adaptive benefit of the proto-Kranz suite of traits.

The refixation mechanism for photorespiratory CO$_2$ in the proto-Kranz species may be nothing more than a single-cell glycine shuttle within the BS that arises when mitochondria become localized to the inner BS wall (Fig. 2). Numerous chloroplasts remain against the outer perimeter of the cell facing the IAS, and thus are presumably assimilating CO$_2$ and oxygenating RuBP. If the mitochondria have moved from a position near an outer chloroplast to the inner edge of the cell, any glycine formed during photorespiration would build up around the outer chloroplasts and diffuse to where GDC
is localized, which in the proto-Kranz species would be in the rank of mitochondria along the centripetal wall of the BS cells (Sage et al., 2013). The diffusion barrier presented by the tonoplast and vacuole would then restrict the efflux of the photorespired CO₂, causing it to accumulate and enhance photosynthesis within chloroplasts in the inner BS. It is also possible that the BS mitochondria receive glycine from the M tissue, which may happen in more than a trivial manner if photorespiration in the M cells exceeds the GDC capacity in the resident mitochondria. This could occur if the GDC capacity in M cells lags behind Rubisco oxygenase activity, which may happen in very hot climates during low CO₂ episodes, or if M GDC expression is reduced in proto-Kranz species. In either case, the increased number of BS mitochondria in proto-Kranz species indicates there is a rise in BS GDC capacity that might draw M glycine into the BS (Sage et al., 2013). This would become an important source of CO₂ for the BS cells of the proto-Kranz species, and could establish conditions where additional reductions in M GDC allow for greater glycine flux to the BS, further enhancing carbon gain and thus facilitating positive selection for a stronger glycine shuttle (Sage et al., 2012; Heckmann et al., 2013).

The potential significance of the proto-Kranz traits is only recently recognized, and few species have been examined for this condition. It is possible that many species from hot climates have enlarged, activated bundle sheaths with a centripetal wall of the BS (Brown and Hattersley, 1989; Monson and Rawsthorne, 2000; Christin et al., 2011; Muhaidat et al., 2011; Sage et al., 2011b, 2012, 2013; Ueno et al. 2003; 2007; Voznesenskaya et al., 2007, 2010, 2013). Glycine decarboxylase is abundant in these mitochondria, such that a dense band of immunolabel against GDC is observed along the inner BS of C₄ species in immunolocalization experiments (Fig. 3; Hylton et al., 1988; Rawsthorne et al., 1988; Ueno et al. 2006; Voznesenskaya et al., 2007; Muhaidat et al., 2011; Sage et al., 2011b, 2013). In the C₃ species with an inner rank of mitochondria, few if any mitochondria are present along the outer periphery of the cell (Muhaidat et al., 2011; Sage et al., 2011b, 2013; Voznesenskaya et al., 2007, 2013). This polarity in mitochondrial placement is one of the distinguishing features in most of the C₂ species examined. The chloroplasts in the inner BS typically line up next to the rank of mitochondria (Figs 2, 4; Monson and Rawsthorne, 2000; Sage et al., 2012, 2013). Rubisco and starch are abundant in these chloroplasts, and it is generally assumed they are photosynthetically engaged (Hylton et al., 1988; Rawsthorne, 1992; Voznesenskaya et al., 2001; Muhaidat et al., 2011; Sage et al., 2013). In many but not all cases, the chloroplasts are abundant enough to form a near-continuous layer between the mitochondria and the vacuole (Holaday et al., 1984; Brown and Hattersley, 1989; Muhaidat et al., 2011; Sage et al., 2013). This arrangement is particularly effective in enhancing the probability of photospired CO₂ being captured and refixed by the inner chloroplasts before it can escape the cell (Rawsthorne, 1992). Many C₃ species also have chloroplasts along the outer BS periphery, but these are not associated with mitochondria as they are in C₄ species (Voznesenskaya et al., 2007; 2010; 2013; Muhaidat et al., 2011; Sage et al., 2011b; Sage et al., 2013). Hence, any photorespiratory metabolites produced by these chloroplasts will have to diffuse into the inner BS for metabolism.

Anatomically, the BS cells of C₄ species are generally more pronounced in leaf cross sections than they are in C₃ species, reflecting in most cases a larger BS cell size (Supplementary Fig. S2; McKown and Dengler 2007; Muhaidat et al., 2011; Sage et al., 2011b, 2013). With the pronounced clump of organelles lining the inner BS, the BS cells of C₄ species exhibit a modest wreath-like appearance that is apparent in cross sections and veins of leaf clearings, including leaves reconstituted from herbarium specimens (Supplementary Fig. S2; McKown and Dengler, 2007; Muhaidat et al., 2011; Sage et al., 2011b; Christin et al., 2011; Khosh ravesh et al., 2012). This characteristic allows for relatively rapid screens for possible new C₄ species using herbarium material, which is important, as living C₃ plants are often unavailable. Carbon isotope screens do not generally identify C₂ species (Sage et al., 2007). C₄ BS cells are rarely as distinctive as C₄ Kranz cells, which are more prominent owing to a larger and denser organelle mass (Figs 2, 4; supplementary Fig. S2).
In most C₃ species, the M tissue is reduced in prominence compared with C₄ relatives, as a result of larger BS cells and in many cases, greater vein density (Supplementary Fig. S2; Ueno et al. 2006; McKown and Dengler, 2007; Muhaita et al., 2011). However, unlike the case with C₃ Kranz, multiple layers of M cells remain around the BS cells and M:BS cell ratios rarely approach the low values observed in C₄ plants (McKown and Dengler, 2007, 2009; Voznesenskaya et al., 2007, 2010; 2013). The ultrastructure of M cells of C₂ species changes little from their C₃ counterparts. C₂ and C₃ M cells of related plants have similar chloroplast numbers, distribution, and size (Stata et al., 2014). GDC is often absent in the M cells, leading to the proposal that activation of the C₂ pathway follows a mutation that knocks out GDC expression in the M mitochondria (Monson and Rawsthorne 2000; Sage et al., 2012). In Cleome, Euphorbia, Heliotropium, Mollugo, and Portulaca, there is no evidence for GDC expression in the M mitochondria of C₂ species (Marshall et al., 2007; Muhaita et al., 2011; Sage et al., 2011b; Voznesenskaya et al., 2007, 2010). In Flaveria, however, the loss of GDC is gradual. C₂ Flaveria species branching lower in the phylogeny still express GDC protein and mRNA in the M mitochondria, whereas C₂ and C₄ Flaveria species branching in a more distal position have negligible GDC expression in M cells (Sage et al., 2013; Schulze et al., 2013).

The three exceptions to the typical C₂ Kranz anatomy described above are the grass Neurachne minor, two Salsola species of the Chenopodiaceae (Sa. arbusculiformis and Sa. divaricata) and the chenopod Sedobassia sedoides. In N. minor, the cells of an inner sheath that sit just inside a relatively empty outer sheath are co-opted to be the site of CO₂ concentration, and presumably, GDC localization (Fig. 4; Supplementary Fig. S2B; Hattersley et al., 1986; Brown and Hattersley, 1989). In these cells, chloroplast and mitochondrial density are high, but there is no apparent pattern in their distribution (Fig. 4D). It is likely that the wall of the inner sheath, and the outer sheath layer of cells, provide a strong barrier to leakage of photorespired CO₂ such that the organelles need not be localized next to each other along the inner cell wall (Brown and Hattersley, 1989). In many succulent chenopods, the leaf anatomy is comprised of multiple layers of M cells around a central core of water storage cells (Voznesenskaya et al., 2001, 2013). The inner M cell layer is co-opted as the site of CO₂ concentration in the C₂ chenopods and the C₄ species that have Salsaloid- and Kochioid-types of Kranz anatomy (Edwards and Voznesenskaya, 2011; Voznesenskaya et al., 2001, 2013). In the C₂ species Salsola arbusculiformis, Sa. divaricata, and Sa. sedoides, the inner Kranz-like layer where GDC is localized occurs adjacent to vascular cells along the periphery of the water storage cells, in what is interpreted to be an intermediate version of the Salsoloid Kranz anatomy (Voznesenskaya et al., 2013; Freitag and Kadereit, 2013). In Sa. arbusculiformis and Sa. divaricata, the BS mitochondria and chloroplasts are very abundant, occupying over a third of the cell-volume (Voznesenskaya et al., 2001, 2013). In Sa. sedoides, many mitochondria line the BS walls adjacent to vascular tissue and other BS cells, but avoid the outer BS facing the intercellular air space (Fig. 4E, F).

The transition from proto-Kranz to C₂-Kranz can be inferred in Flaveria, Heliotropium and Steinchisma, as indicated by the sister position of the proto-Kranz and C₂ species in their respective phylogenies. In Salsola, the proto-Kranz species Sa. montana is present on a close, yet separate branch of the phylogeny than the nearest known C₂ species (Voznesenskaya et al., 2013). In Flaveria and Heliotropium, the changes are similar, being characterized by further enlargement of the BS tissue and increases in organelle size and number, resulting in more chloroplasts associating with the inner mitochondria (McKown and Dengler, 2007; Muhaita et al., 2011; Sage et al., 2013). A reduction or loss of GDC expression is apparent in the M tissue, which when coupled with the larger and more numerous BS mitochondria, explains the reduction in the CO₂ compensation point to values that are half of the C₂ values (Muhaita et al., 2011; Sage et al., 2013). In Steinchisma, the principal change between the proto-Kranz and C₂ species is an increase in chloroplast number and size in the inner BS region. Compared with the proto-Kranz S. laxa, the BS of the C₂ species St. spathellosum (formerly Panicum schenckii) has twice the number of mitochondria, 25% more chloroplasts, and three times as many peroxisomes; however, cell size does not differ (Brown et al., 1983). In considering these examples, it is apparent that the formation of C₂ Kranz from the proto-Kranz condition requires multiple genetic changes and cannot be attributed to a single cause, such as GDC loss in the M tissue. GDC decline in M cells may be an important facilitator of the transition from proto-Kranz to C₂ Kranz, because it may establish a two-tissue glycine shuttle, with subsequent evolutionary selection creating the similar Kranz-like traits that occur repeatedly in the C₂ lineages (Sage et al., 2012; Heckmann et al., 2013; Schulze et al., 2013). In any case, the multiple convergence on C₂ Kranz is strong evidence that this anatomy is specifically adapted for the C₂ pathway, in the same vein that C₄ Kranz is an adaptation for C₄ photosynthesis.

Within the C₂ condition, the major change is the shift from the Type I condition of C₄ photosynthesis only, to the Type II condition of C₂ photosynthesis and an accessory C₄ metabolic cycle (Edwards and Ku, 1987). In Type II species, the C₄ metabolic cycle can account for up to 50% percent of the initial carboxylation capacity, and reduce the CO₂ compensation point of photosynthesis to below 10 μmol mol⁻¹ (Moore et al., 1987b; Monson et al., 1988; Monson and Rawsthorne, 2000). However, there are no major structural changes associated with the transition from the Type I to Type II modes, and the C₂ Kranz type are similar in Type I and Type II forms, as indicated by studies with Flaveria and Mollugo (Kennedy et al., 1980; Holaday et al, 1984; Edwards and Ku, 1987; Monson and Rawsthorne, 2000).

**Kranz anatomy in C₄-like plants**

The C₄-like condition is only confirmed in three species, all of which occur in Flaveria (F. brownii, F. palmerii, and F. vaginata; Monson et al., 1987; Moore et al., 1989; Ku et al., 1991); however, isotopic and anatomical evidence from herbarium specimens indicate additional C₄-like species exist in Blepharis.
(Acanthaceae; McDade, Sage, and Sage, unpublished) and Anticharis (Spergulariaceae; Khoshravesh et al., 2012). Although the diversity of known C₄-like species limits the ability to make broad inferences, the C₄-like species of Flaveria are well studied and thus, for Flaveria at least, provide a detailed observation of the late stages of C₄ evolution. Flaveria palmerii and F. vaginata are in clade A where they are sister to the full C₄ species of Flaveria; F. brownii occurs on a distinct phylogenetic branch, termed clade B and lacks immediate C₄ relatives (McKown et al., 2005). On both clades A and B, Type II C₄ species branch just below the C₄ like species, indicating the C₄-like species arose from type II C₃ ancestors (McKown et al., 2005).

The transition from Type II C₄ to species to the C₄-like condition is marked by a dramatic rise in the activity of the C₄ cycle enzymes, increased water- and Rubisco-use efficiency of photosynthesis, and a large reduction in Rubisco and C₃ cycle activity in the M cells (Fig. 2; Moore et al., 1987b; Ku et al., 1991; Dai et al., 1996; Monson and Rawsthorne, 2000; Kocacinar et al., 2008; Vogan and Sage, 2011). PEPC and NADP-ME activities, for example, are 5-fold higher in the C₄-like species than the C₃ species and are similar to the C₄ values (Ku et al., 1991). Also, in the C₄-like species, the percentage of ¹⁴C label present in aspartate and malate exceeds 67%, compared with 46% in the highest Type II C₃ species (Moore et al., 1987b) and CO₂ compensation points drop to within a few μmol mol⁻¹ of C₄ values (Ku et al., 1991; Dai et al., 1996). These results demonstrate a strong enhancement of the C₄ metabolic cycle and corresponding reduction in the M C₃ cycle in what is considered to be the activation of the C₄ pathway (Sage et al., 2012). C₄-like plants are not considered to be fully developed C₄ species because the localization of Rubisco into the BS is incomplete, the oxygen inhibition of photosynthesis is halfway between C₃ and C₄ values, and the carbon isotope ratios are below the C₄ range, although they are higher than in most C₃ species (Monson et al., 1988; Ku et al., 1991; Monson and Rawsthorne, 2000). In addition, PEPC, Rubisco and carbonic anhydrase may not have fully evolved the kinetic and regulatory properties of the isozymes in their close C₄ relatives (Engelmann et al., 2003; Ludwig, 2011; Ludwig, 2013). The acquisition of these final set of characteristics that distinguish an efficient, fully expressed C₄ pathway occur in a final phase of C₄ evolution termed the optimization phase (Sage et al., 2012).

Structurally, the Kranz anatomy of the C₄-like phase is best described in F. brownii. In F. palmerii and F. vaginata, the structural features are largely C₄ in nature and don’t reveal much about the final phase before the acquisition of full C₄ Kranz anatomy (McKown and Dengler, 2007; Rahman, Sage, and Sage, unpublished). Flaveria brownii retains vestiges of the C₃ condition. The fraction of BS tissue in the leaf is also less in F. brownii than in C₄ leaves (Araus et al., 1999). The bundle sheath ultrastructure of F. brownii is intermediate between Type II C₂ species and C₄ species: chloroplasts are larger and more numerous in F. brownii than in Type II species, but much shorter than in the C₄ Flaveria species such as F. trimervia (Fig. 2; Holaday et al., 1984; Araus et al., 1990). In F. brownii, many BS mitochondria occur along the interior of the centripetal wall of the BS cells, similar to the pattern observed in C₃ species, but markedly different than in F. trimervia, where mitochondria do not form distinct ranks along the inner BS periphery (Fig. 2; Holaday et al., 1984; Brown and Hattersley, 1989; Araus et al., 1990). As with the biochemical and physiological responses, it is safe to conclude that the Kranz anatomy of F. brownii is intermediate between the C₂ and C₄ Kranz modes.

Conclusion

Over the past 40 years, studies with species that exhibit intermediate traits support models that the evolution of C₄ photosynthesis is a staged affair, with the two-celled, photorespiratory glycine shuttle being a critical intermediate step. We propose here that there were four important events that facilitated the evolution of C₄ photosynthesis (Fig. 2). The first is the activation of the C₃ BS, such that in hot environments, high vein density and BS cells become photosynthetically engaged through greater exposure to intercellular air spaces and the acquisition of increased numbers of chloroplasts and mitochondria. These cells are completely C₃, but their enhanced physiological activity enables the formation of weak mechanisms to scavenge photorespiratory CO₂ via the second key event, the migration of mitochondria to the centripetal region of the BS cells. With the reorientation of some chloroplasts to the inner wall to join the mitochondria the proto-Kranz condition arises, and with it, the potential to establish facilitation cascades where the M GDC is reduced while BS GDC and organelle numbers sequentially increase, activating the C₂ photorespiratory CCM. This reduction in M GDC represents the third major event in C₄ evolution. In time, key components of the C₄ metabolic cycle are up-regulated to complement C₃ photosynthesis, and this is proposed to facilitate the next major event, the activation of the full C₄ pathway with the coincident decline of the C₃ pathway in M tissues. Once the C₄-like condition is achieved, evolutionary optimization adjusts many of the photosynthetic components to efficiently operate in the C₄ context, leading to fully developed C₄ photosynthesis. Many C₃–C₄ species from 20 or so lineages support various aspects of this model, although our understanding of the initial and end phases are heavily dependent upon just a few species from a couple of lineages. These handful of species may skew our impression of early and late events, and thus it is important to use phylogenetic analyses to identify and collect addition proto-Kranz and C₄-like species. By doing so, the amount of information available to parameterize models that can analyse and predict trajectories of C₄ evolution will substantially increase.

Supplementary data

Supplementary data are available at JXB online

Figure S1. Transverse sections showing the leaf anatomy of three C₄ species and a C₃ species from the PACMAD clade of grasses, and C₃ and C₄ species from three C₄ lineages of the Chenopodiaceae.
Figure S2. Transverse sections illustrating leaf anatomy of closely related C₄, C₃, and C₂ species from six lineages of C₄ photosynthesis and a Canadian International Development Agency (CIDA) GCIAR-Canada linkage fund grant to TLS and RFS.

Acknowledgements

The authors thank Kate Sault, Matt Staß, Stefanie Sultmanis, and Troy Rennie for technical assistance. This research was supported by grants from the Natural Science and Engineering Research Council of Canada (grant nos. 155258–2008 to TLS and 154273–2012 to RFS) and a Canadian International Development Agency (CIDA) GCIAR-Canada linkage fund grant to TLS and RFS.

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