Evolution of C₄ plants: a new hypothesis for an interaction of CO₂ and water relations mediated by plant hydraulics

Colin P. Osborne¹,* and Lawren Sack²

¹Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK
²UCLA Ecology and Evolutionary Biology, 621 Charles E. Young Drive South, Box 951606, Los Angeles, CA 90095-1606, USA

C₄ photosynthesis has evolved more than 60 times as a carbon-concentrating mechanism to augment the ancestral C₃ photosynthetic pathway. The rate and the efficiency of photosynthesis are greater in the C₄ than C₃ type under atmospheric CO₂ depletion, high light and temperature, suggesting these factors as important selective agents. This hypothesis is consistent with comparative analyses of grasses, which indicate repeated evolutionary transitions from shaded forest to open habitats. However, such environmental transitions also impact strongly on plant–water relations. We hypothesize that excessive demand for water transport associated with low CO₂, high light and temperature would have selected for C₄ photosynthesis not only to increase the efficiency and rate of photosynthesis, but also as a water-conserving mechanism. Our proposal is supported by evidence from the literature and physiological models. The C₄ pathway allows high rates of photosynthesis at low stomatal conductance, even given low atmospheric CO₂. The resultant decrease in transpiration protects the hydraulic system, allowing stomata to remain open and photosynthesis to be sustained for longer under drying atmospheric and soil conditions. The evolution of C₄ photosynthesis therefore simultaneously improved plant carbon and water relations, conferring strong benefits as atmospheric CO₂ declined and ecological demand for water rose.

Keywords: C₄ photosynthesis; C₃ photosynthesis; atmospheric CO₂; plant evolution; drought; hydraulics

1. PHOTOSYNTHETIC CONSERVATISM AND DIVERSITY

Photosynthesis has evolved only once, and every photoautotrophic organism on Earth uses the same ‘C₃ pathway’ [1]. This biochemical cycle employs the enzyme Rubisco to fix CO₂ into a five-carbon acceptor molecule, producing three-carbon organic acids, upon which ATP and NADPH produced from the light reactions are deployed to generate sugars and to regenerate the acceptor molecule. The pigments and proteins involved in C₃ photosynthesis are highly conserved across photosynthetic organisms, and this pathway operates unmodified in the majority of species, termed ‘C₃ plants’. However, the basic C₃ pathway has also been augmented by carbon-concentrating mechanisms (CCMs) in multiple lineages, many of which evolved during the Early Neogene following a massive depletion of atmospheric CO₂ [2-5].

‘C₄ photosynthesis’ is a collective term for CCMs which initially fix carbon into four-carbon organic acids via the enzyme phosphoenolpyruvate carboxylase (PEPC) [6], and then liberate CO₂ from these C₄ acids to feed the C₃ pathway within a compartment of the cell or leaf [7,8] (figure 1a). The compartment is isolated from the atmosphere and resists CO₂-leakage, so that CO₂ is enriched at the active site of Rubisco [12] (figure 1a). Coordination of C₄ and C₃ biochemical pathways requires complex changes to metabolism, and the compartmentation of these pathways usually relies on a specialized leaf anatomy (figure 1b). C₄ photosynthesis is therefore a complex trait based on the transcriptional regulation of hundreds of genes [13], coupled with post-transcriptional regulation [14] and the adaptation of protein-coding sequences [15].

Despite the complexity of C₄ photosynthesis, it has been recorded in more than 60 plant lineages [16]. This striking evolutionary convergence probably arises because the pathway is constructed from numerous pre-existing gene networks [17], and altered levels and patterns of expression of enzymes that are already present in C₃ leaves [18–20]. Many of the known C₄ lineages occur in clusters, suggesting that they share early steps on the evolutionary trajectory towards C₄ photosynthesis, taking an independent path only during later stages of the process [21].

In recent years, evidence from genomics, molecular genetics, physiology, ecology, biogeography,
We begin by reviewing the well-established current hypothesis that atmospheric CO$_2$ depletion, high temperatures and open environments selected for the 
C$_4$ pathway as a means of directly improving photosynthetic rate and efficiency. Our main objective is to introduce a new dimension to these ideas, by proposing that these
same environmental conditions—CO$_2$ depletion, high temperatures and open environments—as well as seasonal drought, would also select for 
C$_4$ photosynthesis for an additional reason; i.e. to compensate for the strain on the plant hydraulic system resulting from excessive demand for water transport. Our hypothesis is consistent 
with recent comparative analyses of ecological niche evolution and plant physiology, which suggest important effects of the 
C$_4$ pathway on plant–water relations. We support our proposal with evidence from the literature, and mechanistic models that integrate the latest understanding of how hydraulics, stomata and photosynthesis are coordinated within leaves. Our central focus is on grasses, but we complement our analysis with additional evidence from eudicots.

2. ENVIRONMENTAL SELECTION ON PHOTOSYNTHETIC EFFICIENCY

Atmospheric CO$_2$ depletion has long been advocated as the primary selection pressure for 
C$_4$ CCMs, through its differential effects on the efficiency of 
C$_3$ and 
C$_4$ photosynthesis [22]. This difference arises because the 
active site of Rubisco is unable to discriminate completely between CO$_2$ and O$_2$, and catalyses the fixation of both molecules [23,24]. The oxygenation reaction generates toxic intermediates that must be metabolized 
via photorespiration to render them harmless and to recover carbon. Oxygenation renders photosynthesis less efficient because it competes directly with carboxylation (CO$_2$-fixation), and because photorespiration consumes the products of photochemistry and liberates CO$_2$. In a 
C$_3$ plant, the ratio of carboxylation to oxygenation (and therefore photosynthetic efficiency) decreases rapidly with declining atmospheric CO$_2$, especially at high temperatures [25]. In contrast, by fixing the bicarbonate ion rather than CO$_2$, the 
C$_4$ pathway does not confuse CO$_2$ with O$_2$ (figure 1a). 
Furthermore, by isolating Rubisco from the atmosphere and concentrating CO$_2$ at its active site [26], it also 
minimizes oxygenation in the 
C$_4$ pathway. However, energy required to run the 
C$_4$ CCM imposes a cost on photosynthetic efficiency under all conditions [27,28]. This means that the efficiency of 
C$_4$ photosynthesis is only greater than the 
C$_3$ type under conditions that support high rates of photorespiration [19]. Low atmospheric CO$_2$ and high temperatures are considered especially important in favouring 
C$_4$ photosynthesis, with a 'crossover threshold' for CO$_2$ of 35–55 Pa and temperatures of 25–30°C (figure 1c) [10,11].

A further physiological difference between 
C$_3$ and 
C$_4$ species arises because the carboxylation reaction of Rubisco is strongly limited by its substrate when CO$_2$ falls below approximately 70 Pa at the active site [29]. Resistances to CO$_2$ diffusion from the atmosphere to the chloroplast mean that this limitation arises when atmospheric CO$_2$ levels fall below approximately 100 Pa. In contrast, significant CO$_2$-limitation only

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Figure 1. Mechanism of 
C$_4$ photosynthesis. (a) Simplified schematic of the 
C$_4$ syndrome, showing how the 
C$_3$ pathway is isolated from the atmosphere in most 
C$_4$ species within a specialized tissue composed of bundle sheath cells (BSC). The 
C$_4$ pathway captures CO$_2$ within mesophyll cells (MC) using the enzymes carbonic anhydrase (CA) and phosphoenolpyruvate carboxylase (PEPC). It then transports fixed carbon to the BSC where decarboxylase enzymes (DC) liberate CO$_2$, which accumulates to high concentrations around Rubisco. (b) Transverse section of a 
C$_4$ leaf, showing the arrangement of MC and BSC. In this picture, the BSC are thicker walled, are stained darker and form rings surrounding the veins (adapted from Watson & Dallwitz [9]). Enlarged BSC relative 
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evolutionary biology and geosciences has enriched our understanding of when, where and how 
C$_4$ photosynthesis evolved. Here, we extend previous work that focused on why the pathway evolved, based on the environmental conditions that drove natural selection.

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occurs in C4 plants at atmospheric CO2 levels of less than 20 Pa [30]. The CO2-saturation of Rubisco in C4 photosynthesis means that in high light environments the enzyme reaches its saturated catalytic rate, maximizing the photosynthetic difference between C3 and C4 species. As a consequence, in open habitats, the ‘crossover threshold’ is raised to higher CO2 concentrations and lower temperatures [31]. Conversely, the cool shade of forest understory environments offers little benefit for the C4 pathway over the C3 type [32], although once C4 evolves in a lineage, descendant species may invade shaded habitats by modifying their tissue costs, allowing them to maintain an advantage in photosynthetic rate over co-occurring C3 species [33].

Water relations have previously been proposed to influence the evolution of C4 photosynthesis in an indirect way. For terrestrial vascular plants, the loss of water is an inevitable cost of photosynthesis, regulated by turgor-mediated decreases in the aperture of stomatal pores. The partial closure of stomata to conserve water in arid and saline soils or dry atmospheric conditions (characterized by high vapour pressure deficit (VPD)) has been hypothesized to select for the C4 pathway via indirect effects on photosynthetic efficiency [34]. Thus, reduced stomatal aperture (i) restricts the CO2 supply to photosynthesis and (ii) decreases transpiration, thereby reducing latent heat loss and raising leaf temperature. Both effects increase photorespiration, depressing the efficiency of C3 photosynthesis, and favouring the C4 type.

Therefore, the general expectation based on physiological evidence is that declining atmospheric CO2 should select for C4 photosynthesis in hot, open and dry or saline environments, where photorespiration is especially high in C3 species [19].

3. ECOLOGICAL DRIVERS OF C4 PATHWAY EVOLUTION

Recent data on the timing of C4 origins and geological history have supported these ideas for the importance of low CO2 and high temperature and irradiance, but also highlight the importance of aridity. First, the modelling of evolutionary transitions from C3 to C4 photosynthesis using time-calibrated molecular phylogenies indicated that C4 origins have all occurred over the past 30 Myr, with no difference in timing between monocot and eudicot lineages (figure 2) [35–37]. Secondly, multiple geological proxies suggested that atmospheric CO2 has remained below 60 Pa for most of this 30 Myr interval (figure 2), after a dramatic decline during the Oligocene (23–34 Ma) that corresponds to the onset of Antarctic glaciation [4]. Therefore, C4 photosynthesis does seem to have evolved in a CO2-depleted atmosphere, within the ranges of uncertainty that are inherent to phylogenetic and geological evidence [4,37].

The hypothesis that high temperatures select for C4 photosynthesis has been supported for 30 years by invoking the observation that species richness of C4 grasses increases along latitudinal and altitudinal temperature gradients [32,38,39]. However, phylogenetic comparative analyses have shown that C4 species do not live in warmer environments than their closest C3 relatives. First, Edwards & Still [40] showed that the absence of C4 species from high altitudes in the predominantly exotic grass flora of Hawaii [39] is better explained by phylogenetic history than photosynthetic pathway. The exclusively C3 lineage Pooidae is most speciose at cool, high elevations. However, species of the PACMAD lineage are most numerous in the warm lowlands, irrespective of whether they use C3 or C4 photosynthesis. Edwards & Smith [41] further investigated this pattern by reconstructing the evolution of temperature niche in the world’s grasses. Their analysis indicated that grasses originated in the tropics. The C3 and C4 species of the PACMAD clade have remained predominantly in these warm environments, irrespective of photosynthetic pathway, while Pooidae have adapted to and radiated in low-temperature environments. Thus, C4 photosynthesis did evolve at high temperatures, but the major innovation that caused ecological sorting of grasses along temperature gradients was the adaptation of certain C3 lineages to cold conditions [41].

Comparative analyses also support the hypothesis that the C4 pathway in grasses evolved in open environments. First, by modelling the rate of evolutionary transitions between C3 and C4 photosynthesis, and shaded and open habitats, Osborne & Freckleton [42] showed that C4 origins were significantly more likely to have occurred in open than shaded environments. Secondly, Edwards & Smith [41] quantified the shift in environmental niche that is correlated with evolutionary transitions from C3 to C4 photosynthesis.
The origins of C₄ photosynthesis were generally associated with migration from an aseasonal tropical niche into a seasonal, sub-tropical one, an ecological shift consistent with a transition from moist tropical forest to drier, more open woodland or savannah habitats [41]. These complementary analyses suggest that C₄ photosynthesis evolved in C₃ species that had migrated out of their ancestral niche in tropical forests, and invaded open sub-tropical woodland or savannah habitats. The C₄ pathway, therefore, seems to be a key adaptation to one of the most important ecological transitions in the evolutionary history of grasses [43], which ultimately enabled the assembly of the tropical grassland biome.

The proportion of C₄ species in eudicot floras increases along gradients of rising aridity [11]. Recent comparative analyses at the regional and global scales show that, in addition to preferring open habitats, C₄ grasses have also sorted into drier environmental niches than their C₃ relatives [40–42]. However, for grasses, there is no evidence that C₄ photosynthesis is more likely to evolve in xeric than mesic habitats [42]. Rather, the distribution of C₄ species in dry areas can be explained by two inferences from comparative analyses: (i) that C₄ origins were accompanied by shifts to a drier ecological niche within the humid sub-tropics [41] and (ii) a greater likelihood that C₄ than C₃ lineages will invade very dry (xeric) environments [42]. In some systems, C₄ grasses tolerate greater aridity than C₃ species of adjacent areas; for example, it has been argued that certain C₄ grasses of the Kalahari occur in areas too dry for the C₃ type to persist [44]. Water relations clearly play an important role in the ecology and biogeography of both C₄ monocots and C₄ eudicots.

A corpus of physiological work and comparative analyses therefore supports the theory of how low atmospheric CO₂ drove selection for improved photosynthetic rate and efficiency in hot and open environments over the last 30 Myr. What has been missing is an understanding of the importance of plant–water relations in differentiating photosynthetic types in such environments.

4. STRAIN ON PLANT–WATER RELATIONS IN A CO₂-DEPLETED ATMOSPHERE

Multiple strands of geological, ecological and physiological evidence indicate that a restriction in water supply and an increase in evaporative demand were important ecological factors during the evolution of C₄ species. First, permanent ice sheets of low CO₂ ‘icehouse’ climate intervals are known to reduce atmospheric moisture levels, with cool temperatures reducing the intensity of the hydrological cycle and increasing climatic seasonality [45]. As a consequence, the CO₂-depleted ‘icehouse’ climate of the last 30 Myr has caused the ecological availability of water to decline across large parts of the Earth’s surface.

Paleontological evidence reveals that open woodland, savannah and grassland vegetation had begun to extend over large areas of the tropics and subtropics by the Miocene (24–6 Ma) [3,46]. By analogy with processes in the modern world, these open tropical and sub-tropical ecosystems are likely to have been generated by seasonal aridity, edaphic conditions that exclude trees (including high salinity), and disturbance by fire and large mammalian herbivores [3,47–49]. Low atmospheric CO₂ may interact with each of these processes by limiting tree growth [50–52]. In fire-prone environments, this limitation means that trees are less likely to become large enough to survive surface fires [51,53]. Indeed, in contemporary mesic savannahs, woody plant cover is apparently increasing in response to rising CO₂ [53]. C₄ plants are generally short-statured herbs, and only trees in exceptionally rare cases (several species of Hawaiian C₄ Euphorbia are fully trees, including rainforest as well as dry forest species; and Haloxylon trees of West Asia have C₄ photosynthetic stems). The central role of seasonal aridity in reducing forest cover therefore means that the early C₄ plants living in open tropical and sub-tropical environments may well have been subjected to fire events and/or episodes of soil drying.

The demand for water imposed by potential evapotranspiration (PET) is also significantly greater for plants in open habitats than in shaded understorey environments. This environmental contrast is caused by a suite of interrelated microclimatic effects associated with tree cover, illustrated in figure 3 using field observations and a model of leaf energy balance and evaporation (appendix A). Micrometeorological data are shown in figure 3a,b for transects crossing a rainforest edge into adjacent pasture in tropical (Mexico) and temperate (New Zealand) localities [54,55]. Closed forest canopies typically intercept greater than 95 per cent of incident shortwave radiation (figure 3a,b). A model of leaf energy balance shows that this markedly reduces the net radiation and therefore the energy available to drive evaporation (latent heat flux) at ground level (figure 3c,d). Shading of the land surface under a forest canopy also causes a decrease in the air temperature at ground level (from 28 to 25°C in the tropical example, and from 10 to 5°C in the temperate example; figure 3). Therefore, the vapour pressure gradient driving transpiration, as indicated by the VPD, also declines (figure 3a,b). Finally, windspeed is lower in forested compared with open environments (figure 3a,b). This reduces the leaf boundary layer conductance to water vapour (figure 3e,f) and further slows the modelled rate of evaporation. The overall effect of this micrometeorological gradient is a massive difference in the PET modelled for leaves in forested compared with open environments (figure 3e,f). In the examples illustrated in figure 3, modelled PET is close to zero in the humid forest understorey, but exceeds 10 mmol H₂O m⁻² s⁻¹ in the adjacent open pasture for both tropical and temperate climates (figure 3e,f).

In fact, the risk of colonizing exposed habitats depends on the actual rate of leaf transpiration (E), which is lower than PET, being determined by the coupling of micrometeorological effects with the leaf stomatal conductance to water vapour (gₛ). The gₛ is controlled by changes in stomatal aperture, which are regulated by leaf water status, photosynthetic rate and chemical signals reflecting the soil water status transmitted from roots to leaves [56]. The gₛ plays a pivotal role in leaf
Gas exchange, limiting both the efflux of water and influx of CO$_2$, and the C$_4$ CCM causes a large shift in this trade-off between carbon gain and water loss. For any given value of $g_s$, the net rate of leaf photosynthetic CO$_2$ uptake ($A$) is greater for C$_4$ than C$_3$ species. This contrast is illustrated in figure 4a for interspecific comparisons of closely related C$_3$ and C$_4$ PACMAD grass species. Measurements were made under modern atmospheric CO$_2$ levels, and in high light and temperature conditions representative of a warm, open environment. The contrast is also modelled in figure 4b using models of leaf gas exchange for idealized C$_3$ [59] and C$_4$ [29] species (see appendix B). In the interspecific comparison, photosynthesis at 30°C can span the same range in C$_3$ and C$_4$ species, with one exceptionally high value of $A$ in the C$_3$ species exceeding the highest values observed in the C$_4$ species (figure 4a) [57]. However, high $A$ for C$_3$ species comes at enormous cost in terms of $g_s$ (figure 4a,b), and elevated rates of CO$_2$-fixation are therefore achieved with far greater water economy in C$_4$ than C$_3$ leaves (i.e. with greater water-use efficiency (WUE), defined as $A$ relative to $E$). The ecological significance of this difference is underscored by the fact that the exceptionally high C$_3$ value of $A$ in figure 4a was measured in the wetland species Phragmites australis, whereas the highest C$_4$ value was in the savannah species Eriachne aristidea [57].

The greater WUE of C$_4$ compared with C$_3$ photosynthesis arises from both differences in stomatal

Figure 3. Micrometeorological gradient spanning the transition from forested to open habitats. Data are shown for (a,c,e) pasture at the edge of tropical rainforest and (b,d,f) temperate rainforest, plotted against distance into the forest (negative values for distance = pasture; positive values = forest). Micrometeorological observations of photosynthetically active radiation (PAR), vapour pressure deficit (VPD) and surface windspeed are shown for (a) Mexico [54] and (b) New Zealand [55]. From these data, net radiation, latent and sensible heat fluxes are calculated using the model outlined in appendix A for the (c) tropical and (d) temperate forests. Calculated values of potential evapotranspiration (PET) and boundary layer conductance ($g_a$) are also shown for the same (e) tropical and (f) temperate localities.

Evolution of C$_4$ plants: a new hypothesis  C. P. Osborne & L. Sack 587
aperture and the kinetic properties of the carboxylase enzymes employed by each pathway (figure 1a). PEPC in C₄ plants is able to fix carbon at a much higher rate than Rubisco in C₃ plants at in vivo substrate concentrations [29]. This allows the C₄ pathway to generate a much steeper air--leaf CO₂ gradient and higher rates of photosynthesis for a given value of \( g_{s} \). The lower \( E \) and greater WUE of C₄ than C₃ plants was first recognized more than 40 years ago [60], although it is subject to ecological adaptation that can lead to significant interspecific variation and thus overlaps in the ranges of ecological adaptation that can lead to significant inter-

pheric CO₂ to sub-ambient levels (figure 5). This dramatically in response to the depletion of atmos-

pheric CO₂ from 100 to 30 Pa equivalent to the Oligocene CO₂ drop (figure 2) results in a rise in \( g_{s} \) from 0.23 to 0.61 mol H₂O m⁻² s⁻¹ in C₃, but only from 0.08 to 0.21 mol H₂O m⁻² s⁻¹ in C₄ species.

We explored the implications of these contrasting stomatal responses to CO₂ in C₃ and C₄ leaves by modelling leaf transpiration and energy balance (appendix A). Simulations with the model made the simplifying assumption that stomata respond only directly to CO₂, with no hydraulic or hormonal feedback on \( g_{s} \). We compared the modelled response of \( E \) to CO₂ under four sets of micrometeorological conditions representing either an open pasture or a shaded rainforest understory, in a humid tropical or temperate climate (using the data from figure 3). Our model simulations showed that falling atmospheric CO₂ drove larger increases of \( g_{s} \) and \( E \) in C₃ than C₄ species (figure 6a,b); this contrast was especially pronounced in open, tropical environments (figure 6a). For the simulations shown in figure 6a, we used a high relative humidity of 80 per cent (VPD = 0.6 Pa), which is typical of the moist tropics and the summer growing season in the humid subtropics (e.g. figure 3a). Under these conditions, simulated \( E \) in the C₃ species exceeded 6 mmol H₂O m⁻² s⁻¹ Mpa⁻¹ at a CO₂ level of less than 40 Pa (figure 6a).

Assuming a leaf-specific whole-plant hydraulic conductance (\( K_{plant} \)) of 12 mmol H₂O m⁻² s⁻¹ Mpa⁻¹ in grasses, and a 50 per cent decline in \( K_{plant} \) under a soil--leaf water potential gradient of −1 Mpa [69], this transpiration rate would be sufficient to cause 50 per cent failure of the hydraulic system. The threshold was not reached in a C₄ leaf under these conditions. In a drier atmosphere of 60 per cent relative humidity (VPD = 1.5 Pa), the threshold for 50 per cent hydraulic failure in the C₃ leaf was reached at CO₂ < 80 Pa, and in the C₄ at < 20 Pa (data not shown). The contrast was yet more pronounced at a VPD of 3.0 kPa, which is typical of hot, arid climates. Here, 50 per cent hydraulic failure

Figure 4. Illustration of the relationships of photosynthesis (A) to stomatal conductance (gs) in C₃ and C₄ species [57,58]. (a) Interspecific comparison of PACMAD grass species under high light and the current ambient CO₂ level (filled symbols, C₃; open symbols, C₄) circles from Taylor et al. [58], photosynthetic photon flux density (PPFD) = 1300 µmol m⁻² s⁻¹, temperature = approx. 25°C, VPD = 1 kPa; triangles from Taylor et al. [57], PPFD = 2500 µmol m⁻² s⁻¹, temperature = 30°C, VPD = 0.8–1.5 kPa). Fitting a general linear model to these data showed that the slope of A on gs was significantly steeper among C₄ than C₃ species (125 versus 29 µmol CO₂ mol⁻¹ H₂O, respectively; F₁,₄₂ = 55.8, p < 0.0001), with a significant quadratic term that shows no interaction with photosynthetic pathway. (b) Intraspecific response simulated for a C₃ and a C₄ species by prescribing variation in gs and modelling A using the approach described in appendix B. Note the saturation response in C₃ and linear response in C₄, indicating the greater sensitivity of A to stomatal closure in C₃, despite achieving higher maximum A at its maximum gs.

Phil. Trans. R. Soc. B (2012)
occurred for the C3 leaf at CO2 \(150\) Pa, and for the C4 leaf at CO2 \(50\) Pa (data not shown). Thus, to avoid failure of the vascular system in open, tropical environments, hydraulic regulation of stomata, investment in hydraulic supply, or a reduction in leaf area \([62]\), would be required at a considerably higher atmospheric CO2 partial pressure in C3 than C4 species. Coupled with the inference that open tropical habitats selected for the C4 pathway in grasses, this observation points strongly to plant–water relations as a potential driver of C4 evolution.

5. HYPOTHESIS FOR A CENTRAL IMPORTANCE OF HYDRAULICS IN C4 EVOLUTION

The current consensus model for the evolution of C4 photosynthesis has been developed over 25 years \([19]\). The evolutionary trajectory from C3 to C4 photosynthesis, via C3–C4 intermediates, is framed in terms of distinct phases for the sake of clarity; however, in reality, these are likely to overlap, and certain developments may occur earlier or later in the sequence. A simplified model of the phases is outlined in table 1, and Sage \([19]\) provides both a comprehensive review of the evidence underpinning this model and the detailed mechanisms proposed. As with the evolution of any complex trait, each step must provide a selective advantage over the previous phase or be selectively neutral.

We propose that atmospheric CO2 depletion and open environments select indirectly for C4 photosynthesis via plant–water relations at two points during the evolutionary sequence (table 1, phases 1 and 3). This mechanism acts in combination with
Table 1. Hypothetical model for the evolution of C₄ photosynthesis, showing three phases that are each observed in extant C₃–C₄ intermediates. The scheme is a simplified version of that presented by Sage [19], incorporating new evidence [70].

phase 1. Evolution of ‘proto-Kranz anatomy’
A reduction in the distance between leaf veins and an enlargement in bundle sheath cells (BSC) (figure 1e) may evolve under dry environmental conditions to enhance leaf water status [19,71,72]. Since photosynthetic activity is limited in the BSC of most C₃ leaves, increases in the number of chloroplasts may initially serve to maintain leaf light absorbance as the BSC occupy a larger fraction of the leaf. An increase in the numbers and asymmetric distribution of mitochondria in the BSC may establish a photorespiratory CO₂ pump that shuttles CO₂ within single BSC (see phase 2). This combination of traits has been termed ‘proto-Kranz anatomy’, and occurs in C₃ species that are closely related to C₃–C₄ intermediates [70].

phase 2. Evolution of a photorespiratory CO₂ pump
Photorespiration liberates CO₂ via a decarboxylation reaction catalysed by the enzyme glycine decarboxylase (GDC). The increasing localization of this enzyme in the BSC mitochondria requires glycine to be shuttled between the mesophyll cells (MC) and the BSC, liberating CO₂ in the BSC and allowing its refixation by Rubisco in this compartment (figure 1b). Efficiency of the glycine shuttle increases greatly if BSC walls are resistant to CO₂ diffusion, thereby concentrating CO₂ in the BSC. This type of photorespiratory CO₂ pump is typical of C₃–C₄ intermediates [19].

phase 3. Evolution of the C₄ cycle
Increases in the PEPC activity of MC may occur initially to scavenge CO₂ that leaks from the BSC, but eventually allows the fixation of CO₂ from intercellular airspaces (figure 1a). Once this occurs, enhancement of decarboxylase enzyme activities in the BSC is needed to recover the acceptor molecule for carbon-fixation (figure 1a). As carbon-fixation by PEPC increases above that of Rubisco, the C₄ cycle is increasingly confined to the BSC, and activities of the C₄ and C₃ cycles are coordinated. Finally, enzymes recruited into the C₄ cycle adapt to their new catalytic environment via changes in turnover rate, substrate affinity and regulation. Changes in stomatal conductance occur during this phase [19].

the well-established effects of atmospheric CO₂ depletion and open environments on photorespiration. A direct role of water relations provides a clear explanation for many of the anatomical changes in the early evolution of C₃–C₄ intermediacy; these are observed in other lineages of C₃, C₄ and CAM species, which indicates that these steps are not rare or extraordinary events, but that C₄ evolution simply co-opts typical steps in adaptation to dry environments.

Evolution of ‘proto-Kranz anatomy’ (table 1, phase 1).
Our hypothesized hydraulic mechanism is based on the vulnerability of C₃ leaves to desiccation and hydraulic failure under conditions of high evaporative demand in hot, open environments. Indeed, as described above, the hydraulic vulnerability of leaves would have increased dramatically as C₃ grass species migrated from the understory of a tropical forest into more open tropical environments. If stomata close to protect the hydraulic system, the plant eventually faces carbon starvation [73,74]. Carbon starvation is also exacerbated by atmospheric CO₂ depletion [75]. These conditions would select for greater hydraulic capacity in C₃ leaves, enabling greater gₛ to achieve rapid rates of photosynthesis in periods when water is abundant, and reducing both the requirement for stomatal closure and the risk of hydraulic failure as stomata partially close in a drying soil or under increasing VPD.

Notably, the anatomical preconditioning required for C₄ is precisely that expected to evolve in C₃ plants under selection for greater tolerance of dry soil, open environments, high VPD and/or low CO₂. Previous studies of adaptation to these conditions within species or across closely related species within given lineages have shown increased vein densities, and thus shorter interveinal distances [76–79]. This higher vein density would permit greater gₛ and higher A during periods with high water availability, to compensate for low CO₂. Indeed, the evolution of greater vein density in the early angiosperms may have allowed them to better cope with low CO₂, while avoiding stomatal closure, contributing to or driving their contemporary dominance of world vegetation [78,80]. The higher vein densities in C₄ than C₃ species may indicate an extension of that trend, i.e. an adaptation to the water deficit that accompanies higher demand from transpiration to maintain photosynthetic rate in low CO₂. In the same way that an increase in vein density may have enabled angiosperms to displace gymnosperms in dominating the world’s forests, this trend in grasses may have provided a competitive advantage over grasses with lower vein densities, contributing to their dominance over large areas of the planet. The high vein densities in species with ‘proto-Kranz anatomy’ led to this feature being co-opted as an early part of the C₄ syndrome, providing enough tissue to later serve as the locus of sequestered C₃ metabolism (figure 1a,b) [19,71,72,81,82].

Similarly, the enlargement of bundle sheath during the early phase of C₄ evolution may have contributed water storage capacitance to the tissue [19,71]. Indeed, the evolution of such tissue in species with ‘proto-Kranz anatomy’ would be an extension of a frequently observed trend in certain plant lineages in which the species adapted to saline and drier climates have more strongly developed achlorophyllous, large-celled tissue. This tissue serves an apparent function for water storage either in the bundle sheath, mesophyll or hypodermal layers, and this trend of greater water storage with aridity is apparent within C₃ lineages with leaves [83,84] and phyllodes [85], but also within C₄ and CAM lineages [86,87] (M. J. Sporck & L. Sack 2011, unpublished data).

Evolution of the C₄ cycle (table 1, phase 3). The evolution of greater WUE is apparently an important step in C₄ evolution. It may be achieved in certain C₃–C₄ intermediates operating a photorespiratory pump (table 1, phase 2) through the enhancement of A for a
Evolution of C₄ plants: a new hypothesis  C. P. Osborne & L. Sack 591

Given $g_s$ (e.g. Heliotropium [88]). However, once the carboxylase activity of PEPC exceeds that of Rubisco (evolution of ‘C₄-like’ plants during phase 3, table 1), further improvements in WUE may occur through reduced $g_s$ (e.g. Flaveria [89]). Thus, evolution towards C₄ probably first involved a suppression of photorespiration (table 1, phase 2) and then increased PEPC activity (table 1, phase 3), but only this last step enabled a reduction of $g_s$. These steps may even occur within C₃ species: populations of the grass Phragmites australis in hot, arid and saline environments show increasing investment in elements required for the C₄ cycle, including PEPC, decarboxylases enzymes and bundle sheath tissues, which may enhance both CO₂-fixation and WUE [90]. Thus, the engagement of CO₂-fixation via PEPC (table 1, phase 3) improves leaf–water relations, allowing a lower value of $g_s$ for a given rate of photosynthesis, and thus a smaller absolute response of $g_s$ to CO₂ depletion in C₄ leaves (figure 5b). This benefit would lead atmospheric CO₂ depletion and open environments to select for the C₄ cycle via plant–water relations, as well as via their effects on photosynthetic efficiency. Together, these physiological differences mean that leaves operating a fully integrated C₄ cycle are less prone than C₃ leaves to hydraulic failure or stomatal closure in hot, open environments. This contrast in sensitivity to water deficit increases dramatically under atmospheric CO₂ depletion.

A greater hydraulic conductance relative to demand, as would arise from the adaptation described, would result in a greater ability to maintain open stomata during soil drying events in C₄ than C₃ leaves, and a lower sensitivity of stomata to increases in VPD and to decreases in CO₂ (see §6). These improvements in water relations provide a plausible physiological basis for the greater likelihood for C₄ than C₃ grass lineages to invade xeric environments [42].

6. HYDRAULIC FEEDBACKS ON LEAF PHYSIOLOGY

Our hypothesis that C₄ evolved not only to improve photosynthetic efficiency per se, but also to reduce water stress by enabling low $g_s$, has additional implications that at first sight lead to inconsistency. While the C₄ species can achieve higher $A$ for a given $g_s$, $A$ declines more rapidly as $g_s$ decreases (figure 4a,b). Because of this greater sensitivity of $A$ to $g_s$, a C₄ plant must keep stomata open to maintain its advantage in $A$ over C₃ plants. At first sight, this problem should render photosynthesis in C₄ plants particularly sensitive to soil drying owing to stomatal closure. As an initial test of our hypothesis for a hydraulics-mediated advantage of C₄ photosynthesis, and to further explore the physiological implications, we used a novel integrated model of leaf photosynthesis, stomatal and hydraulic systems to compare the physiological behaviour of C₃ and C₄ species (appendix B), with a particular focus on conditions of CO₂ depletion (low CO₂), atmospheric water vapour deficit (high VPD) and soil drying (low soil water potential). Given reasonable assumptions, these model simulations supported the hypothesis of a hydraulically based advantage of the C₄ syndrome under high VPD and low soil water potential, enabling stomata to remain open, and maintaining $A$ at higher levels than C₃ species, with greatest differences at low CO₂.

We tested the role of hydraulic conductance in allowing $g_s$ and $A$ to be maintained during drought under varying VPD and atmospheric CO₂ in C₃ and C₄ species. We parametrized the model using data from the literature, and coupled this with photosynthesis models for C₃ and C₄ types (appendix B). Thus, we compared C₃ and C₄ species that differed in $g_s$ (0.23 versus 0.10 mol m⁻² s⁻¹, respectively) but assumed the same responsiveness of $g_s$, and the same vulnerability of the hydraulic system, to declining leaf water potential. We tested three scenarios, with the C₄ species having: the same whole-plant hydraulic conductance ($K_{plant}$) as the C₃ species; double the $K_{plant}$ of the C₃ species; or half the $K_{plant}$ (figure 7 and 8).

When the C₄ species had the same $K_{plant}$ as the C₃, the C₄ was able to maintain $g_s$ at a higher relative level (percentage of its maximum value) as the soil dried; this advantage over the C₃ was especially strong at higher VPD (figure 7). This result is consistent with an experimental comparison of PACMAD grasses under drought, which showed a more sensitive response of $g_s$ to soil water deficits in C₃ species than in closely related C₄ species [57]. Model simulations also showed lower leaf water potential in the C₃ than C₄ species as soil dried, until the point of stomatal closure, where the leaves equilibrated with the soil (figure 7). This contrast is also broadly consistent with comparative experiments on closely related C₃ and C₄ PACMAD grasses, which showed a difference in leaf water potential under moist conditions that was diminished under chronic drought [57,68]. In our model simulations, this advantage was evidently owing to the higher $K_{plant}$ relative to $g_s$ for the C₄ over C₃ species. When the C₄ plant was given double the $K_{plant}$ of the C₃ species, its advantage in maintaining open stomata during soil and atmospheric drought was increased, and when the C₄ plant was given half the $K_{plant}$ of the C₃ species, its advantage was diminished.

The ability of a C₄ species with similar or higher $K_{plant}$ to a C₃ species to maintain open stomata during drought also translated into an ability to maintain $A$ at a higher level. This advantage went beyond compensating for the tendency of $A$ to decline more precipitously with declining $g_s$ in C₄ than C₃ species, described above (figure 4a,b), and typically provided C₄ species with an ability to maintain higher $A$ during mild drought, especially at higher VPD (notably, simulations at yet higher VPDs led to even stronger differences between photosynthetic types). When the C₃ and C₄ species had the same $K_{plant}$, $A$ was higher in the C₄ than the C₃, under low CO₂, but the rank was reversed under high CO₂ (figure 8). Doubling the value of $K_{plant}$ in C₄ compared with C₃ species gave an advantage in $A$, and the ability to maintain photosynthesis during drought. This finding is broadly consistent with an early demonstration that, during the dry season in the Negev desert, the C₄ species Hammmada scoparia showed a shallower decline of $A$ with declining leaf water potential than three C₃ species (Prunus armeniaca, Artemisia herba-alba and Zygophyllum dumosum). At a leaf water potential of −6 MPa, H. scoparia (C₄) had twice the $A$ of these C₃ species.

Phl. Trans. R. Soc. B (2012)
Figure 7. Simulated response of mid-day operating stomatal conductance (as a percentage of maximum for hydrated leaves) and leaf water potential to drying soil at VPD of \((a, b) 1 \text{kPa}\) and \((c, d) 3 \text{kPa}\), for a C3 species and C4 species with identical plant hydraulic conductance \(K_{\text{plant}}\), for a simulated C4 species with double the \(K_{\text{plant}}\) and for a simulated C4 species with half the \(K_{\text{plant}}\) (corresponding to its value of \(g_s\) being approximately half that of the C4 species). Note that the C4 species maintains stomatal opening into drier soil and higher VPD than the C3. Increasing the \(K_{\text{plant}}\) improves this ability in the C4 species, whereas reducing the \(K_{\text{plant}}\) by half leads to very rapid decline of \(g_s\) at high VPD or in a dry soil. \((a–d)\) Dashed line, C3; black solid line, C4; light grey solid line, C4, \(2 \times K_{\text{plant}}\); dark grey solid line, C4, \(0.5 \times K_{\text{plant}}\).

Figure 8. Simulated response of light-saturated photosynthetic rate to a drying soil at low (20 Pa), ambient (40 Pa) or high CO2 (80 Pa) and VPD of \((a) 1 \text{kPa}\) or \((b) 3 \text{kPa}\), for a C3 species and C4 grass species with identical plant hydraulic conductance \(K_{\text{plant}}\), and for a simulated C4 species with double the \(K_{\text{plant}}\), and a simulated C4 species with half the \(K_{\text{plant}}\) (corresponding to its value of \(g_s\) being approximately half that of the C3 species). \((a, b)\) Dashed line, C3; black solid line, C4; light grey solid line, C4, \(2 \times K_{\text{plant}}\); dark grey solid line, C4, \(0.5 \times K_{\text{plant}}\).
Evolution of C₄ plants: a new hypothesis C. P. Osborne & L. Sack 593

species [91]. In contrast, when the C₄ species in our model simulations had reduced $K_{plant}$, this led to a much reduced value of $A$, and the C₃ species showed an advantage of $A$ across all CO₂ levels as soil dried minimally (figure 8). These findings indicate that, theoretically, a reduced $K_{plant}$ in C₄ species would annul any advantage of the C₄ pathway for photosynthesis under drought or low CO₂ in open environments. However, an equal or higher $K_{plant}$ in these conditions would provide an advantage not only in maintaining open stomata, but also in maintaining a high $A$, providing a strong benefit to C₄ species.

What evidence is there that $K_{plant}$ is high relative to $g_s$ in C₄ compared with C₃ species? To-date, no studies have made the necessary direct experimental comparisons. Nonetheless, the two comparative studies of five C₃ and eight C₄ lineages of PACMAD grasses presented in figure 4a (a total of 40 different species) found that C₄ grasses generated a lower soil to leaf gradient in water potential ($\Delta \Psi$) during typical diurnal transpiration than closely related C₃ species [57,58]. Similar patterns have been observed for the C₃ and C₄ subspecies of Alloteropsis semialata in common garden plots at high irradiance and temperature [92]. This finding is consistent with the hypothesis of a higher ratio of hydraulic supply to demand, i.e. a greater $K_{plant}/g_s$ in C₄ than C₃ grass species.

Notably, several studies of C₄ woody eudicots have suggested the opposite situation, measuring reduced stem hydraulic conductance per supplied leaf area ($K_{stem}$) relative to C₃ species [93–95]. This was hypothesized to evolve in the final stages of C₄ evolution after WUE has increased (table 1, phase 3). A reduced $K_{stem}$ would not necessitate a reduction of $g_s$ under well-watered conditions or mild drought. On the other hand, it would likely reduce xylem construction costs, and also possibly reduce the vulnerability to cavitation of stems, and thus their longevity [93–95]. Such adaptation would be especially important for the economics and protection of long-lived woody parts [93]. However, we note that a lower $K_{stem}$ does not necessarily imply a lower $K_{plant}$; indeed, a higher leaf hydraulic conductance ($K_{leaf}$) can easily compensate. Recent work has shown the leaf is a critical bottleneck in the whole-plant hydraulic pathway, accounting for greater than 30 per cent of whole plant resistance, and that leaves have steeper hydraulic vulnerability curves than stem [96]. Consequently, $K_{leaf}$ is a strong determinant of $K_{plant}$, especially when leaves begin to dehydrate during transpiration or incipient drought [97–101]. Such an importance of $K_{leaf}$ in the whole plant pathway is thus consistent with a reduction of $K_{stem}$ in woody dicot C₄ species. It is also consistent with the evolution of high vein density in C₃ species under drier climates (table 1, phase 1), which would serve to increase $K_{leaf}$ and maintain or increase $K_{plant}$ while the evolution of higher water storage capacitance would buffer changes in leaf water potential during high VPD or drought [77,96,102,103]. These modifications would then be co-opted for further evolution of C₃–C₄ intermediates and C₄ species (table 1).

In §5, we argued that the low values of $g_s$ in C₄ species save the hydraulic system from embolism, especially as leaves are heated in open environments, and as stomata are stimulated to open under low CO₂. Our model findings further show that the low values of $g_s$ in C₄ species also reduce stomatal sensitivity to hydraulic feedbacks, allowing stomata to remain open during drought, and photosynthesis to continue, but only if C₄ species have similar or higher $K_{plant}$ than C₃ species, and thus a high hydraulic supply relative to demand. A lower $K_{plant}$ would serve to make C₄ species more sensitive to soil drought, especially under high VPD and low CO₂. The circumstantial evidence we have presented for grass species is consistent with our hypothesis for a high hydraulic supply relative to demand in C₄ species. However, to our knowledge, there are no published data comparing $K_{leaf}$ for C₃ and C₄ plants in general, or for grasses specifically. Such data are essential to test our hypothesis.

7. METABOLIC LIMITATION OF PHOTOSYNTHESIS DURING SEVERE DROUGHT

We hypothesized that the evolution of the C₄ pathway provides significant physiological benefits for carbon-fixation and water conservation in well-watered soil, during the early stages of soil drought, or during transient drought events. However, there is no a priori reason to expect a greater tolerance of severe desiccation in C₄ than C₃ species [104]. In fact, recent comparative analyses of closely related C₃ and C₄ PACMAD grasses suggests the converse; that the C₄ photosynthetic system may be more prone to greater metabolic inhibition under chronic and severe drought events. Experimental evidence from controlled environment, common garden and field investigations all indicate that photosynthetic capacity declines to a greater extent with leaf water potential in C₄ than C₃ grass species after weeks of soil drying, or at very high VPD [57,92,105,106]. Chronic or severe drought may thus diminish, eliminate or even reverse the WUE advantage of C₄ over C₃ species. In the extreme case, photosynthetic capacity is significantly slower to recover after the end of the drought event [106]. Experimental evidence therefore suggests that metabolic limitation of photosynthesis will eventually offset hydraulic benefits of the C₄ syndrome during acute or prolonged drought events. For tolerance of severe drought, tight stomatal control and tissue water storage, as is strongly developed in CAM species, or desiccation-tolerant tissue, are far superior to C₄ metabolism. However, for tolerance of repeated transient droughts in the growing season, C₄ metabolism carries strong advantages, particularly under low CO₂.

8. CONCLUSIONS

We hypothesized that atmospheric CO₂ depletion coupled with high temperatures, open habitat and seasonally dry subtropical environments caused excessive demand for water transport, and selected for C₄ photosynthesis to enable lower stomatal conductance as a water-conserving mechanism. C₄ photosynthesis allowed high rates of carbon-fixation to be maintained.
at low stomatal conductance, and reduced stomatal opening in response to low atmospheric CO₂. These mechanisms served to reduce strain on the hydraulic system. Maintaining a high hydraulic conductance enabled stomatal conductance and photosynthesis to be sustained for longer during drought events. The evolution of C₄ photosynthesis, therefore, rebalanced the fundamental trade-off between plant carbon and water relations, as atmospheric CO₂ declined in the geological past, and as ecological transitions drove increasing demand for water.

Our hypothesis adds the evolution of C₄ photosynthesis to the list of exceptional innovations based on the plant hydraulic system that arose during periods of low CO₂ that have impacted on plant life history, biogeography and the distribution of ecosystems in the deep past, present and future [107]. Previously hypothesized modifications of the water transport system and associated plant features driven by low CO₂ include the evolution of xylem vessels and stomata [108], and the planate leaf [109,110] with high vein density [78]. These innovations permitted more rapid growth and diversification, leading to the succession of dominance from pteridophytes to gymnosperms to angiosperms [78,80,111,112]. The evolution of C₄ photosynthesis, for improved performance in exposed, seasonally dry habitats in low CO₂, thus joins a long line of hydraulic innovations driven by low CO₂ that changed plants and the world.

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APPENDIX A

(a) Model of evaporation from a leaf in a forest understorey or in open pasture

The net radiation balance at the leaf surface (Φₙ₀) is a function of shortwave (S) and longwave (L) radiation fluxes (both W m⁻²):  
Φₙ₀ = S + Lₐ - 2Lₙ + Lₚ,  
(A1)

where 2Lₐ represents longwave radiation emitted upwards and downwards from the leaf, Lₚ the upwards emission from soil and Lₙ the downwards emission from the sky or forest canopy, depending on tree cover. Values of L were calculated according to Stefan’s Law, tracking the temperatures of leaf, soil, tree canopy and sky, by assuming that the lowest layer of leaves in the forest canopy is approximately at air temperature, and the apparent temperature of the clear sky is 20 K lower than that of surface air [113,114]. Heat storage by the leaf was assumed to be negligible, such that Φₙ₀ is dissipated entirely via latent and sensible heat fluxes (AE and H, respectively).

\[ Φₙ₀ = λE + H. \]  
(A2)

Evaporation from the leaf surface (E, mol H₂O m⁻² s⁻¹) was modelled using two variants of the Penman–Monteith equation [115,116]. For the simulations shown in figure 3, we were interested in the potential for evapotranspiration in the absence of any limitation imposed by stomata, and used the original form [115]:  
\[ E = \frac{[sΦₙ₀ + ρₚcpₚVPD]}{λ[s + γ]} ， \]  
(A3a)

where Φₙ₀ is net radiation at the leaf surface (W m⁻²), VPD is the atmospheric VPD (Pa), gₚ is the leaf boundary layer conductance (m s⁻¹) and the remaining parameters are physical properties of air and water: s, the rate of change of saturation vapour pressure with temperature (Pa K⁻¹), ρₚ the density of dry air (kg m⁻³), cₚ, the specific heat capacity of air (J kg⁻¹ K⁻¹), γₚ, the psychrometer constant (Pa K⁻¹) and λ, the latent heat of evaporation for water (J mol⁻¹). The values of s, γₚ, ρₚ and λ were corrected for temperature following Friend [117]. We made the simplifying assumption that the leaf boundary layer conductance is approximately equal for heat and water.

For the simulations shown in figure 6, we were interested in the regulation of E by stomata, and used the modification by Monteith [116]:  
\[ E = \frac{[sΦₙ₀ + ρₚcpₚVPD]}{λ[s + (γₚgₚ/gₖ)]}, \]  
(A3b)

where gₖ is the total leaf conductance for water:  
\[ gₖ = gₚ^{-1} + gₚ^{-1}. \]  
(A4)

and gₚ is a function of leaf width (d = 0.01 m), and wind speed (uₚ m s⁻¹):  
\[ gₚ = 6.62(μ/σ)^{0.5}. \]  
(A5)

Values of gₖ were prescribed for these simulations as a function of atmospheric CO₂, using the equations derived for C₃ and C₄ leaves in figure 5. The sensible heat flux is given by:  
\[ H = (Tₙ - Tₛ)ρₚcpₚgₚ. \]  
(A6)

where Tₙ is the leaf temperature and Tₛ the air temperature (both K). To solve the leaf energy balance, equation (A2) must be rearranged to give H, and equation (A6) to give Tₙ:  
\[ H = Φₙ₀ - λE \]  
(A7)

and  
\[ Tₙ = Tₛ + \frac{H}{ρₚcpₚgₚ}. \]  
(A8)

Equations (A1), (A3), (A7) and (A8) are then solved simultaneously using iteration in R (The R
foundation for Statistical Computing) to yield values for $\Phi_{\text{a}}, E, H$ and $T_1$.

**APPENDIX B**

(a) Modelling of hydraulic-stomatal limitation on photosynthesis in $C_3$ and $C_4$ species

A model based on a few simplifying assumptions was used to determine the response of operating $g_s$ and leaf water potential ($\Psi_{\text{leaf}}$) to declining soil water potential ($\Psi_{\text{soil}}$) and increasing vapour pressure deficit (VPD). The $g_s$ was then used to estimate photosynthesis from a diffusion-biochemistry model (see appendix B). The hydraulic-stomatal model determines $\Psi_{\text{leaf}}$ plant hydraulic conductance ($K_{\text{plant}}$) and $g_s$ at a given $\Psi_{\text{soil}}$ and VPD. First, $\Psi_{\text{leaf}}$ is determined based on steady-state water transport according to the Ohm’s Law analogy [118, 119]:

$$\Psi_{\text{leaf}} = \Psi_{\text{soil}} \frac{(g_s \times \text{VPD})}{K_{\text{plant}}}. \tag{B1}$$

Secondly, a hydraulic response function modelled the vulnerability of $K_{\text{plant}}$ to declining $\Psi_{\text{leaf}}$. This used a linear function to approximate observations between full hydration and turgor loss, as reported for the leaves of grasses and several other taxa [69, 120, 121]:

$$K_{\text{plant}} = K_{\text{max}} + a \times \Psi_{\text{leaf}}, \tag{B2}$$

where $K_{\text{max}}$ and $a$ are constants for given species. We assumed that $K_{\text{plant}}$ showed a similar vulnerability response to leaves [69, 99], calculating $K_{\text{plant}} = 80\% \times$ leaf hydraulic conductance, based on the range shown in previous work on grasses (65% to over 80% of plant resistance in the leaf [69, 101, 118]). Changing these assumptions would not affect the comparative findings of our simulations.

Thirdly, we simulated the decline in $g_s$ with more negative $\Psi_{\text{leaf}}$ as a sigmoidal function:

$$g_s = \frac{g^*}{1 + e^{-(\Psi_{\text{leaf}}-b)/c}}, \tag{B3}$$

where $g^*$ is the maximum value of $g_s$ at $\Psi_{\text{leaf}} = 0$, and $b$ is the $\Psi_{\text{leaf}}$ at 50 per cent stomatal closure. The constant $c$ defines the shape of the sigmoidal curve.

For given $\Psi_{\text{soil}}$ and VPD, equations (B1–B3) were solved simultaneously, minimizing the implicit forms by iteration [122] (Microsoft Visual Basic; Microsoft, Redmond, WA, USA). Using this model, we simulated the response of $g_s$ to $\Psi_{\text{soil}}$ for $C_3$ and $C_4$ species, from 0 to $-2\text{MPa}$, and at VPD of 1 and 3 kPa, using parameters for equations (B1–B3) from the literature, as available (table 2). We also tested scenarios for $C_4$ species with double the $K_{\text{plant}}$ and half the $K_{\text{plant}}$ of the $C_3$ species.

(b) Modelling of photosynthetic rate from diffusion-biochemical limitations for $C_3$ and $C_4$ species during drought under varying vapour pressure deficit and atmospheric $CO_2$

We simulated photosynthetic rate ($A$) and its response to $CO_2$, by first modelling a direct response of $g_s$ to $CO_2$, and then inputting the adjusted $g_s$ values into equations for $C_3$ and $C_4$ photosynthesis.

First, for low and high $CO_2$, we multiplied the $g_s$ values by a factor corresponding to 20 or 80 Pa (1.72 and 0.58, respectively) based on the data and fitted equations of figure 5. This multiplicative adjustment of $g_s$ for $CO_2$ level was applied independently of the $g_s$ response to water status (from which $g_s$ was determined from equations (B1–B3) above). Thus, the model considers a simplified scenario in which the response of stomata to VPD and soil drought on one hand, and to $CO_2$ on the other, is independent and multiplicative, with no hydraulic or hormonal feedback on $g_s$. The assumption is consistent with previous work measuring the effects of $CO_2$ and VPD on $g_s$ in $C_3$ and $C_4$ species [68]. This independence of the responses would put plants in low $CO_2$ at risk of severe leaf dehydration if stomata open strongly when VPD is low, and, indeed, such dehydration has been observed [125]. However, there is evidence that declining soil water availability can interact with the $CO_2$ response, resulting in greater sensitivity of stomata to $CO_2$, to a varying degree across different $C_3$ and $C_4$ species, in part modulated by abscistic acid responsiveness [126, 127]. More work is needed to resolve and to explicitly model the potential interactions of stomatal responses to different factors in $C_3$ and $C_4$ species. For example, the interaction with falling soil water potential during drought would be expected to produce a stronger decline of $g_s$ and of $A$.

In our model, the $g_s$ now adjusted for $CO_2$, was used to predict $A$ based on the equations for $C_3$ and $C_4$ photosynthesis provided by von Caemmerer [29], using parameters from Vico & Porporato [124] (table 2). We assumed that the impact of $\Psi_{\text{leaf}}$ on $A$ was mediated by $g_s$ without any separate, direct impacts on mesophyll conductance or on metabolism itself; these impacts could be added but, without detailed information of differential impacts on $C_3$ and $C_4$ species, would not change the outcome of our scenarios [124].

Thus, for $C_3$ species, $A$ was determined as

$$A = \min(A_c, A_t) - R_d, \tag{B4}$$

where $A_c$ is the Rubisco-limited rate of photosynthesis, $A_t$ is the rate of RuBP-limited $CO_2$ assimilation and $R_d$.

---

Table 2. Parameters used for hydraulic-stomatal-photosynthesis modelling.

<table>
<thead>
<tr>
<th>parameter</th>
<th>units</th>
<th>$C_3$, $C_4$ values</th>
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<tr>
<td>leaf $K_{\text{max}}$</td>
<td>mmol m$^{-2}$ s$^{-1}$ MPa$^{-1}$</td>
<td>15, 15</td>
<td>[69]</td>
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<tr>
<td>$a$</td>
<td>mmol m$^{-2}$ s$^{-1}$ MPa$^{-2}$</td>
<td>$-7.5$, $-7.5$</td>
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<tr>
<td>$b$</td>
<td>MPa</td>
<td>$-1.0$, $-1.0$</td>
<td>[69]</td>
</tr>
<tr>
<td>$c$</td>
<td>MPa</td>
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<td>[69, 123]</td>
</tr>
<tr>
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<td>83, 39</td>
<td>[124]</td>
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<td>$\mu$mol mol$^{-1}$</td>
<td>46, 10</td>
<td>[124]</td>
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<td>[124]</td>
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<td>256, 256</td>
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<td>[124]</td>
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<tr>
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<td>1.66, 0.78</td>
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<td>n.a., 120</td>
<td>[124]</td>
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<td>n.a., 80</td>
<td>[124]</td>
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<td>$V_{W}$</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
<td>n.a., 80</td>
<td>[124]</td>
</tr>
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</table>
is the total mitochondrial rate of respiration. In turn,
\[ A_c = V_{c,max} \frac{C_m - \Gamma^*}{C_m + K_c(1 + (O/K_a))}, \]  
(B 5) where \( V_{c,max} \) is the maximum catalytic activity of Rubisco at current leaf temperature (here considered as optimal); \( C_m \) is the CO2 concentration at the site of photosynthesis in the mesophyll cell (MC); \( \Gamma^* \) is the equilibrium CO2 compensation point for gross photosynthesis; \( K_c \) and \( K_a \) are the coefficients for CO2 and O2 of the Michaelis–Menten kinetics, accounting for competitive inhibition by O2; and \( O \) is the O2 concentration at the site of photosynthesis.

\[ A_f = f_{max} \frac{C_m - \Gamma^*}{4(C_m + 2\Gamma^*)}, \]  
(B 6) where \( f_{max} \) is the maximum potential rate of electron transport. To determine \( A_f \), the equations (B 5) and (B 6) were each equated separately with the diffusion equation:
\[ A = (C_a - C_m) \times g_t, \]  
(B 7) where \( C_a \) is the atmospheric CO2 concentration. The value of \( g_t \) was determined as the conductance to CO2 from the atmosphere to the intercellular space (stomatal conductance to CO2, \( g_s,CO_2 \)) and from the intercellular space to the chloroplast (mesophyll conductance, \( g_m \)) in series:
\[ \frac{1}{g_t} = \frac{1}{g_s,CO_2} + \frac{1}{g_m}. \]  
(B 8) where \( g_s,CO_2 \) was determined as \( g_s/1.6 \) and \( g_m \) as \( g_s \times 1.65 \) [124]. In each case (equations B 6 = B 8, and equations B 7 = B 8), the equations were solved for a given \( g_s \) and \( C_a \) to determine \( C_m \) using the quadratic equation. The values of \( C_m \) were inserted into equations (B 5) and (B 6), respectively, to determine \( A_c \) and \( A_p \) before applying equation (B 4) to determine \( A \).

For the C4 species, a similar approach was used, but the first step involved determining the PEP carboxylation rate (\( V_p \)):
\[ V_p = \min \left( \frac{C_m V_{p,max}}{C_m + K_p}, V_{pr} \right), \]  
(B 9) where \( V_{p,max} \) is the maximum rate of PEP carboxylation, \( V_{pr} \) is an upper bound set by PEP regeneration rate and \( K_p \) is the Michaelis–Menten coefficient of PEP. The \( C_m \) was determined by equating equation (B 9) with equation (B 7) for a given \( g_s \) and \( C_a \). We then used the \( C_m \) and \( V_p \) to determine the \( A_f \) by combining two equations:
\[ A = V_p - L_{bs} - R_d. \]  
(B 10) where \( L_{bs} \) is bundle sheath leakage, given by:
\[ L_{bs} = g_{bs}(C_{bs} - C_m). \]  
(B 11) and \( C_{bs} \) is the CO2 concentration in the bundle sheath. Substituting equation (B 11) into equation (B 10), and making this equation equal to each of equations (B 5) and (B 6) separately (substituting \( C_m \) for \( C_m \) in those equations), allowed them to be solved for \( C_{bs} \). Equations (B 5) and (B 6) were then used to determine \( A_c \) and \( A_p \), and equation (B 4) to determine \( A \).


Evolution of C_4 plants: a new hypothesis


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**GLOSSARY**

A  
net rate of leaf photosynthetic CO2 assimilation

BSC  
bundle sheath cells

CA  
carbonic anhydrase

CAM  
crassulacean acid metabolism

CCM  
carbon-concentrating mechanism

DC  
decarboxylase enzymes

E  
actual rate of leaf transpiration

$g_s$  
leaf stomatal conductance to water vapour

$K_{leaf}$  
hydraulic conductance of leaves

$K_{plant}$  
whole-plant hydraulic conductance

$K_{stem}$  
stem hydraulic conductance

MC  
mesophyll cells

PACMAD  
acronym for the lineage of grasses comprising the subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae

PAR  
photosynthetically active radiation

PEPC  
phosphoenolpyruvate carboxylase

PET  
potential evapotranspiration

PPFD  
photosynthetic photon flux density

RH  
relative humidity

Rubisco  
ribulose-1,5-bisphosphate carboxylase/oxygenase

VPD  
vapour pressure deficit

WUE  
leaf water-use efficiency ($A/E$)

$\psi_{leaf}$  
leaf water potential