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Review

Nature's green revolution: the remarkable evolutionary rise of C_4 plants

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Plants with the C_4 photosynthetic pathway dominate today's tropical savannahs and grasslands, and account for some 30% of global terrestrial carbon fixation. Their success stems from a physiological CO_2-concentrating pump, which leads to high photosynthetic efficiency in warm climates and low atmospheric CO_2 concentrations. Remarkably, their dominance of tropical environments was achieved in only the past 10 million years (Myr), less than 3% of the time that terrestrial plants have existed on Earth. We critically review the proposal that declining atmospheric CO_2 triggered this tropical revolution via its effects on the photosynthetic efficiency of leaves. Our synthesis of the latest geological evidence from South Asia and North America suggests that this emphasis is misplaced. Instead, we find important roles for regional climate change and fire in South Asia, but no obvious environmental trigger for C_4 success in North America. CO_2-starvation is implicated in the origins of C_4 plants 25–32 Myr ago, raising the possibility that the pathway evolved under more extreme atmospheric conditions experienced 10 times earlier. However, our geochemical analyses provide no evidence of the C_4 mechanism at this time, although possible ancestral components of the C_4 pathway are identified in ancient plant lineages. We suggest that future research must redress the substantial imbalance between experimental investigations and analyses of the geological record.

Keywords: atmospheric CO_2 concentration; C_4 plants; plant evolution; stable carbon isotopes

1. INTRODUCTION

Photosynthetic CO_2-fixation has provided the carbon for life on Earth for at least the past 2.7 billion years (Gyr). More than 99% of the history of this ancient process has been dominated by C_3 photosynthesis (figure 1), so-called because its first products are carboxylic acids formed of three linked carbon atoms. But between 25 and 32 million years (Myr) ago, a revolutionary innovation evolved in tropical grasses in the form of a solar-powered carbon dioxide pump based on four-carbon acids ('C_4 photosynthesis'), which boosts photosynthesis in hot conditions. It works by pumping CO_2 from the mesophyll into a specialized ring of bundle sheath cells centred around leaf veins, where an extremely localized version of C_3 photosynthesis operates, bathed in high CO_2 concentrations (figure 1; Hatch 1971). Although, this specialized 'Kranz' anatomy is the norm in the vast majority of C_4 plants, it is not essential, and certain desert species have evolved an alternative form of the pathway in which all elements are packed into single cells (Voznesenskaya *et al.* 2001). A variation on the single-celled carbon concentrating mechanism is called Crassulacean acid metabolism (CAM) and is more widely adopted in drought tolerant species. It operates by temporally separating the activities of a C_4-like pump mechanism and C_3 photosynthesis.

The C_4 photosynthetic pathway is a major evolutionary success, accounting for some 20–30% of terrestrial CO_2-fixation on Earth (Lloyd & Farquhar 1994) and 30% of global agricultural grain production (Steffen *et al.* 2004). Plants utilizing this pathway dominate tropical grasslands and savannahs, and rank among the world's most important crops, including sugarcane (*Saccharum officinarum*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*). And the C_4 revolution is not confined to grasses. In one of the most striking examples of convergent evolution in plants (Conway-Morris 2003), a C_4 carbon-concentrating mechanism has originated in more than 40 independent evolutionary groups (Sage 2004). Paradoxically, its multiple origins belie a complex trait that requires the coordinated expression of at least 20–30 unlinked genes to operate efficiently (Wyrich *et al.* 1998; Furumoto *et al.* 2000).

The C_4 and CAM mechanisms probably arose as a 'fix' for an intrinsic inefficiency in Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase), the enzyme catalysing CO_2-fixation in every plant. This ancient enzyme originated in 'greenhouse' conditions when the Earth's atmosphere contained CO_2 at up to 100 times today's level (Rye *et al.* 1995) and negligible amounts of O_2 (Bekker *et al.* 2004). A CO_2-fixing enzyme in this atmosphere had little requirement for high CO_2-affinity, and gained no advantage from distinguishing between CO_2 and O_2 molecules. However, the ensuing long-term decline in atmospheric CO_2 subsequently

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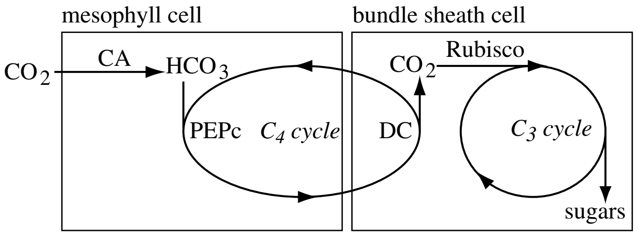

Figure 1. Simple schematic diagram of the C_4 photosynthetic pathway showing compartmentalization of the different enzyme systems involved, and the connection between CO_2-pumping by the C_4 cycle and CO_2-fixation by the C_3 cycle. Abbreviations: CA, carbonic anhydrase; HCO_3, bicarbonate; PEPc, phosphoenolpyruvate carboxylase; DC, decarboxylase enzyme(s); Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

exposed an important problem; Rubisco fixes O_2 as well as CO_2, at a rate depending on the $CO_2 : O_2$ ratio. In today's low $CO_2 : O_2$ atmosphere, O_2-fixation in C_3 plants wastes captured solar energy and causes a net loss of CO_2 via the photorespiration pathway.

C_4 plants overcome this problem using a coupled enzyme system with a much higher affinity than Rubisco for CO_2 (figure 1), first dissolving the gas by using carbonic anhydrase (CA) to form bicarbonate (HCO_3^-), and then fixing it using phosphoenolpyruvate carboxylase (PEPc). The four-carbon products of this fixation diffuse into bundle sheath cells, where CO_2 is released by decarboxylase enzymes (DC, figure 1) and reaches concentrations of 3–8 times those in C_3 photosynthetic cells (reviewed by Kanai & Edwards 1999). Rubisco in C_4 plants, therefore, experiences a saturating CO_2 environment similar to that of C_3 plants growing in ancient 'greenhouse' atmospheres, and photorespiration is minimized (Osmond 1971). Through this mechanism, C_4 plants achieve a substantial photosynthetic advantage over their C_3 contemporaries with falling atmospheric CO_2 and at high temperatures (figure 2; Björkman 1971), as Rubisco becomes increasingly unable to distinguish O_2 from CO_2. However, the C_4 mechanism carries a major cost; its dependence on light to energize the CO_2-pump lowers photosynthetic efficiency relative to the C_3 type, especially in conditions when photorespiration is naturally suppressed, such as high CO_2 and cool temperatures (figure 2).

The use of an energy-dependent system to alleviate photorespiration in C_4 plants leads to a trade-off, with beneficial results for photosynthetic light-use efficiency at high temperatures and a decline in efficiency at low temperatures (figure 2). The point at which costs match benefits is termed the 'crossover temperature' and decreases with CO_2, as photorespiration becomes increasingly problematic for C_3 plants (figure 2). This simple physiological contrast between photosynthetic types was first quantified 30 years ago (Ehleringer & Björkman 1977), and has been invoked subsequently as the 'quantum yield hypothesis' to explain biogeographical patterns of C_4 ecological (Ehleringer *et al.* 1997) and evolutionary success (Ehleringer *et al.* 1991; Cerling *et al.* 1997). Along geographic temperature gradients, the crossover point matches the mean growing season temperature where C_4 grasslands are replaced by C_3 types (Ehleringer *et al.* 1997), both from the equator towards cooler climates and on mountains, where the air cools with altitude (reviewed by Sage *et al.* 1999). In warmer climates, the C_4 type has a photosynthetic advantage, while the C_3 type benefits at cooler temperatures.

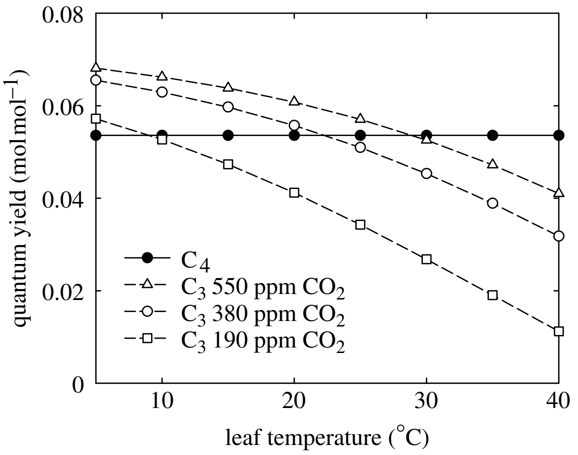

Figure 2. Modelled interaction between temperature and CO_2 on the photosynthetic quantum yields (maximum light-use efficiencies) of C_3 and C_4 plants. Notice that the temperature at which C_3 and C_4 quantum yields cross over declines with falling atmospheric CO_2 concentration.

C_4 plants have clearly evolved an effective solution for the inherent kinetic inefficiency of Rubisco. However, the mechanisms translating this physiological advantage into evolutionary and ecological success remain unclear, and are major unresolved questions in biology. Here, we review recent advances in our understanding of C_4 plant evolution, integrating recent developments across the geological, ecological, physiological and molecular sciences. We begin with the origins of the C_4 photosynthetic pathway, reconciling geological evidence with molecular data on the evolutionary history of plant lineages. Next, we investigate the remarkable picture built-up from isotopic analyses of fossil remains, demonstrating rapid global expansion of C_4-dominated ecosystems, and consider its likely cause. Finally, we present a theoretical analysis of the possible selection pressures for a C_4-type of carbon concentrating mechanism resulting from the unusual atmospheric composition of the Permo-Carboniferous 300 Myr ago (Ma). We investigate the possibility that the necessary environmental and physiological prerequisites were in place for the C_4 mechanism to have evolved 300 Myr earlier than is currently accepted, through a detailed isotopic analysis of fossil plant remains and preliminary physiological measurements of evolutionarily ancient plant lineages.

2. ORIGINS OF C_4 PLANTS

The evolutionary origins of C_4 plants may be elucidated using fossil evidence of Kranz anatomy and an important difference in the stable carbon isotope composition ($\delta^{13}C$) of C_3 and C_4 plants. Rubisco discriminates strongly against the heavy isotope of carbon (^{13}C), relative to its more abundant form (^{12}C). In contrast, the dissolution of CO_2 to form bicarbonate and its subsequent fixation by the CA–PEPc enzyme system slightly favours the heavier isotope (Farquhar 1983). This physiological difference translates into a marked contrast in the $\delta^{13}C$ of C_3 and C_4 plant tissues, which persists long after they have been eaten and incorporated into the bones or teeth of herbivores, or decomposed into biomarkers within geological sediments (Cerling 1999). Analysis of $\delta^{13}C$ in geological materials, therefore, provides an important opportunity for reconstructing changes in C_4 plant abundance on evolutionary time-scales. However, interpreting these data requires care because shifts in the $\delta^{13}C$ of atmospheric CO_2 cause parallel changes in plant tissues.

Kranz anatomy can be identified in well-preserved fossil leaf fragments dating to the Late Miocene, 5–12 Ma. A petrified grass from the Ricardo Formation (12.5 Ma) of California is currently the earliest undisputed C_4 plant, and additionally characterized by a typical C_4 $\delta^{13}C$ signature (Nambudiri *et al.* 1978). A second early example of Kranz anatomy is reported for a silicified grass from the Ogallala Formation (5–7 Ma) in Kansas (Thomasson *et al.* 1986). Several older C_4 grass species are claimed from the Fort Ternan locality in Kenya (14 Ma) on the basis of cuticle morphology (Dugas & Retallack 1993), but internal leaf anatomy is not preserved, and their photosynthetic type remains in question (Cerling 1999). These fossils highlight a major difficulty in identifying fossil plants on the basis of anatomical features; in most cases these are simply not preserved, an issue especially acute for C_4 plants, which inhabit seasonally dry environments where fossilization is unlikely. Direct evidence of Kranz anatomy is therefore rare, and the origins of C_4 photosynthesis must be inferred by integrating data from stable carbon isotope analyses and investigations of plant molecular genetics.

Despite intriguing isotopic evidence from the Cretaceous (90 Ma, Kuypers *et al.* 1999), general consensus currently places the earliest origins of C_4 photosynthesis within the family Poaceae (grasses) (Kellogg 2000). Grasses first appear in the fossil record as pollen in the Palaeocene (55–60 Ma), with additional, more equivocal records of grass-like pollen in the latest Cretaceous (70 Ma; Jacobs *et al.* 1999). Estimates of when C_4 photosynthesis arose in this group come from molecular genetic techniques, in which evolutionary histories are retraced by comparing differences in DNA sequences between species. Since most DNA mutations are selectively neutral, with no effect on Darwinian fitness, they accumulate over time by 'genetic drift' and therefore indicate evolutionary distance between species. By using the number of mutations between species and a mutation rate calibrated using fossils, the 'molecular clock' technique dates the appearance of C_4 photosynthesis in the grass sub-family Panicoideae at 25–32 Ma (Gaut & Doebley 1997). However, phylogenetic data are unable to distinguish with confidence whether this event was followed by multiple reversions back to the C_3 type, up to six further C_4 origination events, or the persistence of genes allowing flipping between types (Kellogg 2000; Duvall *et al.* 2001, 2003; Guissani *et al.* 2001). Molecular reconstructions also confirm three additional, and independent, origination events occurring earlier than 25 Ma, within the cluster of related grass lineages Aristidoideae, Eriachneae and Chloridoideae, although these are not precisely dated at present (Kellogg 2000, 2001). Each origination co-opted a slightly different set of biochemical pathways to achieve a functional C_4 cycle (Sinha & Kellogg 1996), and followed an adaptation to open habitats in grass groups whose ancestors were confined to forest shade habitats, like today's bamboos (Kellogg 2001). However, the location of this evolutionary innovation remains mysterious, because high diversity and ancient origins in the grasses make geographic centres of C_4 evolution hard to pinpoint (Sage 2004).

C_4 photosynthesis also proliferated within the Cyperaceae (sedges), and numerous families of Eudicots, including the Asteraceae (daisies), Brassicaceae (cabbages), Euphorbiaceae, but especially the Chenopodiaceae and related Amaranthaceae, where 13 independent origination events are postulated (Kadereit *et al.* 2003). Molecular genetic evidence points to 37 independent evolutionary origins for C_4 photosynthesis outside the grasses (Sage 2004), starting as early as 14–21 Ma in the Chenopodiaceae (Kadereit *et al.* 2003). Biogeographical analysis of diversity in these groups suggest diverse centres of C_4 origin located across the world, in southern Texas-central Mexico, central Asia, sub-tropical Africa and sub-tropical South America (Sage 2004).

Molecular genetics, therefore, predicts that the carbon isotope signature of C_4 grasses should be picked up from the Oligocene (23–35 Ma) onwards, with C_4 Eudicots contributing soon afterwards (from 14 to 21 Ma). The signal is faint, but present in carbonates from fossil soils (palaeosols) dating to 23 Ma in the southern Great Plains of North America (Fox & Koch 2003). After ruling out a number of potential biases, reconstructions for this region suggest that C_4 plants made up 12–34% of the biomass from 23 to 7 Ma in the Late Miocene (Fox & Koch 2003), and grew in open woodland ecosystems (Strömberg 2004). Similar claims from East Africa of a persistent, but relatively low, abundance of C_4 plants in a savannah or woodland ecosystem from 15 to 7 Ma are disputed, on the grounds of conflicting evidence from mammalian teeth and possible misidentification of palaeosols (Cerling 1999). What is clear, however, is that prior to 9 Ma there is no isotopic signature of a C_4-dominated ecosystem anywhere in the world. Data from Africa, South America, the Indian sub-continent, and China all show ecosystems comprised entirely of C_3 plants, although a low C_4 presence is difficult to exclude against a varying C_3 $\delta^{13}C$ background (Cerling *et al.* 1997). For up to 25 Myr from their inferred origins, C_4 plants were rare or absent from the tropics. The C_4 revolution was a long-time coming.

3. CO_2 AND THE RISE OF C_4 PLANTS TO ECOLOGICAL DOMINANCE

C_4 plants came to dominate terrestrial ecosystems abruptly in the Late Miocene (5–8 Ma). A revolutionary expansion of C_4 plants is identified by major shifts in $\delta^{13}C$ (figure 3) across the southern US Great

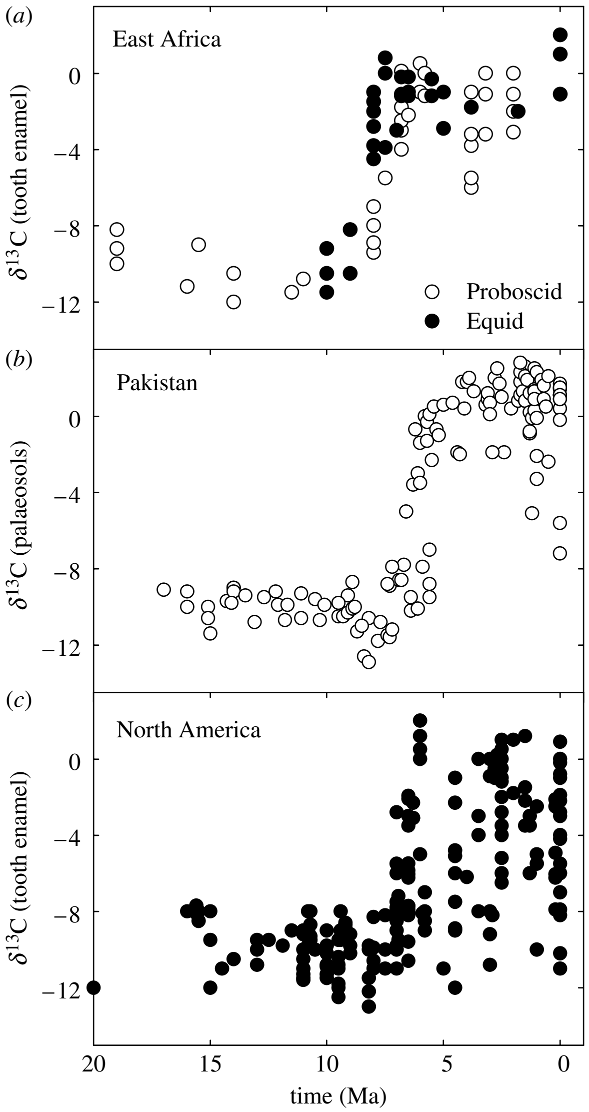

Figure 3. Increase in $\delta^{13}C$ from palaeosols and tooth enamel showing apparent synchronicity in the transition to C_4-dominated terrestrial ecosystems across continents. Data for (*a*) from Cerling *et al.* (1997), (*b*) from Quade & Cerling (1995) and (*c*) from Passey *et al.* (2002).

Plains, Argentina, Bolivia, India, Pakistan, Nepal and Kenya (reviewed by Cerling *et al.* 1997). Isotopic changes are recorded in palaeosol carbonate, organic matter, and in the diets of large mammals and flightless birds via tooth, bone and egg shell $\delta^{13}C$ (figure 3; reviewed by Cerling *et al.* 1997; Cerling 1999). The revolution continued into the Pliocene (2–5 Ma), with later C_4 expansions in the northern US Great Plains (Cerling *et al.* 1997), China (Ding & Yang 2000), Chad (Zazzo *et al.* 2000), and across East Africa (Levin *et al.* 2004). It transformed ecosystems from the tropical to the warm temperate climate zones across four continents, with C_4 biomass increasing from near zero to more than 80% of vegetation in just 2–4 Myr (figure 3).

The near-synchronous expansion of C_4 plants across diverse geographical regions in the Late Miocene points towards a global trigger for the phenomenon, and led Ehleringer, Cerling and co-workers (Ehleringer *et al.* 1991; Cerling *et al.* 1997) to propose the net decline in atmospheric CO_2 over the last 150 Myr as the mechanism. According to this proposal, falling CO_2 gradually lowered the crossover temperature of C_3 plants (figure 2) until it fell below tropical temperatures, making C_4 photosynthesis progressively more advantageous and allowing C_4 plants to achieve ecological dominance in ever-cooler climates (Ehleringer *et al.* 1991; Cerling *et al.* 1997). Circumstantial evidence for this decline in crossover temperature with CO_2 comes from a slight asynchrony in the timing of C_4 expansion, with dominance achieved first in hot, equatorial Kenya, followed by the southern Great Plains and Pakistan (20–37° N), and finally the northern Great Plains (40–43° N; Cerling *et al.* 1997). Further indirect support is provided by the inverse relationship between tropical C_4 plant abundance from $\delta^{13}C$ records during recent ice age (glacial) cycles (Ehleringer *et al.* 1997), and fluctuations of atmospheric CO_2 from 180 p.p.m during glacial intervals to 280 p.p.m during interglacial periods (Petit *et al.* 1999).

The CO_2 starvation mechanism rapidly achieved widespread acceptance, but geological data are now beginning to challenge its proposed role in C_4 success (Keeley & Rundel 2003). Palaeo-CO_2 reconstructions from three independent proxies indicate low CO_2 concentrations for at least 15 Myr before the Late Miocene expansion of C_4 grasslands (figure 4; Pagani *et al.* 1999; Pearson & Palmer 2000; Royer *et al.* 2001). The inferred levels of CO_2 vary between 180 and 320 p.p.m (figure 4), and correspond to crossover temperatures of less than 10–22 °C (figure 2), well within tropical temperature limits. According to the CO_2 hypothesis, the C_4 mechanism would, therefore, have presented a substantial photosynthetic advantage in the tropics as early as the Oligocene (23 Ma). Critically, we note that CO_2 was not declining during the Late Miocene period of ecological change, and one proxy even indicates an increase at 8 Ma (figure 4; Pagani *et al.* 1999). Further evidence from the last glacial cycle also challenges the primary role of CO_2 as a driver of ancient C_4 successes, with uncoupling of C_4 plant abundance from CO_2 being attributed to lower summer rainfall (Huang *et al.* 2001; Scott 2002).

On the basis of our assessment, we reject the hypothesis of CO_2-starvation as the proximate driver of Miocene C_4 expansions. However, we still see C_4 physiology as an adaptation to low CO_2 atmospheres, because it only provides a photosynthetic advantage at tropical temperatures of less than 30 °C when CO_2 concentrations are lower than 500 p.p.m (figure 2; Ehleringer *et al.* 1997). Consequently, although declining CO_2 was not the direct trigger for Miocene expansions of C_4 plants, a decrease in its concentration was a necessary pre-condition for this widespread C_4 success (Sage 2001). Three independent palaeo-CO_2 proxies all show a drop in CO_2 from a high point of greater than 1000 p.p.m in the mid-Cretaceous to its Early Miocene low (Royer *et al.* 2004), and a new record based on phytoplankton $\delta^{13}C$ now points to a sharp drop in CO_2 from between 1000 and 1500 p.p.m to around 300 p.p.m during the Oligocene (23–35 Ma; Pagani *et al.* 2005), coincident with continental ice-sheet initiation on Antarctica (Zachos *et al.* 2001). This decrease corresponds to a fall in crossover temperature from greater than 40 to 17–21 °C (figure 2), and

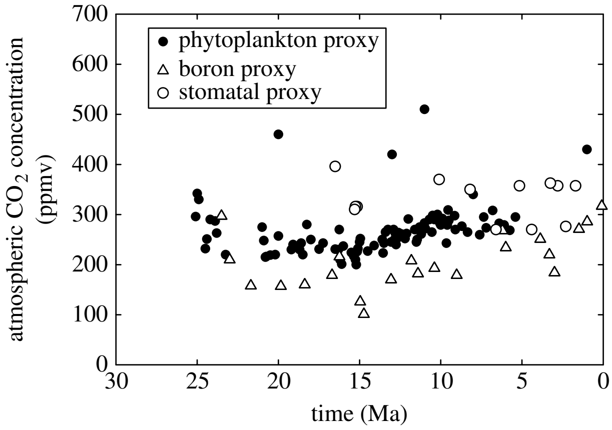

Figure 4. Atmospheric CO_2 trends over the past 35 Myr, as reconstructed from three different proxies (data from Pagani *et al.* 1999; Pearson & Palmer 2000; Royer *et al.* 2001).

matches almost exactly with the dates of C_4 grass origins suggested by molecular clocks.

Declining CO_2 in the Oligocene may, therefore, have selected for the first C_4 plants (Keeley & Rundel 2003), but verification of this proposal must await the publication of further molecular clock dates and more palaeoclimate evidence. When did C_4 photosynthesis originate in the grass lineages Aristidoideae, Eriachneae and Chloridoideae? How closely do these dates coincide with the drop in palaeo-CO_2 concentrations, and what other environmental factors changed during the Oligocene, when major upheaval occurred in the climate system? CO_2 may yet have a part to play in C_4 evolution, but current evidence suggests that it was not directly responsible for the rise of C_4 plants to dominance in the Miocene and Pliocene (figure 4).

4. ALTERNATIVE MECHANISMS FOR C_4 SUCCESS

Explaining the Late Miocene expansion of C_4 grasslands requires mechanisms other than CO_2. In seeking these, we begin by considering the environmental and biotic factors promoting dominance by C_4 plants in modern ecosystems. All are regional rather than global in their sphere of influence, and stem from continental climate change or the interaction between disturbance and grassland ecosystem development. To investigate the relative merits of these explanations, we examine evidence in the geological record for the mechanistic drivers of C_4 success in the Indian subcontinent and North America.

(a) *Competition, climate and disturbance*

Two primary factors control the distribution of C_4-dominated ecosystems in the modern world; the limits of forest distribution set by rainfall and disturbance, and geographical gradients of temperature and rainfall. Since C_4 plants are predominantly herbaceous, only rarely achieving the stature of shrubs or small trees (Sage 2001), they are rapidly overtaken in height by forest trees or savannah shrubs and suppressed by shading. Most C_4 plants cannot tolerate shading of sunlight to below 25% of its open sky value, possibly because of anatomical (Ogle 2003), phylogenetic, environmental or physiological constraints (reviewed by Sage & Pearcy 2000). Closed forest, therefore, precludes ecosystem dominance by C_4 plants, but is replaced by open woodland, savannah or grassland in seasonally arid climates because of water-shortage and disturbance by fire (Bond *et al.* 2005). The latter is especially common in monsoonal climates, where the dry season is followed by a high incidence of lightning strikes accompanying convective rainfall (Keeley & Rundel 2003), and favours C_4 grasses with their ability to grow more rapidly than C_3 grasses in hot, open conditions after a recent burn (Knapp & Medino 1999).

Herbivores may also tip the balance between forest and grasslands, and can be classified on the basis of their foodstuff; specialist grazers eat only grasses, while dedicated browsers prefer forbs, shrubs and trees, avoiding the intense dental wear caused by the silica bodies (phytoliths) in grass leaves. Browsing and other damage to woody vegetation by large herbivores promotes grasses, through an important interaction with fire in savannah ecosystems (Bond *et al.* 2005). However, grazing can permit forest recovery through the removal of grass biomass, which lowers the amount of fuel for fires and decreases their intensity (Briggs *et al.* 2002). Shifts to seasonal climates, fire and herbivory may therefore all be implicated in the loss of forest vegetation that was a critical prerequisite for Miocene C_4 success.

Creation of open woodland, savannah or grassland removes light-limitations on C_4 plants, and their occurrence then depends on temperature, with the relative abundance of C_4 relative to C_3 grasses increasing with minimum summer temperatures (reviewed Sage *et al.* 1999). However, seasonally arid conditions mean that the effects of temperature on grassland distribution are mediated strongly by the amount of precipitation and timing of rainfall events, which constrain the length of the growing season through soil moisture availability.

C_4 grasses typically dominate regions receiving rainfall from monsoonal weather systems during hot summer conditions, when the suppression of photorespiration provides a photosynthetic advantage over the C_3 pathway (figure 2). Their CO_2-concentrating mechanism also allows C_4 leaves to achieve higher photosynthetic rates at lower stomatal conductances than in C_3 species, thereby conserving water in hot conditions when evaporative demand is high (reviewed Long 1999). This efficient use of water throughout the life cycle of C_4 plants must offer a major selective advantage over C_3 species in open, seasonally dry habitats. In contrast, C_3 grasses tend to populate regions where rains fall during cool winter weather (reviewed by Sage *et al.* 1999), a period when C_4 species are susceptible to damage caused by chilling (reviewed Long 1983). Together, these interacting physiological responses to rainfall and temperature are the likely cause of the strong correlations between the total representation of C_4 species in a flora, annual or summer rainfall, and minimum or mean summer temperature at the continental scale (e.g. Hattersley 1983). However, the direction of these correlations differs significantly between C_4 physiological sub-types

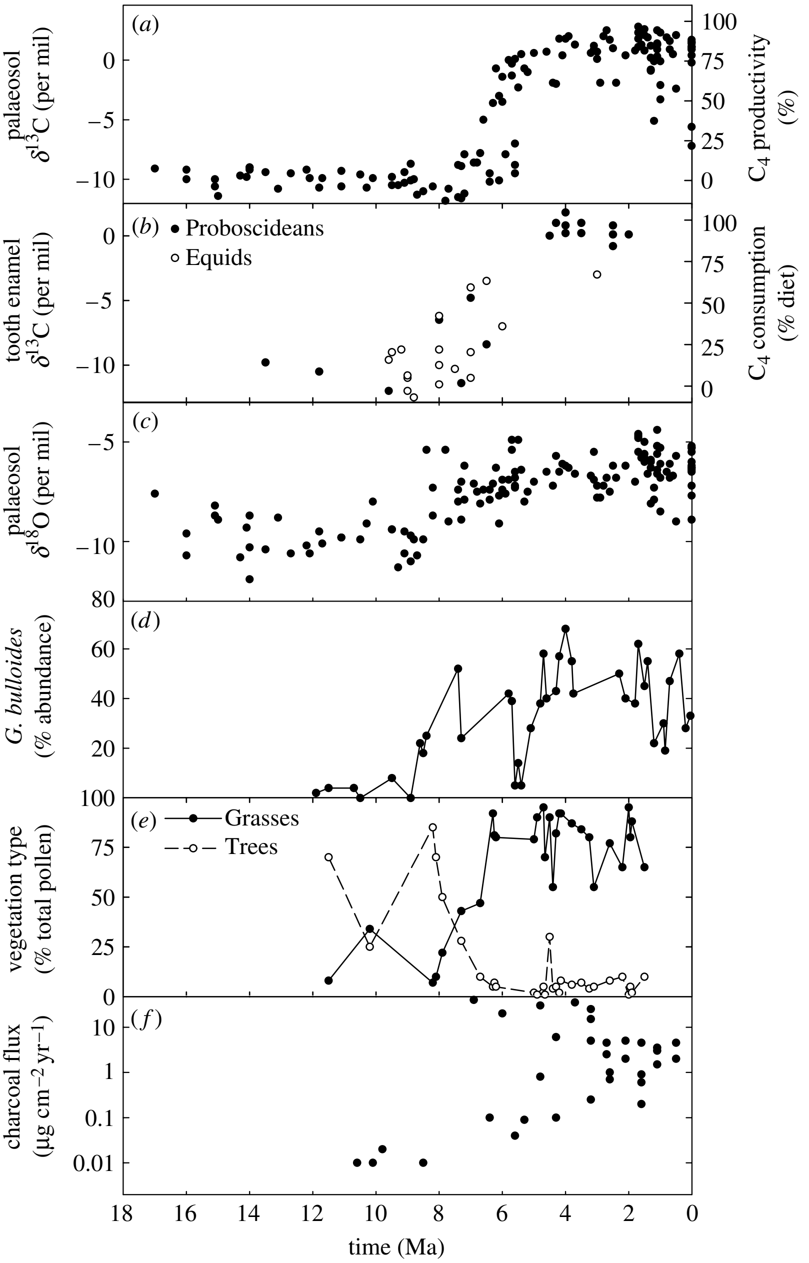

Figure 5. Geological evidence from the Indian subcontinent of ecosystem dynamics and climate change through the Late Miocene and Pliocene: (*a*) δ^{13}C of palaeosol carbonates (data from Quade & Cerling 1995) and inferred C$_4$ plant productivity (following Fox & Koch 2003), (*b*) δ^{13}C of tooth enamel from Equids (horses) and Proboscideans (elephant-like mammals) (data from Quade & Cerling 1995) and the inferred proportion of diet comprised of C$_4$ plant biomass (following Passey *et al.* 2002), (*c*) δ^{18}O of palaeosol carbonates (data source as (*a*)), (*d*) abundance of *Globigerinoides bulloides* in the Arabian Sea (data from Zhisheng *et al.* 2001), (*e*) vegetation type inferred from pollen abundance (data from Hoorn *et al.* 2000), and (*f*) charcoal flux to North Pacific sediments (data from Keeley & Rundel 2003).

(Hattersley 1992) and phylogenetic groups (Taub 2000), for reasons that are poorly understood.

Modern plant biogeography points to important roles for temperature, rainfall patterns and disturbance in controlling C$_4$ plant distributions, although the precise interactions between these variables remain unclear. In seeking the primary cause of Miocene C$_4$ expansion, we must therefore examine both the geological history of climatic- and disturbance-mediated loss of forest cover, and the development of seasonal palaeoclimate regimes.

(b) *Indian subcontinent*

The Siwalik formation stretches through Pakistan, northwest India and Nepal, and contains one of the best-studied geological records of C$_4$ success in the Late Miocene. Its fossils and sediments richly document the vegetation type, fauna, photosynthetic type and climate of the Himalayan foothills and Ganges floodplain from the Early Miocene through the Pliocene, with Pacific Ocean sediments offering complimentary evidence of fire frequency (figure 5).

Measurements of δ^{13}C from palaeosol carbonates in the Siwalik formation and organic sediments in the Bay of Bengal securely date the timing of the C$_4$ revolution in this region (figure 5*a*). C$_4$ plants began to increase in abundance from 7.7 Ma in Pakistan (figure 5*a*), 7.0 Ma in Nepal and the Bengal Fan, and 6.0 Ma in northern India. This increase gathered pace rapidly, resulting in the complete replacement of C$_3$ vegetation across the region and ecosystem dominance by C_4 plants by 5.5 Ma (reviewed by Quade *et al.* 1995). Timing of the transition is paralleled by an increase in the $\delta^{13}C$ values of herbivore tooth enamel (figure 5*b*), demonstrating major changes in the diet of mammals with the shift in vegetation type (Cerling *et al.* 1997).

Expansion of C_4 grasslands in the Indian subcontinent has been attributed to the initiation or intensification of the Indian monsoon (Quade *et al.* 1995). This climatic mechanism is supported by geological evidence from the stable oxygen isotope ratio ($\delta^{18}O$) of palaeosol carbonates, which show significant increases from 8.5 to 6.0 Ma, arriving ahead of the shift in $\delta^{13}C$ by 0.5–1.0 Ma (figure 5*c*). The $\delta^{18}O$ of soil carbonates depends on complex interactions between temperature, the $\delta^{18}O$ of rainwater and the rate of evaporation from the soil surface. The Miocene increase in $\delta^{18}O$ could, therefore, result from warming, a larger proportion of rainfall in the summer rather than winter and/or a more southerly source of water in rainfall, all consistent with the initiation or a major intensification of the Indian monsoon. This interpretation is further supported by significant increases in the abundance of marine organisms such as *Globigerinoides bulloides* at 8.5 Ma (figure 5*d*), indicative of greater monsoon-driven upwelling in the Arabian Sea, and by the initiation of dust deposition in China (evidence reviewed by Zhisheng *et al.* 2001).

Climate model simulations demonstrate that sudden uplift of the Tibetan Plateau at *ca* 8 Ma could be responsible for the onset of the Indian monsoon (Zhisheng *et al.* 2001). However, the timing of this mechanism is disputed, and new palaeo-elevation estimates suggest that uplift was largely complete by 15 Ma (Spicer *et al.* 2003; Currie *et al.* 2005), pointing to a more subtle interaction between tectonic activity and climate. Moreover, $\delta^{18}O$ data from the Himalayan foreland now suggest constant monsoon intensity in this area from 10.7 Ma, with a general decline in rainfall from *ca* 8 Ma (Dettman *et al.* 2001). Whatever drove the change, 8.5 Ma was marked by a rapid transition to a drier climate, with most evidence pointing to a shift from year-round moist conditions to a summer-wet, winter-dry monsoon climate.

The transition to a monsoonal climate had major impacts on vegetation in the Indian subcontinent. Prior to this climatic shift, in the Early to Middle Miocene (8–18 Ma), plant fossils and pollen from the Lower Siwalik indicate a landscape comprising wet tropical evergreen forests in the lowlands, and moist deciduous forest with patches of pines at higher altitudes (figure 5*e*; Hoorn *et al.* 2000). Pollen demonstrates that grasses were present (figure 5*e*) and probably confined to gaps or forest margins, but palaeosol isotopes cannot resolve their photosynthetic type against the background signal from forest vegetation (Jacobs *et al.* 1999; Hoorn *et al.* 2000). These interpretations of Middle Miocene vegetation are confirmed by the presence of mouse deer and lorises, small mammals confined to dense forest today (reviewed by Jacobs *et al.* 1999).

Monsoonal conditions caused major changes in vegetation, characterized by a significant increase in grass and shrub pollen through sediments from the Late Miocene (6.5–8 Ma), accompanied by a decline in forest plant groups (figure 5*e*). This indicates a gradual transition from forest to open savannah that began at higher altitudes and spread down to the floodplain (reviewed by Jacobs *et al.* 1999; Hoorn *et al.* 2000). It was accompanied by the appearance of savannah and grassland large mammal taxa, including giraffes, grazing ungulates and hippopotamus (reviewed by Cerling *et al.* 1997; Jacobs *et al.* 1999). Complimentary pollen records show the complete loss of woody vegetation at this time, and the consequent establishment of C_4 grasslands on both the Ganges floodplain and Himalayan foothills (figure 5*a*,*e*).

Ecological expansion of C_4 grasslands and the immigration of large mammals to the northern Indian subcontinent followed the rapid loss of C_3 woody vegetation from 8 to 5.5 Ma. As the winter drought intensified under monsoonal conditions and forest cover declined, fire also seems to have played a major part in the transition to grassland. Cores of sediment drilled from the deep-ocean floor in the northwest Pacific indicate a greater than 1000-fold increase in charcoal abundance during the Late Miocene (figure 5*f*). The charcoal probably originated in South Asia and includes charred grass cuticle fragments (Keeley & Rundel 2003, 2005). It suggests dramatic increases in fire frequency, intensity or extent accompanying the Indian monsoon, greatly amplifying the negative effects of climate change on tree cover (discussed by Keeley & Rundel 2003). Our review of terrestrial and marine geological evidence, therefore, implicates the initiation/intensification of the Indian monsoon, drought-mediated loss of forest cover, and a re-enforcing feedback promoted by fire as the reasons for C_4 expansion rather than any change in CO_2 concentration (figure 5*c*–*f*).

(c) *North America*

Investigations in the Great Plains region of North America have provided abundant records of fossil teeth and soils (palaeosols) for the Miocene and Pliocene offering unrivalled information on the coevolution of herbivore diet and C_4 plants. However, the emerging picture from North American fossils is more complex than for the Indian subcontinent, and the role of climate is less clear. In the Great Plains, the evolutionary emergence of grazers, the appearance of species with a specialized diet of C_4 plants, and the rise of these plants to dominance in Great Plains ecosystems occurred asynchronously (Fox & Koch 2003).

Replacement of closed forest by woodland or savannah ecosystems on the Great Plains began in the Early Miocene (15–23 Ma; reviewed by Jacobs *et al.* 1999), some 15-Myr earlier than the equivalent change on the Indian subcontinent (§4*b*). It was accompanied by a period of continuous diversification in the grasses, as they adapted to the new open habitats (Jacobs *et al.* 1999; Kellogg 2001), and the coevolution of grazing mammals. By 15 Ma *ca* 25% of mammals were typically hypsodont grazers, with tooth morphology indicating a mixed diet for 35% and browsing diet for 40% of the remaining species (figure 6*a*). By the height of the Middle Miocene Climatic Optimum (15–17 Ma; Zachos *et al.* 2001), these communities were more

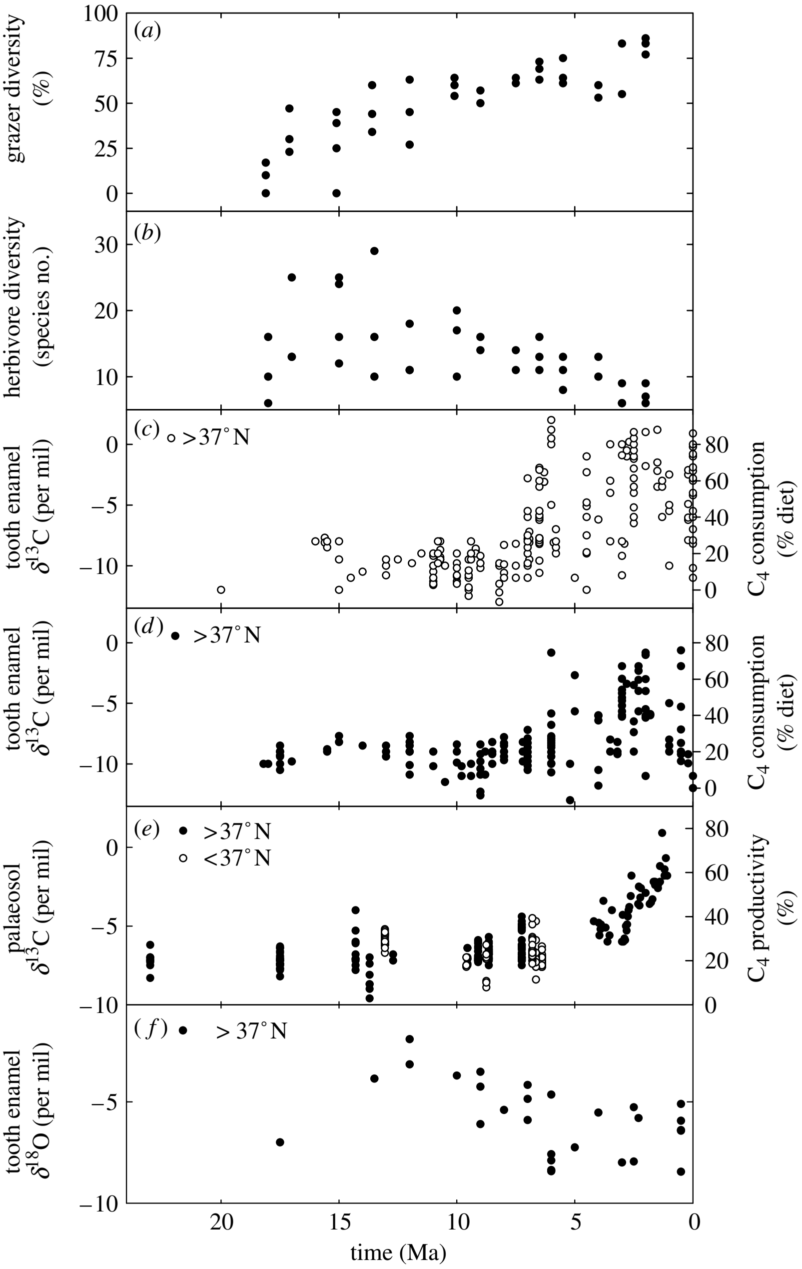

Figure 6. Geological evidence from the Great Plains of North America of ecosystem dynamics and climate change through the Late Miocene and Pliocene. Data sources are: (*a*) and (*b*) Janis *et al.* (2000); (*c*) Cerling *et al.* (1997), Latorre *et al.* (1997) and Passey *et al.* (2002), (*d*) Cerling *et al.* (1997) and Passey *et al.* (2002), (*e*) Fox & Koch (2003), and (*f*) MacFadden *et al.* (1999) and Passey *et al.* (2002).

diverse than those of modern East Africa (figure 6*b*; Janis *et al.* 2000).

Analyses of δ^{13}C from tooth fossils of the Middle Miocene indicates that the new species of specialized grazers subsisted primarily on C_3 grasses at sites across the Great Plains (figure 6*c*,*d*). A slight positive bias in these δ^{13}C values can be interpreted as either a drought response in the C_3 food plants or a diet composed of up to 25% C_4 grasses (Passey *et al.* 2002). The latter interpretation is consistent with δ^{13}C evidence from palaeosols (figure 6*e*), suggesting a 12–34% contribution by C_4 grasses to plant productivity (Fox & Koch 2003), and analyses based on the taxonomic identification of fossil phytoliths, indicating the dominance of C_3 grasses with a lesser C_4 component (Strömberg 2004). The scenario of savannah ecosystems populated by C_3 grasses contrasts with the situation in the Indian subcontinent, where the creation of open woodland and savannah starting at 8.5 Ma permitted the immediate dominance of ecosystems by C_4 grasses. Clearly, the loss of woody vegetation in North America that began sooner, at 23 Ma, did not occur at a time when conditions favoured the dominance of C_4 grasses.

δ^{13}C values unequivocally indicating a diet composed of C_4 plants are first detected 6.6 Ma in horse teeth from the Coffee Ranch formation in Texas (figure 6*c*; Passey *et al.* 2002), 9 Myr after the peak of mammalian diversity (figure 6*b*; Janis *et al.* 2000), and more than 2 Myr after the equivalent change in Pakistan (figure 5*b*). The transition to a predominantly C_4 diet was complete in some Texan animals by 6 Ma, but others continued to feed on C_3 plants or had a mixed diet (figure 6*c*; Cerling *et al.* 1997). The cause of such a long delay in the evolution of grazers like the modern bison, with their ability to subsist on a specialized C_4 diet, remains unclear (Passey *et al.* 2002).

Comparison of the transition from C_3-based to C_4-dominated diets (figure 6*c*,*d*) and the equivalent change in regional plant productivity (figure 6*e*) is complex because the region spans a wide latitudinal (climatic) gradient. Data from fossil teeth indicate that the dietary transition occurred more rapidly in the southern Great Plains (figure 6*c*), being followed more slowly in the northern part of the region (figure 6*d*). This observation suggests an interaction with temperature, where C_4 plants rose to dominance earlier in warmer climates (Cerling *et al.* 1997). Palaeosol $\delta^{13}C$ data from the latest Miocene and Pliocene are not yet available for the southern Great Plains (figure 6*e*), but values from the northern part of the region (figure 6*e*) match the late rise in $\delta^{13}C$ of fossil teeth (figure 6*d*). Some indication of a delay between the origin of C_4-based diets and increasing C_4 plant productivity requires further investigation. It is also interesting to note that Late Miocene horses co-occurring with thermophile tortoises had a higher proportion of C_4 grasses in their diets (Passey *et al.* 2002), perhaps suggesting heterogeneity in the Great Plains regional climate, and a greater abundance of C_4 grasses in warmer areas (Passey *et al.* 2002). However, while these arguments are entirely reasonable, they cannot be the full explanation. It is unclear, for example, why C_4 plants only come to dominate North American ecosystems in the globally cool Pliocene, instead of during the Middle Miocene Climatic Optimum (15–17 Ma) when global temperatures were warmer. Part of the answer would seem to lie with the role played by regional climatic warming or changing patterns of precipitation.

Our understanding of regional climate change accompanying ecological transitions in diet and vegetation is incomplete at present, but some insights have been obtained from analyses of $\delta^{18}O$ from tooth enamel. $\delta^{18}O$ values decline in the Middle to Late Miocene (13–5 Ma), and remain relatively stable from the Pliocene onwards (less than 5 Ma; figure 6*f*), most likely reflecting a combination of physiological and behavioural changes in the animals and climatic change (Passey *et al.* 2002). These patterns are also seen in palaeosol carbonates during the Late Miocene and Pliocene, once the effects of latitude have been removed by statistical procedures (Fox & Koch 2004). Decreases in tooth and palaeosol $\delta^{18}O$ during the Late Miocene are consistent with the global cooling trend shown for the same period by $\delta^{18}O$ in deep-sea sediments (Zachos *et al.* 2001). However, the stabilization of these terrestrial values in the Pliocene appears to be at odds with the ocean records, which suggest increased ice volume and continued global cooling over the past 5 Myr. Relative stability in the $\delta^{18}O$ of North American mammal tissues therefore points to regional warming set against a background of global cooling, or climatic change involving a warming of rainfall, caused either by an increase in summer rains or switch to a more southerly source of rainwater. Although speculative, this idea is consistent with the climatic change expected to accompany the narrowing and closure of the ocean straits between North and South America during the latest Miocene and Pliocene. Ocean–atmosphere model simulations suggest that an open sea passage through Central America led to a collapse of the North Atlantic thermohaline circulation (Maier-Reimer *et al.* 1990). Narrowing and closure of these straits would have reinstated circulation (Mikolajewicz and Crowley 1997), an event associated in climate model simulations with warming and increased humidity over the Great Plains (Vellinga and Wood 2002). Perhaps an increase in summer moisture drove a Late Miocene transition from arid C_3 grassland with a minor C_4 component, to a more mesic C_4 prairie ecosystem (Keeley & Rundel 2005)?

5. ARE C_4 PLANTS TEN-TIMES OLDER THAN WE THINK?

The origin of C_4 plants during the Oligocene is consistent with the C_4 photosynthetic pathway evolving as an adaptation to CO_2 starvation (§3), with climate playing an important part in determining the relative performance of C_3 and C_4 plants in the field (§4). From these observations, the question arises as to whether plants experienced environmental conditions driving the selection of a C_4 carbon-concentrating mechanism earlier in their evolutionary history (Beerling 2005). In this section, we assess the theoretical potential for variations in atmospheric composition and climate to select for the C_4 photosynthetic pathway in the Late Palaeozoic and Mesozoic (65–365 Ma), and report results from an isotope-based survey of fossil plants, designed to search for evidence of its early origination.

Geochemical models of Earth's atmospheric evolution clearly indicate large variations in atmospheric CO_2 and O_2 throughout the 470 Myr interval that plants have colonized the continents (figure 7*a*; Berner 2005). Model predictions of CO_2 variations are generally well-supported by independent evidence based on analyses of a wide range of fossil materials, including soils, leaves of land plants, and the isotopic composition of molecular biomarkers of marine phytoplankton metabolism (Crowley & Berner 2001; Berner 2005). Corresponding variations in atmospheric O_2 occurred largely because, on multimillion-year time-scales, the two cycles are coupled through the burial of organic carbon both on land and in the oceans (Berner 2005). The models show a striking decrease in atmospheric CO_2 and an increase in O_2 during the Late Palaeozoic (figure 7*a*; 320–500 Ma), which, through different mechanisms, resulted from the rise of vascular land plants and the spread of rooted forests (Berner 2005). Evolutionary trends towards increased vegetation activity accelerated the extent and rate of silicate rock weathering, the primary sink for CO_2 in the long-term, and caused a massive increase in sedimentary organic carbon burial, most obviously manifested as the formation of extensive Carboniferous coal deposits (Berner 2005).

Identifying times favouring C_4 over C_3 photosynthesis during the Phanerozoic (the past 570 Myr) requires consideration of the influence of both atmospheric composition and climate. We calculated

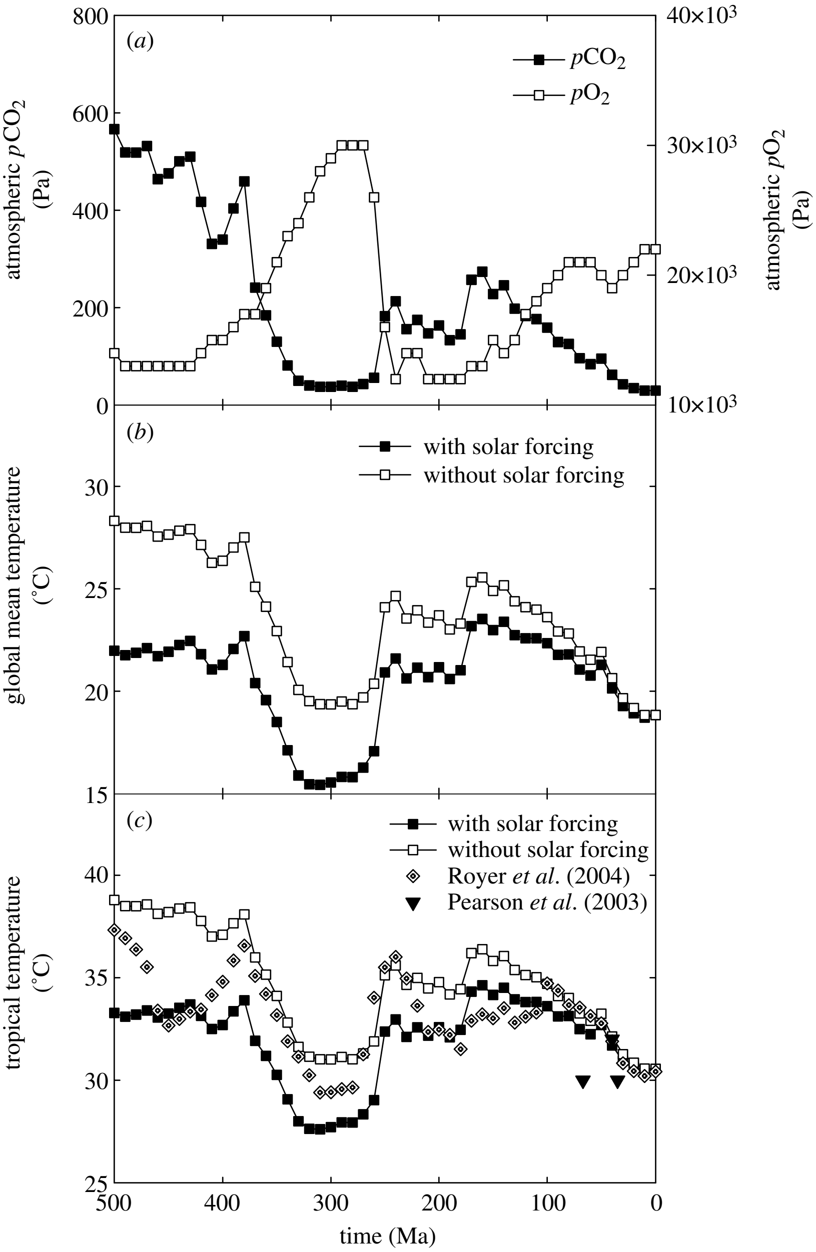

Figure 7. Variations in: (*a*) the partial pressure of atmospheric CO_2 and O_2 over the past 500 Myr of the Phanerozoic, (*b*) calculated changes in global mean surface temperature, and (*c*) calculated changes in the tropics. Also shown in (*c*) are estimates of tropical temperatures from oxygen isotope ratios of fossil foraminifera shells (Pearson *et al.* 2001) and calculations from marine calcium carbonate fossils (Veizer *et al.* 2000) corrected for the CO_2-effects on ocean pH (Royer *et al.* 2004).

global changes in climate through the Phanerozoic to provide a basis for this assessment, by modifying a simple zero-dimensional model of planetary energy balance that collapses latitude, altitude and longitude to give a single global mean value for a given atmospheric CO_2 concentration (figure 7*b*; Beerling & Woodward 2001). The calculations include the direct effects of CO_2, via the atmospheric greenhouse effect, and the effect of the estimated *ca* 5% increase in the Sun's output over the past 500 Myr (Caldeira & Kasting 1992). Calculated changes in global mean surface temperature strongly track the concentration of atmospheric CO_2 (figure 7*a*,*b*), and are sensitive to changes in solar forcing, especially in the early part of the Palaeozoic, when the global climate is 5 °C cooler than expected from a simple consideration of the greenhouse effect alone (figure 7*b*). The drastic decline in atmospheric CO_2 caused by the rise of vascular land plants leads to a pronounced global climatic cooling (320–500 Ma) as evidenced by the widespread Permo-Carboniferous continental glaciation (Crowell 1999; Royer *et al.* 2004).

However, C_4 plants are usually distributed in sub-tropical or tropical climates, where temperatures exceed the crossover temperature for photosynthetic efficiency (figure 2). We approximated long-term trends in tropical land surface temperature (30° N to 30° S) over the past 500–Myr using a conversion factor based on results from general circulation models of the climate system at different times in Earth history and a range of atmospheric CO_2 concentrations (figure 7*c*; Otto-Bliesner 1995; Valdes & Crowley 1998; Otto-Bliesner *et al.* 2000; Beerling & Woodward 2001). The approach is necessarily approximate, but predicted

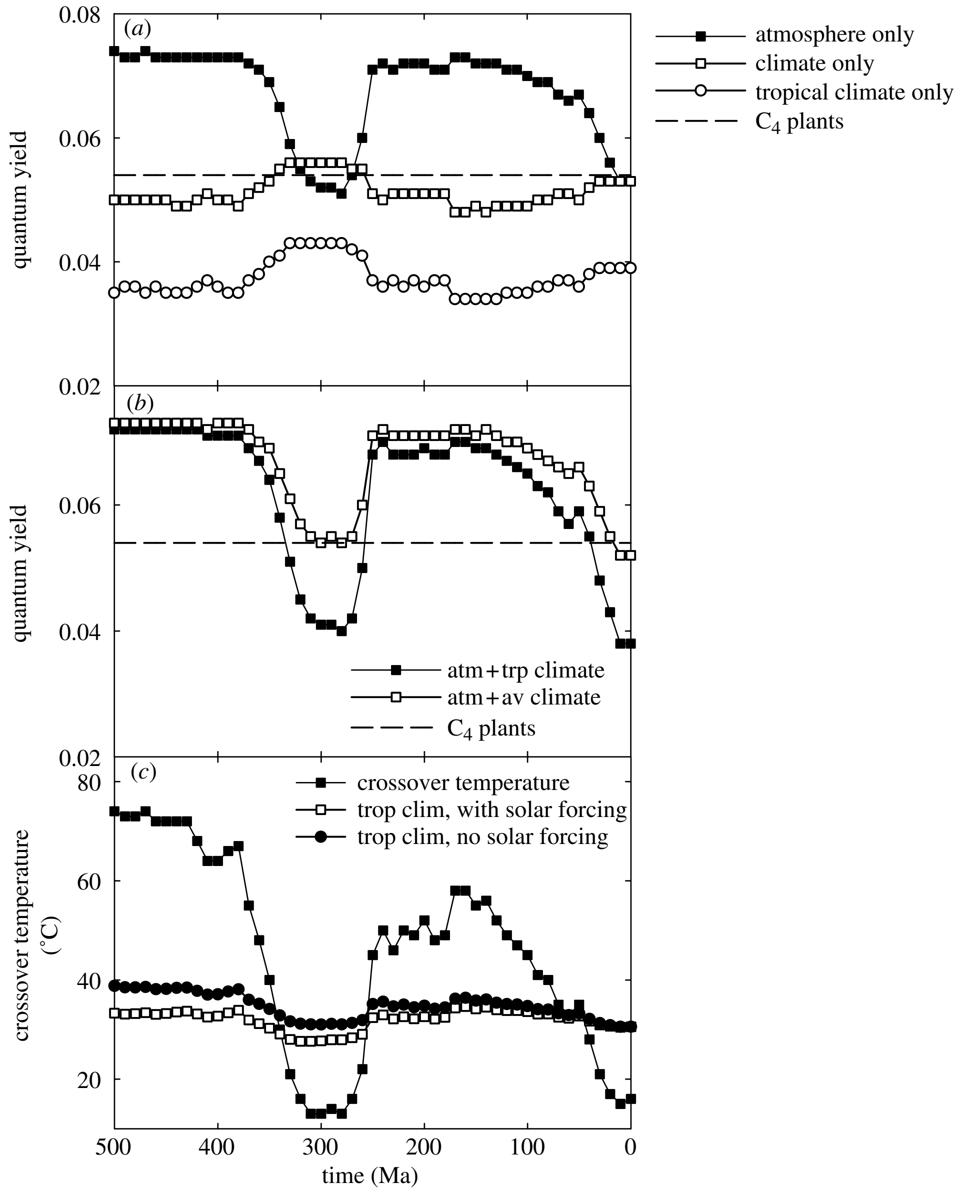

Figure 8. Calculated changes in: (*a*) quantum yield of C_3 and C_4 plants through the past 500 Myr of the Phanerozoic, (*b*) the effects of CO_2 in combination with either tropical climate (filled squares) or global mean climate (open squares) on quantum yield, and (*c*) the calculated crossover temperature for C_4 and C_4 plants in comparison with the tropical climate (with and without the effects of solar forcing) shown in figure 7.

temperatures can be compared with those obtained by analysis of shallow marine calcium carbonate fossils (Veizer *et al.* 2000), after correction for the effects of seawater pH (Royer *et al.* 2004), and the oxygen isotope composition of foraminifera from the tropical oceans during past warm climate intervals (Pearson *et al.* 2001). Comparison of observed and predicted trends in tropical land surface temperatures shows a reasonable degree of agreement and provides support for the approach (figure 7*c*).

We determined the likely effects of these changes in atmospheric composition (figure 7*a*) and climate (figure 7*c*) on C_3 and C_4 plant performance using the quantum yield approach (figure 2; Ehleringer *et al.* 1991, 1997; Cerling *et al.* 1997). Long-term variations in atmospheric composition produced a marked drop in the predicted quantum yield of C_3 plants during the Permo-Carboniferous (245–365 Ma) that was only partially offset by the effects of a cooler climate (figure 8*a*). Further analyses of the combined influence of variations in atmospheric composition and tropical climate revealed the Permo-Carboniferous and Late Tertiary (less than 50 Ma) to be the only times throughout the entire *ca* 470 Myr history of terrestrial plant life when C_3 quantum yield dropped below that of C_4 plants (figure 8*b*). Calculation of the crossover temperature (figure 2) reinforces this view. The temperatures at which C_4 plants attain higher quantum yields than their C_3 counterparts far exceed the highly conserved high temperature limits for photosynthesis (40–50 °C; Larcher 1994) for most of the Phanerozoic except during the Permo-Carboniferous and the Tertiary (figure 8*c*). This result is quite robust, being insensitive to uncertainties in our approach to calculating tropical temperatures (figure 8*c*). Since C_4 photosynthesis did actually arise in the Tertiary (§2), these results and those from modelling photosynthesis (Beerling 2005) suggest the Permo-Carboniferous as a time when environmental conditions could have strongly promoted the evolutionary selection of C_4 plants.

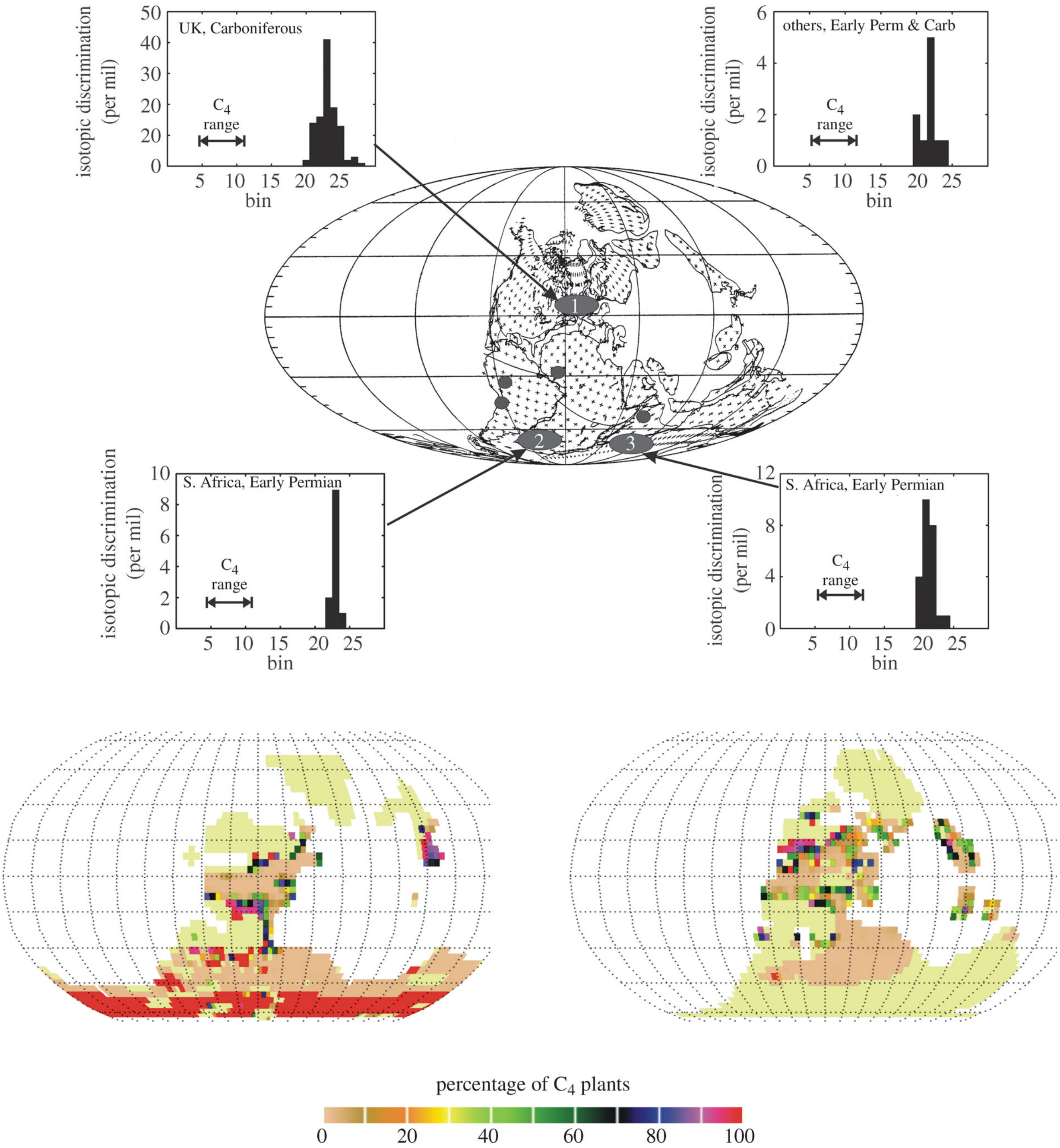

Figure 9. Simulated global distribution of C_4 plants using a generalized dynamic global vegetation model (Beerling & Woodward 2001) and either the land surface climatology from the UGAMP (lower left map) or NCAR (lower right map) GCMs. Central picture shows the palaeogeography of the Late Carboniferous (Westphalian; Scotese & McKerrow 1990) and the main regions/localities from which fossil plants were sampled for stable carbon isotope analysis (see appendix A for details). The frequency histograms depict the calculated values of carbon isotope discrimination and, for comparison, the values expected if plants operated with the C_4 photosynthetic pathway.

To identify possible geographic regions of C_4 plant occurrence in the Permo-Carboniferous we extended our analysis to the global scale (Beerling & Woodward 2001). We achieved this aim using a process-based generalized dynamic global vegetation model forced with two contrasting climates of the Late Carboniferous (*ca* 305 Ma), simulated by different general circulation models (GCMs); the UK Universities Global Atmospheric Modelling Programme (UGAMP) and the National Centre for Atmospheric Research (NCAR) GCM (Valdes & Crowley 1998; Otto-Bliesner & Shields, unpublished). The global simulations are made assuming the C_4 photosynthetic pathway had evolved, predicting regions where C_4 plants dominated the vegetation.

Our simulations indicate consistent tropical regions where C_4 plants could have persisted, regardless of the underlying land surface climatology (figure 9). The simulated presence of C_4-dominated vegetation in the high latitudes is also indicated with the warmer, drier high latitude climate of the UGAMP GCM but not the with the much colder NCAR GCM climate (figure 9). It is possible that an increased frequency of ancient wildfires due to the high O_2 content of the atmosphere would have allowed C_4 plant distributions to expand considerably through the removal of forest cover (section 4; Beerling & Woodward 2001). However, although a high O_2 atmosphere may have increased the probability of ignition of a fuel source (Watson *et al.* 1978), there is no evidence that it enhanced the spread of fire (Wildman *et al.* 2004). Indeed, Wildman *et al.* (2004) reported from their thermochemistry and flame-spread experiments that, with moisture contents typical of forest floor fuel sources, there was no sustained burning in an atmosphere of between 21 and 35% O_2.

To examine the possibility of C_4 plant occurrence this far back in time, we conducted an isotopic survey of Late Carboniferous and Early Permian plant fossils from three key regions (Beerling 2005), encompassing the tropics and the high southern latitudes (appendix A). Plant materials were sampled from the collections of the British Geological Survey, Keyworth, and the Natural History Museum, London, and supplemented with Early Permian plant remains from the northern Prince Charles Mountains, East Antarctica (McLoughlin & Drinnan 1997), kindly supplied by Stephen McLoughlin (University of Melbourne, Australia). From these sources, we sampled and analysed foliage and stem material of Late Carboniferous lycopsids, the dominant plant group in the tropics (DiMichele *et al.* 2001), as well as a number of other herbaceous and woody plant groups (figure 9, group 1; appendix A). We also sampled the foliage and bark of the short woody plant *Glossopteris* which dominated the high southern latitudes in the Early Permian (Chaloner & Lacey 1973), from two different regions, Antarctica and South Africa (groups 2 and 3, figure 9; appendix A). The isotopic survey was completed by a small number of samples from a scattered set of localities encompassing areas (Peru, India, Brazil, Russia and Ghana) of predicted C_4 occurrence. Results are expressed in terms of isotopic discrimination (Δ) to remove the effects of the $\delta^{13}C$ of atmospheric CO_2, which were estimated using a smoothed marine carbonate $\delta^{13}C$ curve (Veizer *et al.* 1999), assuming equilibrium between these reservoirs.

We find no isotopic evidence of C_4 photosynthesis in any Late Carboniferous or Early Permian fossil plants remains, irrespective of location (figure 9). This is in agreement with previous reports of no clear C_4 isotopic signature in fossil plants pre-dating the Tertiary (Bocherens *et al.* 1994; Beerling 2005). Two studies have interpreted spurious isotopic values as indicative of C_4 or CAM plants (Wright & Vanstone 1991; Jones 1994). Wright & Vanstone (1991) sampled thin clay layers between thick marine limestone sequences, interpreted as exposed surfaces where the calcification of soils into rhizolith crusts or root mats had occurred. However, it is doubtful whether these clays, and the postulated soil carbonates associated with them, formed in a manner suitable for faithfully preserving $\delta^{13}C$, and the claim was unsupported by any anatomical information (Cerling 1999). The study of isotopic charcoal remains of putative gymnosperms and lycopods from Donegal, Ireland requires further investigation to better resolve the nature of the anomalous values (Jones 1994).

We conclude that the atmospheric composition of the Late Carboniferous and Early Permian would have provided a powerful selective pressure for the evolution of C_4 photosynthesis, yet isotopic surveys of fossil plants have so far failed to detect any evidence of the pathway at this time. We recognize, of course, that more exhaustive isotopic surveys may yet reveal the presence of the C_4 photosynthetic pathway in the Permo-Carboniferous. However, the lack of evidence for its existence leads us to ask in §7 more fundamentally whether an essential physiological precursor of C_4 photosynthesis is absent in ancient plant lineages dating from the Permo-Carboniferous.

6. ARE C_4-LIKE ENZYME SYSTEMS PRESENT IN ALL PLANTS?

The realization that some modern C_3 plants actually operate with components of the C_4 photosynthetic pathway (Hibberd & Quick 2002) suggests a hypothesis for explaining the absence of the syndrome in Permo-Carboniferous taxa; if ancient plant lineages lack this component pathway, they may lack an essential pre-condition for evolving C_4 photosynthesis.

Hibberd & Quick (2002) reported that tobacco (*Nicotiana tabacum*) and celery (*Apium graveolens*), both typical C_3 plants, use C_4-type enzymes to strip CO_2 from four-carbon organic acids dissolved in the xylem sap. The authors fed these species with the C_4 organic acid malate and reported its assimilation into sugars by the green photosynthetic cells located within the stems. The pathway they described requires C_4-type decarboxylase enzymes to produce CO_2 from malate, and Rubisco to fix it into sugars. Both were present in the vascular bundles of tobacco and celery, with the decarboxylases at much higher levels than in leaf cells. The system appears to help the plants conserve carbon, by converting malate produced by respiration in the roots into sucrose and starch for growth (Raven 2002).

Perhaps a pathway for recapturing respired CO_2 that would have otherwise been lost to the atmosphere represents a first step in C_4 plant evolution (Monson 1999)? It is possible then that early ferns and gymnosperms, the two groups that predominated in the Late Palaeozoic, lacked the necessary anatomy or physiology to evolve even this first step towards the C_4 photosynthetic pathway (Raven 2002). To investigate this possibility, experiments were conducted with two species of plants from ancient lineages of ferns (*Osmunda regalis*) and gymnosperms (*Ginkgo biloba*; Palmer & Quick, unpublished). Following the methodology of Hibberd & Quick (2002), ^{14}C labelled malic acid was fed in a 0.1 mM malate carrier solution to cut petioles of *O. regalis* and *G. biloba* for 30 min in the light. Plants were then flash frozen, heated in 70% ethanol to remove soluble sugars and exposed to X-ray film.

The results of this preliminary investigation (figure 10; Palmer & Quick, unpublished data) reveal an accumulation of ^{14}C in insoluble material within cells associated with the vascular tissue. These data are consistent with the idea that CO_2 can be removed from malate in the xylem transpiration stream and fixed by nearby photosynthetic cells. Further support for this possibility was obtained by imaging the distribution of

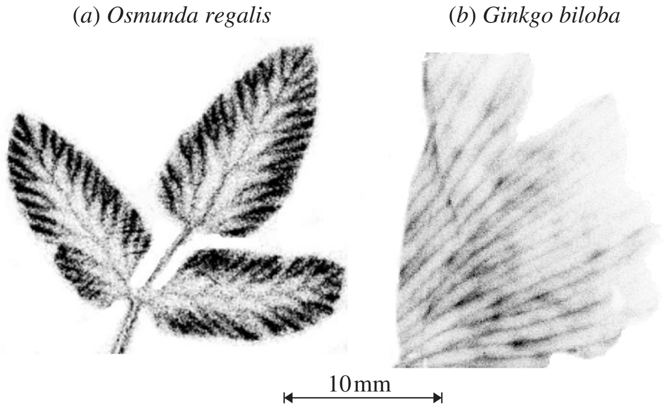

Figure 10. Photographic evidence showing the incorporation of ^{14}C-labelled malate into insoluble compounds in leaves of (*a*) *Osmunda regalis* and (*b*) *Ginkgo biloba* (Palmer & Quick, unpublished data). Note the ^{14}C activity concentrated around vascular tissues.

chlorophyll in the photosynthetic cells of thin sections of *O. regalis* and *G. biloba* petioles. Under fluorescence microscopy, abundant chlorophyll was detected preferentially surrounding the xylem and phloem tissue in the petioles of both species (Fletcher & Quick, unpublished data). Although activities of the decarboxylase enzymes within the petioles of these two species still need to be assayed, these preliminary datasets would seem to support earlier speculation that photosynthesis in vascular tissue was common in the past (Raven 2002).

7. FUTURE DIRECTIONS

This review has identified several key uncertainties in our understanding of C_4 plant evolution that point to future research priorities. Extensive isotopic evidence from fossil materials clearly indicates major changes in the proportion of plants with the C_4 photosynthetic pathway over the past 30 Myr of the Tertiary. Up until now these regional-scale patterns of C_4/C_3 vegetation dynamics across the Earth's surface have been interpreted largely on the basis of the quantum yield model, in conjunction with inferred changes in the global environment. However, our review indicates that neither the switch to C_4 dominated ecosystems in North America nor on the Indian sub-continent are adequately explained in this way, with evidence instead pointing to important roles for the pattern of seasonal rainfall. A clear example of how misleading the quantum yield model can be comes from CO_2 enrichment experiments in a tallgrass prairie community, which show an increase in abundance of C_4 over C_3 grasses via a feedback involving plant water relations (Owensby *et al.* 1999). New approaches are, therefore, required to understand what actually happened in the past and why. We suggest that a significant change in direction is required with new foci on experiments with C_3 and C_4 plants grown under manipulated environmental conditions, and on approaches for better quantifying the role of disturbance from sedimentary records.

Experiments are required to investigate how the differential physiological responses of C_3 and C_4 species to climate and CO_2 translate into ecological success. Crucially, the emphasis must shift from models centred on physiology towards ecology, focusing on the processes linking leaf physiology with differential growth, resource-use, and survival responses in C_3 and C_4 plants. This approach will lead to a better understanding of competition, reproductive fitness and survivorship, factors directly responsible for evolutionary and ecological success. We currently know little about the differences between C_3 and C_4 species in their seasonal responses of photosynthesis and the timing of growth and reproduction in regions with contrasting climatic regimes. Recent experimental work has highlighted important interactions between high CO_2 responses in C_3 and C_4 plants and the efficiency of carbon, water and nutrient-use. However, it is unclear how these responses change under CO_2-starvation, and how they interact with plant tolerance of extreme temperatures and drought. To comprehensively address these uncertainties, a programme of experiments must aim to establish the key processes by linking controlled environment, common garden and field-based approaches.

Detailed studies in the Indian sub-continent and charcoal abundance in sediment cores from the Pacific Ocean suggest an association between the occurrence of fire and the expansion of ecosystems dominated by C_4 plants. A key priority for future research must be to investigate the strength of this likely interaction, through more detailed isotopic and morphological examination of combustion products in well-dated, high-resolution ocean sediment cores. Careful selection of ocean cores with footprints originating from contrasting continental sources must first identify whether the coevolution of C_4 ecosystems and fire is a general phenomenon. The issue of whether increasing fire frequency is specifically associated with the rise of C_4 grasslands *per se*, or with the replacement of forest by grasslands in general also needs to be addressed. A test of this issue can be achieved by comparison of the evidence for fire history in the Indian subcontinent and North America, with their contrasting histories of grassland evolution.

Further back in time, beyond the Tertiary, it is still far from clear why unusual atmospheric conditions did not select for C_4 photosynthesis. Current ideas concern the evolution of high vein densities in plants experiencing drought from water deficit or salinity as a prerequisite for C_4 Kranz anatomy (Sage 2001).

Whether these anatomical properties are confined to certain groups of flowering plants is unclear. A major direction for future research must, therefore, be identification of the developmental genetic and environmental controls over vascular tissue production in leaves, and the extent to which these can operate in non-flowering plants.

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APPENDIX A

Carbon isotope composition ($\delta^{13}C$) and isotopic discrimination ($\Delta^{13}C$) of Carboniferous and Permian fossil plants.

fossil specimen	specimen number	locality	age	material analysed	$\delta^{13}C$ (‰)	$\Delta^{13}C$ (‰)
A. British Geological Survey collections, Keyworth						
Lycopsids						
Bothrodendron minutifolium	RC2872	South Yorkshire, UK	Westphalian B	foliage	−22.5	20.9
B. minutifolium	51813	Kent, UK	Westphalian	foliage	−23.0	21.4
Cyperites eileitus	4168	South Yorkshire, UK	Westphalian B	foliage	−22.4	20.8
Lepidostrobophyllum alatum	RC4838	Somerset, UK	Westphalian D	foliage	−22.1	20.5
L. intermedium	RC3134	Staffordshire, UK	Westphalian	foliage	−21.9	20.2
Lepidophyllum sp.	6127	Lancashire, UK	Westphalian	foliage	−22.0	20.3
Lepidodendron gracile	5284	UK	Westphalian	foliage	−22.3	20.7
L. lanceolatum	JP218	Monmouthshire, UK	Westphalian	foliage	−22.9	21.2
L. lycopodioides	18239	UK	Westphalian	foliage	−22.2	20.6
Sigillariophyllum bicarinatelm	4907	South Yorkshire, UK	Westphalian B	foliage	−23.7	22.1
Sigillariostrobus rhombractiatus	1177	South Yorkshire, UK	Westphalian B	foliage	−22.0	20.3
Lepidodendron aculeatum	2640	South Yorkshire, UK	Westphalian A	bark	−23.4	21.8
	4033	South Yorkshire, UK	Westphalian A	bark	−23.8	22.2
	4034	South Yorkshire, UK	Westphalian A	bark	−24.8	23.3
	7105	South Yorkshire, UK	Westphalian A	bark	−25.1	23.6
	3821	South Yorkshire, UK	Westphalian A	bark	−23.7	22.1
	2729	South Yorkshire, UK	Westphalian A/B	bark	−23.5	21.9
	2643	South Yorkshire, UK	Westphalian A/B	bark	−24.4	22.9
	3892	South Yorkshire, UK	Westphalian C	bark	−24.9	23.4
	1009	South Yorkshire, UK	Westphalian C	bark	−26.0	24.5
	2482	Ayrshire, UK	Westphalian A	bark	−27.7	26.3
	2481	Warwickshire, UK	Westphalian A/B	bark	−24.1	22.5
	1765	Somerset, UK	Westphalian C/D	bark	−26.1	24.6
	5686	Trane Coll., UK	Westphalian	bark	−27.7	26.3
L. berwickense	2775	Berwickshire, UK	Westphalian	bark	−24.2	22.6
L. camertion	3246	Somerset, UK	Westphalian C	bark	−24.4	22.9
L. canobianum	3127	Dumfrieshire, UK	Carboniferous limestone series	bark	−23.7	22.1
L. feistmantel	4946	South Yorkshire, UK	Westphalian B	bark	−24.3	22.8
L. fusiforme	4984	South Yorkshire, UK	Westphalian B	bark	−24.0	22.4
L. gaudryi	5157	South Yorkshire, UK	Westphalian B	bark	−27.4	26.0
	4877	Lanarkshire, UK	Westphalian	bark	−24.3	22.8
L. jaraczwskii	4878	South Yorkshire, UK	Westphalian A/B	bark	−23.7	22.1
	1414	Gartness Colliery, Aidrie, UK	Westphalian A	bark	−24.6	23.1
	4616	Lancashire, UK	Westphalian A	bark	−23.4	21.8

(*Continued.*)

fossil specimen	specimen number	locality	age	material analysed	δ^{13}C (‰)	Δ^{13}C (‰)
L. jaschei	4288	Herbertshire, UK	Scottish Carboniferous limestone series	bark	−24.8	23.3
	4289	Herbertshire, UK	Scottish Carboniferous limestone series	bark	−24.6	23.1
	2459	Stirlingshire, UK	Scottish Carboniferous limestone series	bark	−24.1	22.5
	3043	Stirlingshire, UK	Scottish Carboniferous limestone series	bark	−24.0	22.4
	2452	Stirlingshire, UK	Scottish Carboniferous limestone series	bark	−24.0	22.4
L. lansburgi	1549	Ayrshire, UK	Westphalian A	internal stem	−26.0	24.5
L. lycopodoides	1468	South Yorkshire, UK	Westphalian A/B	bark	−22.8	21.2
	2717	South Yorkshire, UK	Westphalian A/B	bark	−22.0	20.3
	2787	Low Moor, Yorkshire, UK	Westphalian A/B	bark	−23.9	22.3
	1868	Cadedy Coll., Yorkshire, UK	Westphalian	bark	−24.1	22.5
	1869	Cadedy Coll., Yorkshire, UK	Westphalian	bark	−26.0	24.5
L. mannabachense	2474	Yorkshire, UK	Westphalian A/B	bark	−25.3	23.8
	2473	South Yorkshire, UK	Westphalian A/B	bark	−24.5	23.0
L. nathorsti	5751	Berwickshire, UK	Westphalian	bark	−24.8	23.3
	2957	Berwickshire, UK	Westphalian	bark	−24.4	22.9
	2954	Berwickshire, UK	Westphalian	bark	−23.2	21.6
L. obovatum	4868	South Yorkshire, UK	Westphalian A	bark	−24.6	23.1
	2641	South Yorkshire, UK	Westphalian A	bark	−24.3	22.8
	2644	South Yorkshire, UK	Westphalian A/B	bark	−24.5	23.0
	3362	Lancashire, UK	Westphalian	bark	−24.4	22.9
	3367	Lancashire, UK	Westphalian	bark	−24.0	22.4
	3366	Lancashire, UK	Westphalian	bark	−25.1	23.6
	6141	Lancashire, UK	Westphalian	bark	−23.6	22.0
	2475	Lancashire, UK	Westphalian	bark	−24.0	22.4
	2469	Northumberland, UK	Westphalian A	bark	−26.0	24.5
	2470	Northumberland, UK	Westphalian A	bark	−23.4	21.8
L. ophiurus	413	Staffordshire, UK	Westphalian A/B	internal stem	−24.2	22.6
L. peachii	2466	Durham, UK	Westphalian	bark	−24.4	22.9
L. rimosum	3894	South Yorkshire, UK	Westphalian C	bark	−25.5	24.0
	5155	South Yorkshire, UK	Westphalian C	bark	−25.7	24.2
	4894	South Yorkshire, UK	Westphalian A/B	bark	−23.9	22.3
L. rhodeanum	2494	Grange Colliery, UK	Carboniferous Limestone series	bark	−24.3	22.8
L. simile	4893	South Yorkshire, UK	Westphalian A/B	bark	−23.6	22.0
	4892	South Yorkshire, UK	Westphalian A/B	bark	−25.9	24.4
	4728	South Yorkshire, UK	Westphalian	bark	−25.3	23.8
	6372	Staffordshire, UK	Westphalian	bark	−26.5	25.1
	6106	Devon, UK	Westphalian	bark	−23.2	21.6
L. spitsbergense	6509	Spitsbergen	Westphalian	bark	−25.9	24.4
L. tijoui	3946	Holland	Westphalian	bark	−22.3	20.7
L. veltheimianum	29	Lothian, UK	Scottish Carboniferous limestone series	bark	−24.7	23.2
	3482	Lothian, UK	Scottish Carboniferous limestone series	bark	−23.7	22.1
	4828	Manor Powis Colliery, UK	Carboniferous limestone series	bark	−25.5	24.0
	60	Grange Colliery, UK	Carboniferous limestone series	bark	−25.1	23.6

(*Continued.*)

fossil specimen	specimen number	locality	age	material analysed	δ^{13}C (‰)	Δ^{13}C (‰)
	82	Grange Colliery, UK	Carboniferous limestone series	internal stem	−28.3	27.0
	71	Grange Colliery, UK	Carboniferous limestone series	stem scars	−24.2	22.6
	61	Grange Colliery, UK	Carboniferous limestone series	stem scars	−24.8	23.3
	88	BurghLee Pit, UK	Carboniferous limestone series	internal stem	−23.5	21.9
	65	Rafstoch Quarry, Stirling, UK	Carboniferous limestone series	bark	−25.3	23.8
	67	Rafstoch Quarry, Stirling, UK	Carboniferous limestone series	bark	−24.2	22.6
	68	Rafstoch Quarry, Stirling, UK	Carboniferous limestone series	bark	−25.9	24.4
	2624	Rafstoch Quarry, Stirling, UK	Carboniferous limestone series	bark	−24.0	22.4
	48	Rafstoch Quarry, Stirling, UK	Carboniferous limestone series	bark	−25.5	24.0
	46	Northumberland, UK	Westphalian	bark	−24.7	23.2
L. wedekindi	2157	South Yorkshire, UK	Westphalian B	bark	−25.7	24.2
Lepidocystis vesicularis	1363	Pennsylvania, USA	Westphalian A	bark	−23.8	22.2
Lyginoendron landsburgii	3220	Ayrshire, UK	Westphalian	bark	−22.7	21.1
Sphenopsids						
Sphenophyllum emarginatum	76043	Staffordshire, UK	Westphalian	foliage	−27.2	25.8
S. majus	RC2848	South Yorkshire, UK	Westphalian B	foliage	−24.4	22.9
Horsetails						
Annularia fratens	88	UK	Westphalian	foliage	−22.7	21.1
A. radiata	4275	Limburg, Holland	Westphalian	foliage	−23.1	21.5
A. stellata	75930	Somerset, UK	Westphalian D	foliage	−24.3	22.8
free sporing plants with gymnospermous wood						
Archaeopteris reussi	RC2823	Staffordshire, UK	Westphalian	foliage	−24.1	22.5
Seed ferns						
Alethopteris grandiniodes	75930	Somerset, UK	Westphalian D	foliage	−23.3	21.7
A. lonchitica	RC2137	Cumbria, UK	Westphalian	foliage	−23.8	22.2
A. rubescens	250	Somerset, UK	Westphalian D	foliage	−22.3	20.7
Aulacotheca dixiana	76261	UK	Westphalian	foliage	−23.0	21.4
Neuropteris acuminata	RC2858	South Yorkshire, UK	Westphalian B	foliage	−22.4	20.8
N. flexuosa	14254	Gloucstershire, UK	Westphalian	foliage	−24.0	22.4
N. macrophylla	14272	Somerset, UK	Westphalian D	foliage	−23.4	21.8
N. tenuifolia	14231	UK	Westphalian	foliage	−23.3	21.7
Neuropteris spp.	RC2976	Somerset, UK	Westphalian D	foliage	−21.5	19.8
Sphenopteris affinis	5190	Lothian, UK	Westphalian	foliage	−23.6	22.0
S. obtusifolia	RC3335	Staffordshire, UK	Westphalian	foliage	−25.3	23.8
Tree ferns						
Acitheca polymorpha	RC3080	Somerset, UK	Westphalian D	foliage	−23.0	21.4
Pecopteris arborescens	GSD3241	Somerset, UK	Westphalian D	foliage	−21.5	19.8
Coniferophytes						
Cordaites principalis	RC2881	South Yorkshire, UK	Westphalian B	foliage	−21.6	19.9

(*Continued.*)

fossil specimen	specimen number	locality	age	material analysed	δ^{13}C (‰)	Δ^{13}C (‰)
B. Natural History Museum, London						
Lycopsods						
Brasilodendron pedoanus	v-230	Rio Grande, Brazil	Permian	bark	−21.6	19.5
B. pedoanus	v230 g	Rio Grande, Brazil	Permian	bark	−21.3	19.2
Paikhoia	v-53069	Russia	Permian	bark	−23.6	21.6
Lepidodendropsis lissonia	v-25932	Peru	Lower Carboniferous	bark	−23.0	20.5
L. sekoidiensis	v-57018	Ghana, West Africa	Lower Carboniferous	bark	−23.1	20.6
Glossopterids						
Glossopteris sp.	v-13459	Buckley Island, Antarctica	Permian	foliage	−25.6	23.7
Glossopteris sp.	v-52409	Theron Mts, Antarctica	Permian	foliage	−23.0	21.0
Glossopteris sp.	v-61704	Antarctica	Permian	stem	−23.4	21.4
Glossopteris sp.	—	Estcourt, South Africa	Permian	foliage	−23.5	21.5
Glossopteris sp.	v-3132	South Africa	Permian	foliage	−24.4	22.4
Glossopteris sp.	v-7348	South Africa	Permian	foliage	−24.1	22.1
Glossopteris sp.	v-7126	Nagpur, India	Permian	foliage	−24.8	22.9
Glossopteris (*hormonia*?)	—	Tanzania, southern Africa	Permian	stem	−24.1	22.1
Lidgettonia africana	v-34639	South Africa	Permian	foliage	−23.3	21.3
Lidgettonia	v-34637	South Africa	Permian	stem	−24.4	22.4
Lidgettonia	v-34637	South Africa	Permian	foliage	−23.9	21.9
Schzamann gondiscum	v-12948	Zimbabwe, southern Africa	Permian	foliage	−24.6	22.7
S. gondiscum	v-12948	Zimbabwe, southern Africa	Permian	foliage	−24.5	22.6
Vertibraria indica	v-24436	Australia	Permian	stem	−24.9	23.0
Vertebratia	v-7597	Zimbabwe, southern Africa	Permian	stem	−23.9	21.9
Gymnosperm woods						
Anarcticoxylon preisty	v-13490	Priestley Glacier, Antarctica	Permian	wood	−23.3	21.3
Dadoxylon	v-20451	Antarctica	Permian	wood	−22.7	20.7
Dadoxylon	v-20451	Antarctica	Permian	wood	−22.8	20.8
Dadoxylon	v-20450	South Victoria Island, Antarctica	Permian	wood	−22.9	20.9
Gangamopteris sp.	v-61712	East Antarctica	Permian	wood	−24.0	22.0
unidentified sp.	v-20451	Victoria Land, Antarctica	Permian	stem	−22.7	20.7
unidentified sp.	v-52411	Theron Mts, Antarctica	Permian	stem	−22.9	20.9
unidentified sp.	v-13464	Buckley Island, Antarctica	Permian	wood	−22.9	20.9
unidentified sp.	v-11272	Antarctica	Permian	stem	−23.0	21.0
unidentified sp.	v-61728	Droning Maud Land, E. Antarctica	Permian	stem	−23.6	21.6
C. collections of S. McLoughlin						
Glossopteris sp.	PCM 1-B	Prince Charles Mountains	Permian	leaf mats	−22.0	19.9
Glossopteris sp.	PCM 2-B	Prince Charles Mountains	Permian	leaf mats	−23.1	21.1
Australoxyon mondii	PCM 1-C	Prince Charles Mountains	Permian	wood	−21.4	19.3

(*Continued.*)

fossil specimen	specimen number	locality	age	material analysed	δ^{13}C (‰)	Δ^{13}C (‰)
Glossopteris sp./*A. mondii*	PCM 15-B	Prince Charles Mountains	Permian	leaves and wood	−21.6	19.5
Glossopteris sp./*A. mondii*	PCM 9-A	Prince Charles Mountains	Permian	leaves and wood	−22.2	20.1
charcoal	PCM 1-A	Prince Charles Mountains	Permian	wood	−22.9	20.9
charcoal	PCM 15-A	Prince Charles Mountains	Permian	wood	−21.5	19.4
charcoal	PCM 2-A	Prince Charles Mountains	Permian	wood	−21.3	19.2
Glossopteris sp./*A. mondii*	HV6-133	Homevale Station, Bowen Basin	Permian	leaves and wood	−22.3	20.3
Araucarioxylon sp.	HV6-154	Homevale Station, Bowen Basin	Permian	wood	−23.4	21.4
Glossopteris sp. /*Araucarioxylon*	HV2-140	Homevale Station, Bowen Basin	Permian	leaves and wood	−23.4	21.4

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