New Phytologist

Review

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The evolution of C_4 photosynthesis

Author for correspondence:
Rowan F. Sage
Tel: +1 416 978 7660
Fax: +1 416 978 5878
Email: r.sage@utoronto.ca

Received: 3 July 2003
Accepted: 16 October 2003

doi: 10.1046/j.1469-8137.2004.00974.x

Rowan F. Sage

Department of Botany, University of Toronto, 25 Willcocks Street, Toronto, Ontario M5S 3B2, Canada

Contents

Summary

Key words: carbon concentration, C_3–C_4 photosynthesis, *Flaveria*, macroevolution, photorespiration, photosynthesis.

C_4 photosynthesis is a series of anatomical and biochemical modifications that concentrate CO_2 around the carboxylating enzyme Rubisco, thereby increasing photosynthetic efficiency in conditions promoting high rates of photorespiration. The C_4 pathway independently evolved over 45 times in 19 families of angiosperms, and thus represents one of the most convergent of evolutionary phenomena. Most origins of C_4 photosynthesis occurred in the dicots, with at least 30 lineages. C_4 photosynthesis first arose in grasses, probably during the Oligocene epoch (24–35 million yr ago). The earliest C_4 dicots are likely members of the Chenopodiaceae dating back 15–21 million yr; however, most C_4 dicot lineages are estimated to have appeared relatively recently, perhaps less than 5 million yr ago. C_4 photosynthesis in the dicots originated in arid regions of low latitude, implicating combined effects of heat, drought and/or salinity as important conditions promoting C_4 evolution. Low atmospheric CO_2 is a significant contributing factor, because it is required for high rates of photorespiration. Consistently, the appearance of C_4 plants in the evolutionary record coincides with periods of increasing global aridification and declining atmospheric CO_2. Gene duplication followed by neo- and nonfunctionalization are the leading mechanisms for creating C_4 genomes, with selection for carbon conservation traits under conditions promoting high photorespiration being the ultimate factor behind the origin of C_4 photosynthesis.

Abbreviations

CA, carbonic anhydrase; GDC, glycine decarboxylase; PCA, photosynthetic carbon assimilation; PCR, photosynthetic carbon reduction; PEPCase, phosphoenolpyruvate carboxylase; PG, phosphogylcolate; PPDK, pyruvate orthophosphate dikinase; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase; WUE, water-use efficiency.

© *New Phytologist* (2004) **161**: 341–370

I. Introduction

C_4 photosynthesis is a series of biochemical and anatomical modifications that concentrate CO_2 around the carboxylating enzyme Rubisco. Many variations of C_4 photosynthesis exist, reflecting at least 45 independent origins in 19 families of higher plants. C_4 photosynthesis is present in about 7500 species of flowering plants, or some 3% of the estimated 250 000 land plant species (Sage *et al.*, 1999a). Most C_4 plants are grasses (4500 species), followed by sedges (1500 species) and dicots (1200 species). C_4 photosynthesis contributes about a quarter of the primary productivity on the planet, and a large fraction of the primary production humans consume – either directly as plant material or indirectly via animal products – is derived from C_4 crops and pasture grasses (Lloyd & Farquhar, 1994; Brown, 1999). C_4 grasses and sedges dominate nearly all grasslands in the tropics, subtropics and warm temperate zones, and are major representatives of arid landscapes from the temperate zones to the tropics (Archibold, 1995; Sage *et al.*, 1999b). Because of enhanced water and nutrient-use efficiency, C_4 plants are also capable of growing in habitats that may be too harsh for C_3 species, such as rock outcrops (Fig. 1) and hypersaline or

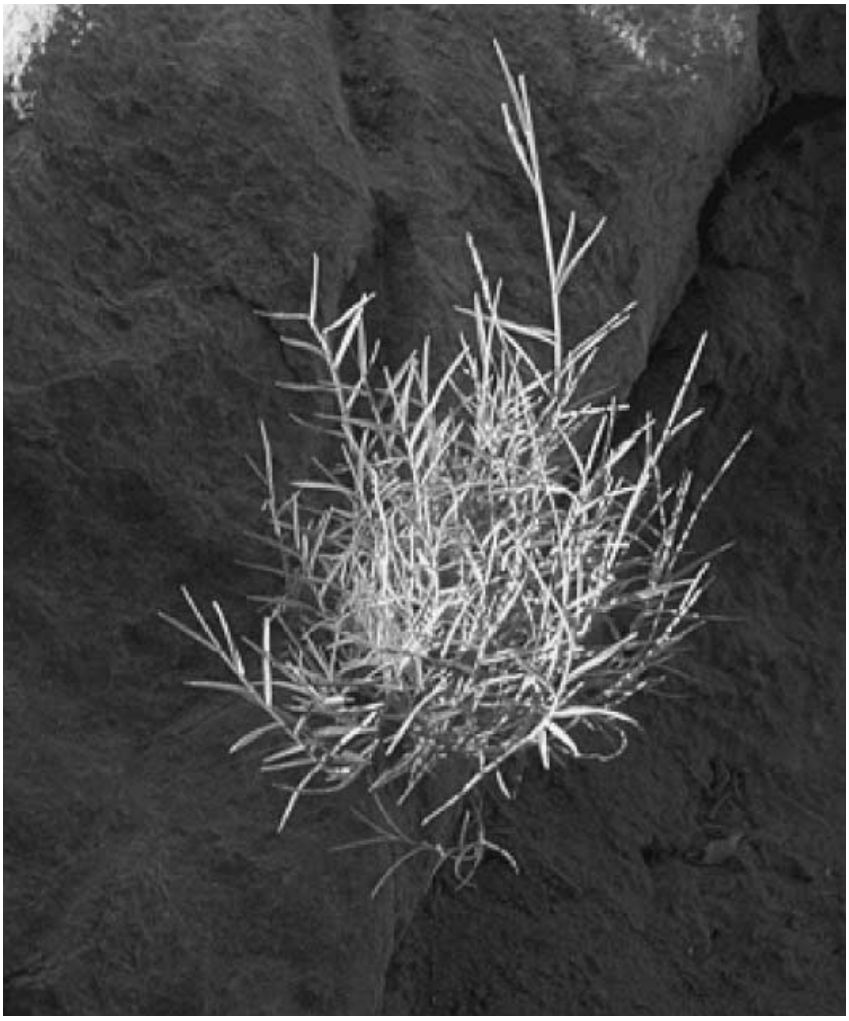

Fig. 1 C_4 photosynthesis allows plants to grow in habitats that may otherwise be too harsh, as indicated by this C_4 *Muhlenbergia* sp. growing on a rock face in Zion National Park, Utah, USA. Photo by R.F.S.

arid soils of low latitude (Schulze *et al.*, 1996). As a result, complex ecosystems existed where there may otherwise have been bare ground.

In recent years there has been widespread interest in the evolutionary diversification of C_4 plants. Geologists are interested because C_4 photosynthesis affects atmosphere, climate and biotic systems through geological time (Lloyd & Farquhar, 1994; Cerling *et al.*, 1997; Pagani *et al.*, 1999; Royer *et al.*, 2001; Ehleringer *et al.*, 2004). Zoologists and anthropologists care because C_4 plants influenced the evolution of mammals and hominids (MacFadden, 1997; van der Merwe & Tschauner, 1999; Harris & Cerling, 2002; Janis *et al.*, 2002). C_4 crops and weeds have also affected historical trends, as shown by the rise of Mesoamerican civilizations based on maize, and the expansion of the transatlantic slave trade based on cane sugar (Hobhouse, 1999; van der Merwe & Tschauner, 1999). In the future, human-induced global change will favor widespread expansion of C_4 grasslands at the expense of forests (Sage & Kubien, 2003). Climatologists and policy makers are taking note because C_4 grassland expansion alters regional climate and reduces air quality and biodiversity via effects on fire cycles and surface albedo. Finally, as a convergent phenomenon C_4 photosynthesis is an excellent model for complex trait evolution in response to environmental change (Monson, 2003).

Given the significance of C_4 plants, it is important to understand the evolution of the C_4 pathway. Much of what we know about C_4 photosynthesis was discovered in a 15 yr burst of research that followed the discovery of the pathway in the mid-1960s (Edwards & Walker, 1983; Osmond, 1997; Hatch, 1999). By the 1980s, the main features of the pathway were identified, its taxonomic distribution described, and the ecological importance understood (Edwards & Walker, 1983). Since then there has been substantial accumulation of information from numerous disciplines, such that we can now propose plausible hypotheses about the mechanisms, timing and environmental imperatives of C_4 plant evolution. In this review, I synthesize the current understanding of C_4 plant biology to provide a comprehensive overview of the evolution of the C_4 pathway. I begin by reviewing the characteristics that define C_4 photosynthesis, which is necessary both for background review and to update our concepts in light of recent discoveries of single-celled patterns of C_4 photosynthesis. I then address in turn four commonly asked questions of C_4 evolution: (1) Why did C_4 plants evolve? (2) Where did they evolve, in terms of both taxonomic distribution and ecological habitat? (3) How did they evolve? (4) When did they evolve? I finish with some thoughts on C_4 photosynthesis in the future, when human activities may alter patterns of C_4 evolution.

II. What is C_4 photosynthesis?

C_4 photosynthesis is not a single metabolic pathway. It is a series of biochemical and structural adjustments that have exploited phosphoenolpyruvate carboxylase (PEPCase) and other existing enzymes to concentrate CO_2 around Rubisco (Fig. 2). In all versions of C_4 photosynthesis, the initial step is the fixation of inorganic carbon by PEPCase, followed by the movement of the resulting four-carbon acids to an interior compartment where Rubisco is localized (Hatch, 1987; Kanai & Edwards, 1999). Here, CO_2 is released by the decarboxylation of the four carbon acid, and its concentration rises to a level that nearly saturates the Rubisco active site (von Caemmerer, 2000). The decarboxylation reaction also produces a three-carbon acid, which diffuses back to the compartment where PEP carboxylase is located. If necessary, the three-carbon acid is converted to pyruvate, which is then phosphorylated to regenerate PEP.

While all C_4 plants share a common theme, the specific means by which CO_2 concentration occurs can vary substantially between the different evolutionary lineages (Edwards & Walker, 1983; Kanai & Edwards, 1999). The only enzymatic step common to all versions of C_4 photosynthesis is the initial carboxylation reaction catalyzed by PEP carboxylase to yield oxaloacetic acid (OAA). Three decarboxylation enzymes (NADP-malic enzyme, NAD-malic enzyme and PEP carboxykinase) have been identified, and their relative abundance is the basis for naming the three biochemical subtypes of C_4 photosynthesis. If NADP-malic enzyme is used, OAA is converted to malate which then diffuses to the interior compartment (Fig. 2). Pyruvate is formed during the decarboxylation reaction, and this returns to the outer compartment to be phosphorylated back to PEP. If NAD-ME is used, OAA is transaminated to asparatate which then diffuses to the interior compartment (Fig. 2). Pyruvate is also formed during the NAD-ME decarboxylation reaction, but this is transaminated to alanine, which then returns to the outer compartment where it is converted to pyruvate and phosphorylated to yield PEP. PEP carboxykinase-type plants form PEP during the decarboxylation reaction, and this can return directly to the outer compartment for carboxylation by PEPCase (Leegood & Walker, 1999).

Anatomically, C_4 photosynthesis requires the modification of C_3 leaf structure to form the inner compartment where Rubisco is localized and CO_2 can be concentrated (Dengler & Nelson, 1999). In most C_4 plants this results in the formation of a wreath-like cell arrangement, termed Kranz anatomy (Fig. 2). In the textbook pattern of Kranz anatomy an outer layer is derived from mesophyll cells, while the inner layer is derived from any of a number of cell layers that are near or within the vascular bundle. PEP carboxylase is localized in the outer, mesophyll layer, and thus the region where the initial carboxylation step occurs is termed the mesophyll tissue, or the photosynthetic carbon assimilation (PCA) tissue. Commonly, a layer of parenchyma cells around the vascular bundles have been incorporated into the inner layer of the Kranz anatomy, thus this layer is commonly referred to as the bundle sheath. Rubisco and many of the Calvin cycle enzymes are localized in this inner layer, and for this reason it is often

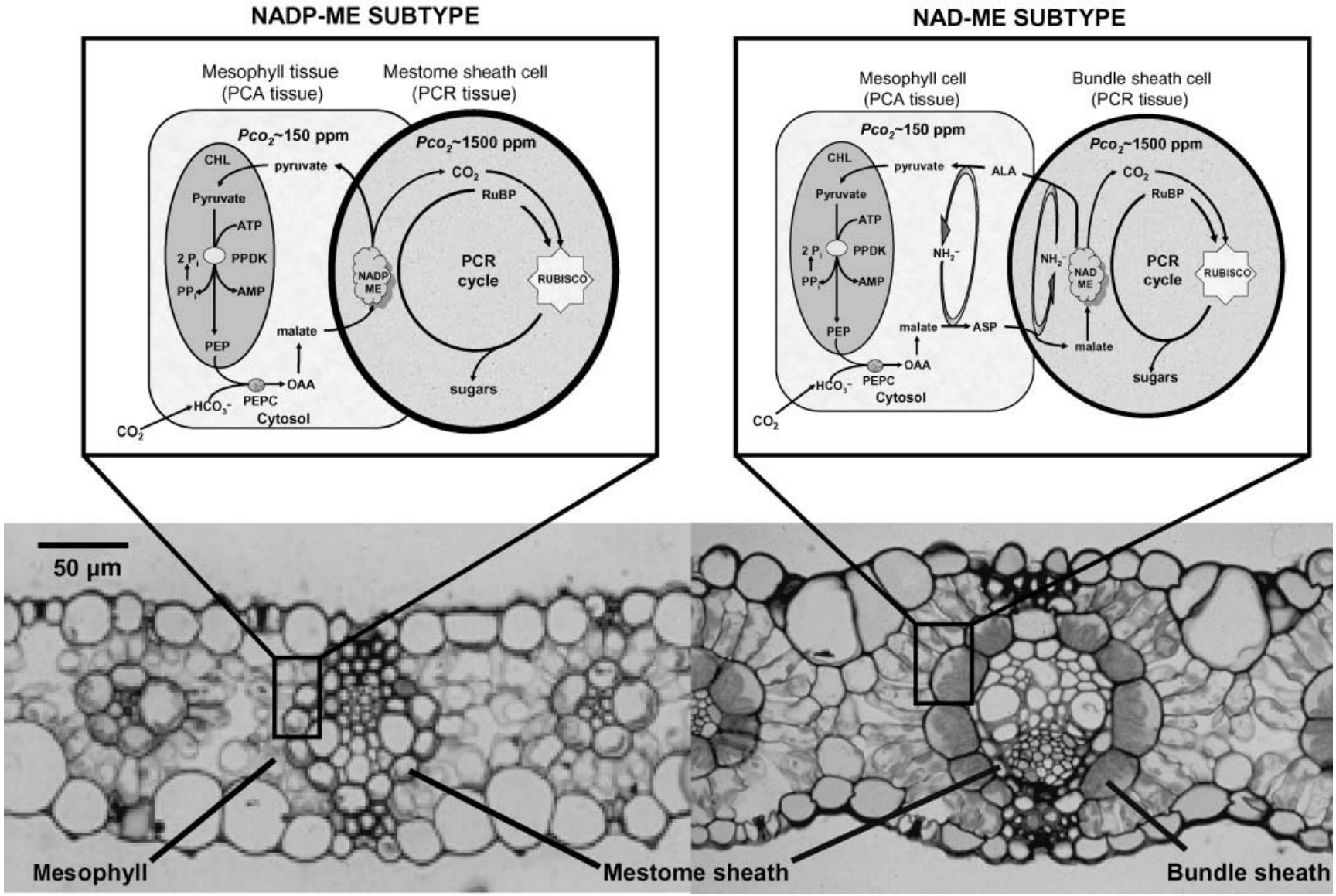

Fig. 2 Leaf structure and C_4 metabolic pathways of the NADP-ME grass *Themada triandra* with the PCR tissue in the mestome sheath (left panel) and the NAD-ME grass *Panicum effusum* with the PCR tissue in the bundle sheath layer that resides outside the mestome sheath (right panel). In *Themada* no true bundle sheath layer is present, although the mestome sheath is commonly referred to as the 'bundle sheath' tissue. ASP, aspartate; ALA, alanine; OAA, oxaloacetate; CHL, chloroplast; PEPC, PEP carboxylase; PPDK, pyruvate, phosphate dikinase; PP_i, pyrophosphate. NH_2^- and circular arrows indicate the presence of transanimation cycles in the mesophyll and PCR tissue. Micrographs by Professor Nancy Dengler, University of Toronto (by permission).

termed the photosynthetic carbon reduction tissue (PCR; Dengler & Nelson, 1999). In the different evolutionary lines, tissues other than the bundle sheath proper have been modified to form the PCR tissue (Fig. 2; Dengler & Nelson, 1999; Soros & Dengler, 2001). In certain C_4 grasses the mestome sheath forms the PCR tissue. The mestome sheath is an outer layer of cells in the vascular bundle that is developmentally distinct from anatomically correct bundle sheath cells. (Mestome sheath and other cells within vascular bundles are derived from procambium or vascular meristematic tissue. Bundle sheath proper is derived from the ground meristem, the meristematic tissue that forms the nonvascular, nonepidermal cells in a leaf.) In numerous sedge lines (eleocharoid, fimbristyloid and chlorocyperoid), a layer of vascular parenchyma cells inside of the mestome sheath forms the PCR tissue; the mestome sheath itself is nonphotosynthetic (Soros & Dengler, 2001). Rhynchosporoid sedges, in contrast, have the PCR enzymes in the mestome sheath while the bundle sheath proper is incorporated into the PCA tissue. In dicots, the PCR tissue is usually derived from the bundle sheath proper. In succulent forms, however, the PCR tissue can be derived from a layer of parenchyma cells that surround large, chloroplast-free water-storage cells that, in turn, enclose the vascular tissue (Dengler & Nelson, 1999; Pyankov *et al.*, 2001b; Kadereit *et al.*, 2004).

In total, over 15 distinct types of Kranz-leaf anatomy have been identified in the various dicot and monocot lineages of C_4 photosynthesis (Dengler & Nelson, 1999; Kadereit *et al.*, 2003). In all these cases the outer wall of the PCR compartment is thought to have been modified to reduce the rate of CO_2 efflux and thus trap CO_2 inside. The outer wall of the PCR compartment in some grasses is often impregnated with suberin, presumably to enhance the resistance of the wall to CO_2 efflux. Suberization of the outer PCR wall is not a requirement for C_4 photosynthesis, because many NADP-ME and all NAD-ME type species lack the suberin barrier (Dengler & Nelson, 1999). In species without a suberin barrier there is a tendency for chloroplasts to occur on the inner, or centripedal, side of the bundle sheath cell. By doing so, the large vacuole of the bundle sheath cell helps slow CO_2 escape.

Recently, the phenomenon of single-celled C_4 photosynthesis has been identified in a number of species. In the aquatic monocots *Hydrilla verticillata* and *Egeria densa* (both Hydrocharitaceae), a C_4 biochemical cycle operates intracellularly by collecting CO_2 from the cytoplasm and concentrating it into the chloroplasts (Reiskind *et al.*, 1997; Casati *et al.*, 2000; Bowes *et al.*, 2002). Rubisco and NADP-ME are localized in the chloroplasts, and PEPCase in the cytosol of each photosynthetic cell. No obvious diffusion barrier is present and apparently much CO_2 leaks out of the chloroplast, as indicated by quantum yield values in *H. verticillata* that are half the normal C_3 values (Spencer *et al.*, 1994). Even with this inefficiency, C_4 photosynthesis in *H. verticillata* is considered adaptive because it increases carbon gain at very low CO_2 levels that frequently occur in warm, freshwater ponds (Bowes *et al.*, 2002; Maberly & Madsen, 2002).

Other aquatic plants such as *Sagittaria subulata* (Alismataceae), various green algae and some diatoms are proposed to operate intracellular C_4 cycles, although there is some uncertainty about how well these enhance CO_2 levels in the chloroplast (Reinfelder *et al.*, 2000; Johnston *et al.*, 2001; Bowes *et al.*, 2002). To consider algae and aquatic plants as true C_4 plants, they should meet the criteria of using PEPCase to supply virtually all of the CO_2 that Rubisco uses in RuBP carboxylation. This is one of the principal criteria in distinguishing between C_3–C_4 intermediates and fully developed C_4 plants (Monson, 1989a). *Hydrilla verticillata* has a high degree of C_4 cycle activity in support of C_3 cycle activity, and thus warrants being considered a C_4 plant. Further work is required before other species can be considered to operate C_4 photosynthesis, rather than simply running a weak C_4 cycle that marginally enhances C_3 photosynthesis.

In terrestrial plants, two single-celled C_4 species have been identified, *Bienertia cycloptera* and *Borszczowia aralocaspica* (Fig. 3; Freitag & Stichler, 2000, 2002; Voznesenskaya *et al.*, 2001b, 2002). Both are in the Suaedoid tribe of the Chenopodiaceae, and both independently arose from C_3 ancestors in the genus *Suaeda* (Schütze *et al.*, 2003). The mechanism by which the single-celled C_4 pathway operates in the two species show marked differences. In *Borszczowia*, the photosynthetic cells are arrayed in tightly packed columns around a central core of succulent tissue (Fig. 3a,c; Freitag & Stichler, 2000). PEP carboxylation and regeneration occur at the distal ends of the cell exposed to the intercellular air spaces. The C_4 acids produced must diffuse from here to the opposite, proximal end of the cell where they are decarboxylated (Voznesenskaya *et al.*, 2001b). An elongated vacuole provides high resistance to CO_2 efflux and thus CO_2 accumulates at the proximal end of the cell where Rubisco is localized. In this regard, the general layout of C_4 photosynthesis in *Borszczowia* is similar to Kranz-type C_4 plants, the major difference being the lack of a cell wall segregating the PCA and PCR compartments (Sage, 2002b).

Bienertia cycloptera exhibits a radical departure from Kranz anatomy. Here, photosynthetic cells resemble thick barrels, and each cell is exposed on all sides to the intercellular air spaces (Fig. 3b,d; Freitag & Stichler, 2002; Voznesenskaya *et al.*, 2002). PEP carboxylase is localized in cytoplasmic pockets at the periphery of each cell, while Rubisco and the decarboxylating enzymes are located in chloroplasts that are bunched together in a cytoplasmic core in the center of the cell (Voznesenskaya *et al.*, 2002). A large vacuole separates the inner and outer cytoplasm, and is thought to be the diffusive barrier that slows CO_2 leakage out of the core cytoplasm. Thin cytoplasmic strands run through the vacuole and connect the inner and outer cytoplasm compartments. It is along these strands that metabolites diffuse between the PCA and PCR compartments (Voznesenskaya *et al.*, 2002).

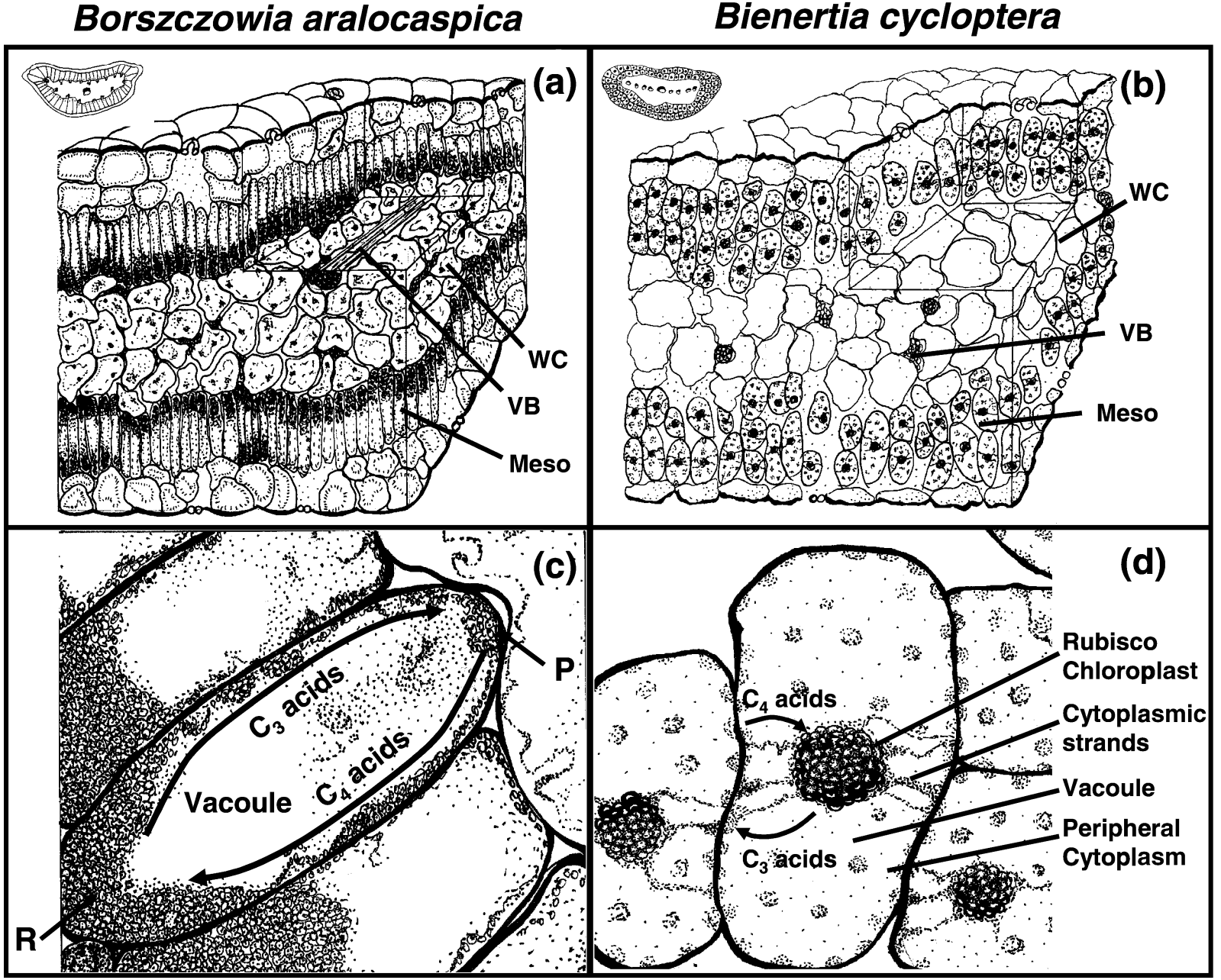

Fig. 3 Leaf anatomy of the two single-celled C_4 plants, *Borszczowia aralocaspica* (a,c) and *Bienerta cycloptera* (b,d). (a,b) Whole-leaf cross sections (insets) and three-dimensional drawings of the leaf anatomy; (c,d) structure of individual cells with the pathway of organic acid flux in each cell highlighted. Meso, mesophyll; P, region where PEPCase is located; R, region where Rubisco is located; VB, vascular bundles; WC, water-storage cells. Developed from Voznesenskaya *et al*. (2001b, 2002) and Freitag & Stichler (2000, 2002); drawings by Katherine Sage.

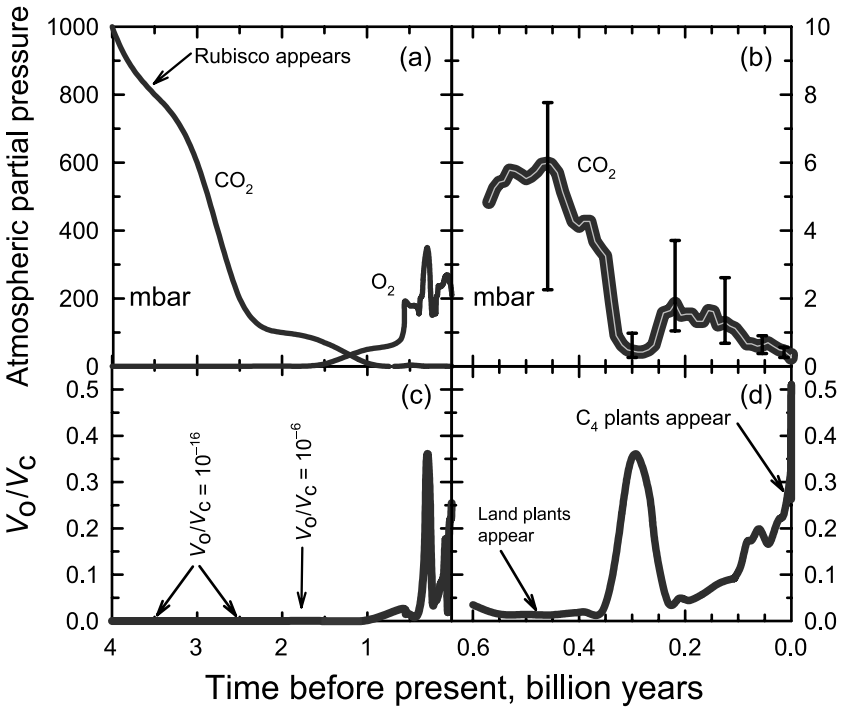

Fig. 4 Profiles of modeled atmospheric CO_2 and O_2 partial pressures (in mbar) over the history of the earth (a,b), and corresponding estimates of relative oxygenation potential for C_3 photosynthesis (c,d). Atmospheric CO_2 levels were modeled over the past 4 billion years (a) and 0.6 billion years (b); atmospheric O_2 levels were modeled over the past 4 billion years (data from Berner 1994). Oxygenation potential is modeled as the ratio of RuBP oxygenation to carboxylation (V_O/V_C) corresponding to the gas levels shown in (a,b) and assuming a C_3 Rubisco (from spinach) at 30°C. from Sage (1999) by permission.

III. Why did C_4 photosynthesis evolve?

In all photosynthetic organisms, only Rubisco catalyzes the net fixation of CO_2 into organic molecules. Rubisco and the C_3 mode of photosynthesis evolved early in the history of life (Hayes, 1994), and apparently were so successful that competing forms of net photosynthetic carbon fixation have gone extinct, assuming they ever existed. In high CO_2 atmospheres, Rubisco operates relatively efficiently. However, the active site chemistry that carboxylates RuBP can also oxygenate it, producing one molecule of PGA and one of phosphoglycolate (Andrews & Lorimer, 1987). Phosphoglycolate (PG) is metabolically useless and toxic if it accumulates in the cell (Ogren, 1984). Converting PG to useful metabolites is thus essential in land plants, yet it requires substantial metabolic energy and results in the loss of 25% of the carbon entering the pool of PG molecules (Ogren, 1984; Douce & Heldt, 2000). Collectively, RuBP oxygenation and the metabolism of PG are termed photorespiration. In the current atmosphere, photorespiration can inhibit photosynthesis by over 30% at warmer temperatures (> 30°C) (Jordan & Ogren, 1984; Sharkey, 1988; Ehleringer *et al.*, 1991).

Throughout most of Earth's history, RuBP oxygenation was negligible due to elevated CO_2 and low O_2 levels in the atmosphere (Fig. 4; Sage, 1999). For oxygenase activity to become significant, O_2 concentrations in solution have to exceed CO_2 concentrations by about 10-fold (Jordan & Ogren, 1984). Because of the different solubility of CO_2 and O_2, a 10-fold difference in solution concentration occurs when atmospheric partial pressures of O_2 are about 100 times greater than CO_2 partial pressures at 30°C (von Caemmerer & Quick, 2000). Atmospheric conditions favoring significant levels of photorespiration probably did not occur before 400 million yr ago because earlier CO_2 levels were many times greater than today, while O_2 levels were generally much lower (Fig. 4; Berner & Kothavala, 2001). Only during the Carboniferous period (280–340 million yr ago) and in the past 35 million yr have atmospheric conditions favored significant levels of photorespiration.

By the Carboniferous, all plants used Rubisco for the net carboxylation step of photosynthesis, and Rubisco was well integrated into the primary metabolism of the plant. Because of this integration, the likelihood of evolutionarily solving the photorespiratory problem within the context of C_3 photosynthesis was probably nil. Even if a novel carboxylase could be produced, it would probably be useless because the plant would lack the metabolic pathways to regenerate acceptor molecules and process the carboxylation products.

PEP carboxylase (PEPCase) is the other major carboxylase in C_3 plants, serving a vital function in moving carbon from the glycolytic pathway into the Krebs cycle (Chollet *et al.*, 1996). In its current configuration, PEP carboxylation does not allow for net CO_2 fixation into carbohydrate, because the carbon added to PEP is lost as CO_2 in the Krebs cycle. For PEPCase to evolve into a net carboxylating enzyme, fundamental rearrangements in carbon flow would also be required, while the existing role of PEPCase would have to be protected or replaced in some manner.

Evolving a Rubisco that is free of oxygenase activity also appears unlikely because the active site biochemistry is

constrained by similarities in the oxygenase and carboxylase reactions (Andrews & Lorimer, 1987; Roy & Andrews, 2000). The rate of photorespiration in plants = $0.5/S_{rel}$ (O/C) where S_{rel} is the specificity of Rubisco for CO_2 relative to O_2; O is the O_2 concentration in the stroma; and C is the CO_2 concentration in the stroma (Jordan & Ogren, 1984; Sharkey, 1988). Accordingly, evolution of a photorespiratory-free Rubisco would involve an increase in S_{rel}. Evolution has produced Rubisco enzymes with varying S_{rel}; however, limits may have been reached as C_3 plants express Rubisco enzymes with a relatively narrow range of S_{rel} values (Roy & Andrews, 2000). One of the disadvantages of a Rubisco with high relative specificity is it has a slow catalytic turnover rate, k_{cat} (Andrews & Lorimer, 1987). The relatively narrow range of S_{rel} in C_3 plants probably reflects a balance between selection for enhanced S_{rel} and high k_{cat} (Sage, 2002a).

In the absence of further improvements to Rubisco, the other solution to the photorespiratory problem is to enhance the stromal concentration of CO_2 or to reduce O_2. Reducing O_2 is unlikely due to unfavorable energetics. For example, lowering stromal O_2 levels by 1000 p.p.m., from 210 000 to 209 000 p.p.m., would have no significant effect on photorespiration, but would greatly enhance the ATP cost of photosynthesis, assuming it costs 1 ATP per O_2 pumped. By contrast, because of the higher specificity of Rubisco for CO_2 relative to O_2, pumping CO_2 into the stroma is 80–100 times more effective per ATP spent, in terms of the relative effect on photorespiration. (In C_3 plants S_{rel} is 80–100, meaning that 80–100 carboxylations will occur for each oxygenation at equal concentrations of CO_2 and O_2. The reason oxygenation is a problem today is because CO_2 levels in the atmosphere are much lower than O_2 levels; von Caemmerer & Quick, 2000.) Increasing CO_2 around Rubisco by 1000 p.p.m. would nearly eliminate oxygenase activity, and under circumstances of high photorespiration could justify the additional energy costs required to operate a CO_2 pump (von Caemmerer, 2000).

In addition, all the enzymes required for carbon concentration are present in C_3 species, serving a variety of functions in carbohydrate and nitrogen metabolism. PEP carboxylase is ubiquitous in eukaryotic organisms, as it plays a central role in carbon flow into the Krebs cycle, in pH control within cells, and in the mobilization of carbohydrate stores into a range of biosynthetic precursors (Chollet *et al.*, 1996). In plants, PEP carboxylase is used in a range of turgor-driven movements, most notably the opening of stomata, and is important in the acquisition and assimilation of mineral nutrients (Cockburn, 1983; Johnson *et al.*, 1996). The decarboxylation enzymes of C_4 photosynthesis appear in a range of metabolic roles in C_3 plants. PEP carboxykinase occurs in oil and resin ducts, vascular tissues, guard cells, and sink tissues of fruits and roots (Leegood & Walker, 2003). It also metabolizes fats to carbohydrates in germinating seeds (Rylott *et al.*, 2003). NADP-ME and NAD-ME are important in organic acid metabolism of C_3 cells where they perform a variety of housekeeping roles (Wedding, 1989; Edwards & Andreo, 1992; Drincovich *et al.*, 2001). NADP-ME, for example, is important in wound responses (Casati *et al.*, 1997), fruit ripening, gluconeogenesis, and recycling of organic acids leaving the vasculature (Edwards & Andreo, 1992; Hibberd & Quick, 2002). Instead of evolving novel enzymes, CO_2 concentration requires changes in the kinetics, regulatory set points, and tissue specificity of existing enzymes. This pattern of exploiting existing biochemistry rather than inventing new enzymes is the general rule in complex trait evolution (Doebley & Lukens, 1998).

Given these considerations, it is no surprise that the primary means of compensating for photorespiration in land plants has been the layering of C_4 metabolism over existing C_3 metabolism. All C_4 plants operate a complete C_3 cycle, so in this sense the C_4 pathway supplements, rather than replaces, C_3 photosynthesis. Because it uses existing biochemistry, the evolutionary trough that must be crossed to produce a C_4 plant is relatively shallow, and could be bridged by a modest series of incremental steps.

IV. Evolutionary lineages of C_4 photosynthesis

C_4 photosynthesis occurs in three families of monocots – grasses (Poaceae), sedges (Cyperaceae), and Hydrocharitaceae – and 16 dicot families, assuming the Amaranthaceae and Chenopodiaceae are treated as distinct families (Table 1; Figs 5–8). (Recent treatments merge the Chenopodiaceae into the Amaranthaceae (Soltis *et al.*, 2000). More recent systematic studies indicate the traditional separation is justified, however (Kadereit *et al.*, 2004). The traditional separation into Amaranthaceae and Chenopodiaceae is used here.) Phylogenetic analyses clearly show that each of these families arose from C_3 ancestors, such that it can be safely concluded that the C_4 pathway independently evolved in each family (Kellogg, 1999). Within many of the families with C_4 species, multiple independent origins are also apparent (Table 1). Grasses are estimated to have 11 independent lineages, and four are known for the Cyperaceae (Fig. 6). The Asteraceae has three independent lineages, and two appear certain in the Zygophyllaceae (Fig. 7). Two lineages may also occur in the Portulacaceae and the Sesuvioideae tribe of the Aizoaceae (Fig. 7), while 10 lineages are apparent in the Chenopodiaceae and three are probable in the Amaranthaceae (Fig. 8).

Multiple origins also occur within genera (Table 1; Fig. 8). In *Salsola* (Chenopodiaceae), two C_4 origins are supported (Pyankov *et al.*, 2001a; 2001b; Kadereit *et al.*, 2004), and two are suspected in *Portulaca* (Portulacaceae). The most prolific genus for evolving C_4 photosynthesis is *Suaeda*, whose species tend to be halophytes of semiarid regions. Some 100 *Suaeda* species are known, of which 60 are C_4 (Sage *et al.*, 1999a). Four C_4 lineages rise from ancestral C_3 *Suaeda* species (Schütze *et al.*, 2003). One C_4 line of *Suaeda* species has 'schoberia' (conospermoid)-type Kranz anatomy, while a second

Table 1 Postulated evolutionary lineages of C_4 photosynthesis, with Kranz type and biochemical subtype if known

Family	Lineage	Representative genera	Kranz anatomy/subtype	Reference[a]
Monocots				
Poaceae	1. Aristideae	*Aristida*, *Stipagrostis*	NAD-ME	1
	2. Chloridoideae	*Centropodia* and all Chloridoideae	Classic NAD-ME, PCK	1, 2
	3. Eriachneae	*Eriachne*, *Pheidochloa*	Eriacnioid NAD-ME	1
	4. Andropogoneae	*Andropogon*, *Zea*, *Sorghum*	Classic NADP-ME	3
	5. Paniceae I	*Paspalum-Thrasya* clade	Classic NADP-ME	3
	6. Paniceae II	*Leptocoryphium* clade	Classic NADP-ME	3
	7. Paniceae III	*Axonopus/Ophiochloa* clade	Classic NADP-ME	3
	8. Paniceae IV	*Streptostachys/Panicum priontis/ Andropogon*	Classic NADP-ME	3
	9. Paniceae V	*Echinochloa* clade	Classic NADP-ME	3
	10. Paniceae VI	*Digitaria* clade	Classic NADP-ME	3
	11. Paniceae VII	*Panicum/Setaria/Urochloa*	All classic subtypes	3
Cyperaceae	12. Abilgaardieae	*Fimbrystylis*, *Crosslandia*	Fimbrystyloid, NADP-ME	4
	13. Cypereae	*Cyperus*, *Kyllinga*, *Mariscus*	Chlorocyperoid, NADP-ME	4
	14. *Eleocharis*	*Eleocharis*	Eleocharoid and Fimbristyloid/NAD-ME	4
	15. Rhynchosporeae	*Rhyncospora*, *Syntrinema*	Rhynchosporoid and chlorocyperoid/ NADP-ME	4
Hydrocharitaceae	16. Hydrilleae	*Hydrilla*, *Egeria*	Single-cell NADP-ME	5
Eudicots				
Acanthaceae	17. *Blepharis*	*Blepharis* section *Acanthodium*	Atriplicoid/unknown	6
Aizoaceae	18. Sesuviodeae I	*Sesuvium*, *Cypselea*	Atriplicoid/unknown	7
	19. Sesuviodeae II	*Zayleya*, *Trianthema*	Atriplicoid/NADP-ME	7
Amaranthaceae	20. Aervineae	*Aerva*	Atriplicoid/unknown	8
	21. Amarantheae	*Amaranthus*	Atriplicoid/NAD-ME	8
	22. Gomphreneae	*Gomphrena*, *Alternanthera*	Atriplicoid/NADP-ME	8
Asteraceae	23. Helenieae I	*Flaveria*	Atriplicoid/NADP-ME	9
	24. Helenieae II	*Pectis*	Unknown/NADP-ME	10
	25. Heliantheae	*Isostigma/Chrysanthellum*	Atriplicoid and Suaedoid/unknown	10
Boraginaceae	26. *Heliotropium*	Section *Orthostachys*	Atriplicoid/NAD-ME	11
Brassicaceae	27. *Cleome*	Section *Gynandropsis*	Unknown	12
Caryophyllaceae	28. *Polycarpaea*	*Polycarpaea*	Atriplicoid/NAD-ME	12
Chenopodiaceae	29. Atripliceae	*Atriplex*	Atriplicoid/NAD-ME	13
	30. Salicornieae	*Halosarcia*	Halosarcia/NAD-ME	13
	31. Camphorosmeae	*Bassia*, *Kochia*, *Chenolea*	Kochoid/NADP-ME	13
	32. Salsoleae I	NAD-ME *Salsola*, *Climacoptera*	Salsoloid/NAD-ME	13
	33. Salsoleae II	*Salsola/Haloxylon*	Salsoloid/NADP-ME	13
	34. Salsoleae III	*Girgensohnia*, *Noaea*, *Ofaiston*	Salsoloid/NADP-ME	13
	35. Suaedoideae I	*Suaeda*	Salsina/NAD-ME	13, 14
	36. Suaedoideae II	*Suaeda*	Schoberia/NAD-ME	13, 14
	37. Suaedoideae III	*Bienertia*	Single-cell/NAD-ME	13, 14
	38. Suaedoideae IV	*Borszczowia*	Single-cell/NAD-ME	13, 14
Euphorbiaceae	39. *Chaemacyce*	*Chamaesyce*	Atriplicoid/NADP-ME	12
Gisekiaceae	40. *Gisekia*	*Gisekia*	Atriplicoid/NAD-ME	15
Molluginaceae	41. *Mollugo*	*Mollugo*	Atriplicoid/NAD-ME	16
Nyctaginaceae	42. *Boerhavia*	*Boerhavia/Allionia/Okenia*	Atriplicoid/NAD-ME	12, 15
Polygonaceae	43. *Calligonum*	*Calligonum*	Salsoloid/unknown	12
Portulaceae	44. *Portulaca*	*Portulaca grandifolia*	Atriplicoid/NAD-ME	16
	45. *Portulaca*	*Portulaca oleracea*	Atriplicoid/NADP-ME	16
Scrophulariaceae	46. *Anticharis*	*Anticharis*	Unknown	12
Zygophyllaceae	47. *Tribulus*	*Tribulus/Kallstroemia/Tribulopsis*	Atriplicoid/NADP-ME	17
	48. *Zygophyllum*	*Zygophyllum simplex*	Salsoloid/unknown	17

[a]Main references: (1) GPWG (2001); (2) Hilu & Alice (2001); (3) Giussani *et al*. (2001); (4) Soros & Bruhl (2000); and Muasya *et al*. (2002); (5) Bowes *et al*. (2002); (6) Vollesen (2000); R.F. Sage, unpublished; (7) Hassan, Thiede and Liede, unpublished; R.F. Sage, unpublished; Hartmann (1993); (8) Kadereit *et al*. (2004); R. Sage, T. Sage and Pearcy, unpublished; (9) Kopriva *et al*. (1996); (10) Kellogg (1999), Karis & Ryding (1994); (11) Frohlich (1978); Sage and R.F. Sage, unpublished; (12) Sage *et al*. (1999a); (13) Kadereit *et al*. (2004); Schütze *et al*. (2003); (15) Cuénoud *et al*. (2002); R.F.S., unpublished; (16) Guralnick & Jackson (2001); (17) Sheahan & Chase (1996); R.F. Sage, unpublished. Additional subtype and anatomy information from Rathnam *et al*. (1975); Carolin *et al*. (1978); Pyankov *et al*. (2001b). 'R.F. Sage, unpublished' refers to results of isotopic screens of herbarium samples in the families indicated.

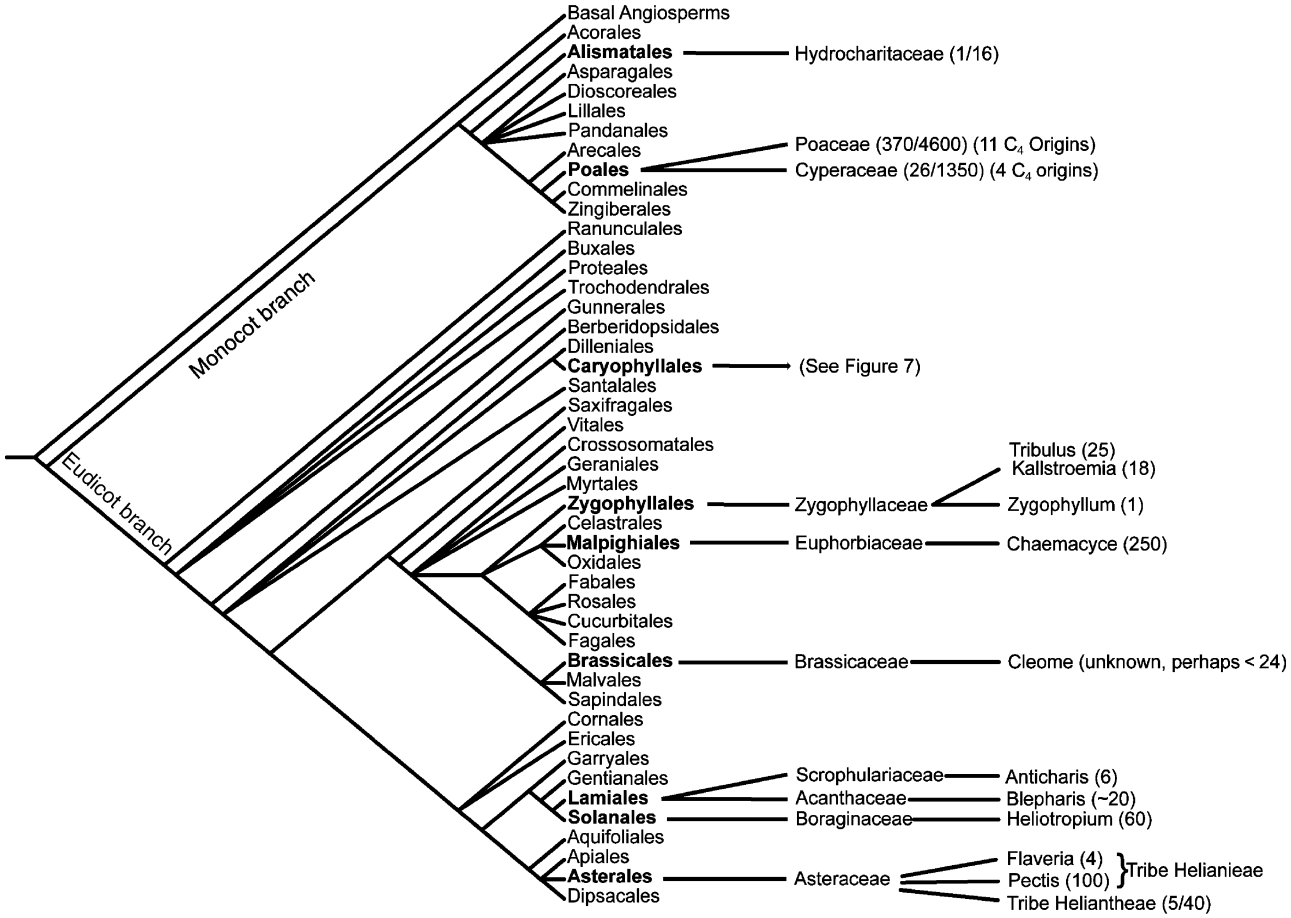

Fig. 5 Distribution of C_4 photosynthesis in the taxonomic orders of the angiosperms. Angiosperm orders with C_4 photosynthesis are shown in bold. Lines to the right of these orders indicate families and principal C_4 genera within a lineage. Numbers in parentheses refer to estimates of genera/species numbers or, where relevant, just species numbers. Adapted from Stevens (2003) by permission.

has 'salsina' (suaedoid) Kranz anatomy. The other two C_4 lineages arising from *Suaeda* are the single-celled C_4 species *Bienertia cycloptera* and *Borszczowia aralocaspica*. Nonphotosynthetic characteristics clearly place these species in the Suadoideae tribe of the Chenopodiaceae, while the unique single-celled C_4 anatomy distinguishes them from each other and all other C_4 species, demonstrating independent evolution of the C_4 pathway (Freitag & Stichler, 2002; Schütze *et al.*, 2003).

V. Where did C_4 photosynthesis evolve?

The identification of the C_4 lineages allows for an assessment of the regions and habitats where C_4 photosynthesis evolved. Centers of C_4 origin are indicated by (1) the geographic distribution of species expressing intermediate traits between C_3 and C_4 photosynthesis; (2) the location of the greatest taxonomic diversity within a C_4 lineage; and (3) the location of the nearest C_3 relatives (Powell, 1978; Pyankov *et al.*, 2001b). This approach works well in the dicots, where most lineages have relatively low diversity and appear to be of recent origin (Ehleringer *et al.*, 1997; Pyankov *et al.*, 2001b). In grasses and sedges, the higher number of species and greater age of the C_4 pathway create a more complex picture and points of origin are uncertain at this time.

From the distribution of C_4 dicots and their relatives, it is apparent that the 30 or so lineages are associated with one of five general centers of C_4 diversity that occur in the arid tropics, subtropics and warm temperate zones (Fig. 9). In North America, the main center corresponds to the arid zone stretching from southern Texas into central Mexico. With reasonably high confidence, the origin of five C_4 dicot lineages can be located in this region. In *Flaveria*, for example, this region has the greatest species and functional type diversity, with C_3, C_4 and most intermediate species (Powell, 1978). In addition, the nearest relative to *Flaveria*, the genus *Sartwellia*, occurs in the area. The nearest C_3 relative of C_4 *Chamaesyce* (Euphorbiaceae) occurs in southern Texas, indicating that C_4 photosynthesis arose here in this group (Webster *et al.*, 1975). C_4 *Heliotropium* section *Orthostachys* also appears to have arisen in the Mexican center due to high diversity of this section in northern Mexico and the presence of C_3–C_4

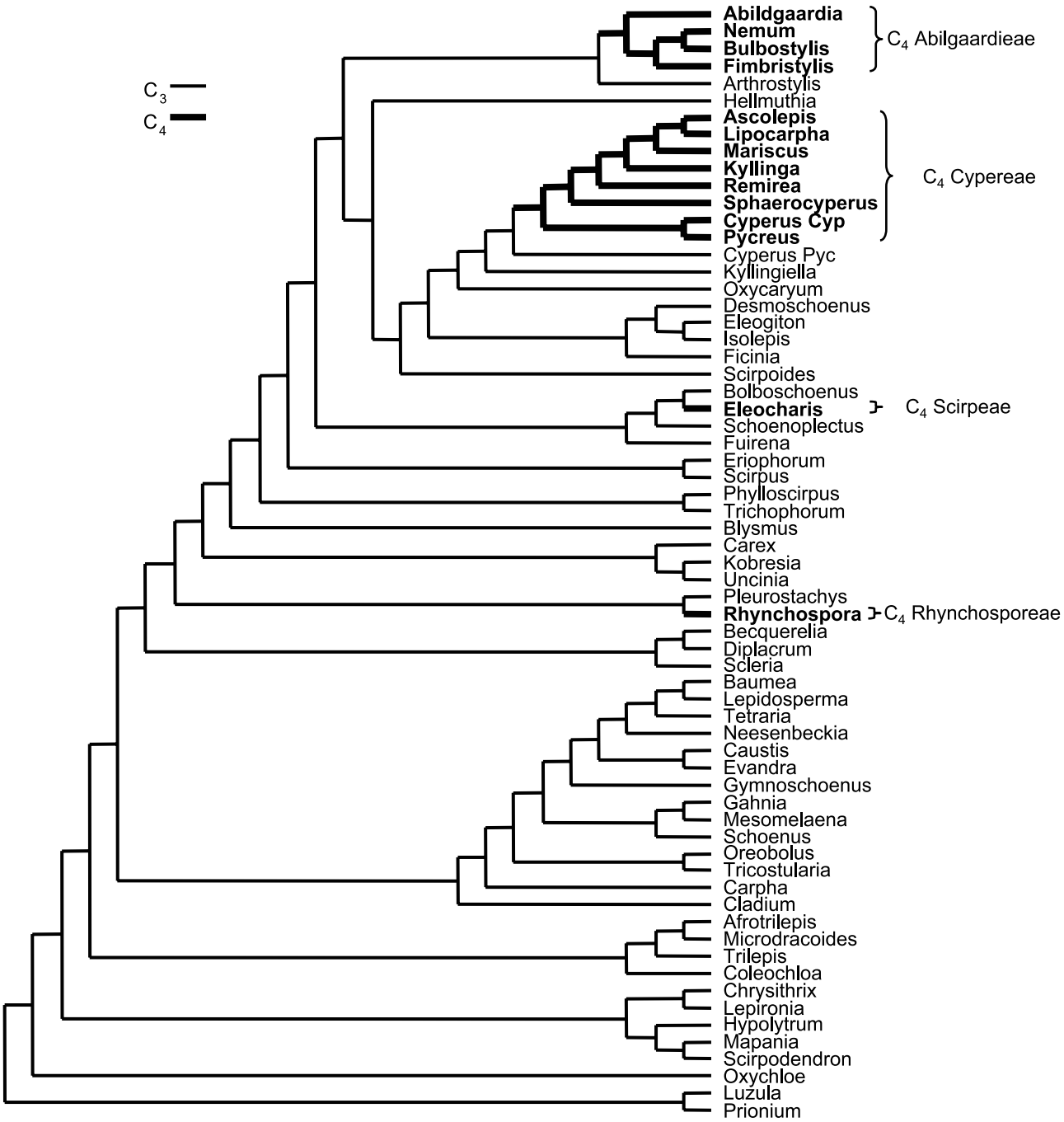

Fig. 6 Distribution of C_4 photosynthesis in the Cyperaceae. Genera containing C_4 species are shown in bold text; bold lines indicate C_4 lineages. From Soros & Bruhl (2000), reprinted by permission. Abbreviations: Cyp, Cyperus type; Pyc, Pycnostachys type.

intermediate species such as *Heliotropium convolvulaceum* (Frohlich, 1978). The 10 origins in the Chenopodiaceae are centered in central Asia, a region with vast interior basins and dry, salinized soils (Pyankov *et al.*, 2001a; 2001b; Kadereit *et al.*, 2003; Schütze *et al.*, 2003). In Africa, C_4 photosynthesis occurs in a number of families at very low diversity, for example, Scrophulariaceae (*Anticharis* with six C_4 species); Acanthaceae (*Blepharis* with fewer than 25 C_4 species); Gisekiaceae (*Gisekia* with five C_4 species); and Molluginaceae (*Mollugo* with 2–3 C_4 species) (Fig. 7; Table 1). Australia, by contrast, has no obvious C_4 origins among the dicots, despite being the driest of continents. Australia has a relatively diverse assortment of C_4 *Heliotropium* and *Polycarpaea* (Caryophyllaceae) species, but the center or origin for these groups is probably elsewhere, based on the presence of both C_3 and C_4 species of these genera in the Americas in the case of

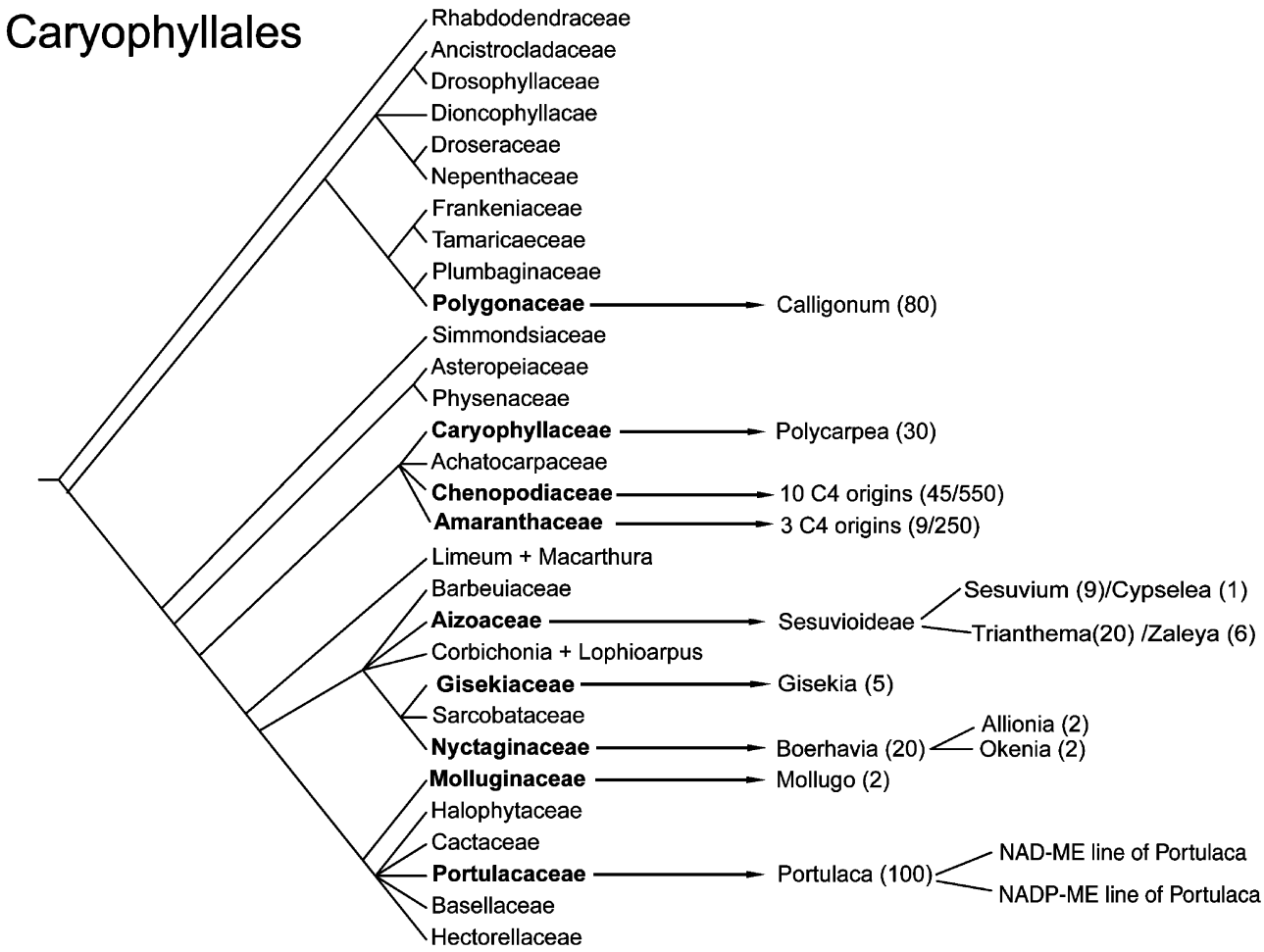

Fig. 7 Distribution of C_4 photosynthesis in the Caryophyllales. Families containing C_4 species are shown in bold, with arrows pointing to the principal genera. Numbers of C_4 genera are shown before a slash; C_4 species number appear after a slash, or in the case of listed genera, are indicated within parenthesis. Modified from stevens (2003) based on data in Schütze *et al*. (2003), Sage *et al*. 199a, and recent isotope screens of herbarium materials (R. Sage, unpublished).

Heliotropium, or Arabia and north-east Africa in the case of *Polycarpaea* (R.F.S., unpublished). Instead of being a center of origin, Australia may instead be a region where C_4 species arrived from elsewhere and extensively diversified.

VI. How did C_4 photosynthesis evolve?

1. Environmental imperatives

From the time of its initial discovery, C_4 photosynthesis has been described as an adaptation to hot, dry environments (Osmond *et al*., 1982; Hattersley, 1983). Since 1991, C_4 photosynthesis has also been hypothesized to be an adaptation to CO_2 deficiency, with low CO_2 of recent geological time being a major selection pressure (Ehleringer *et al*., 1991, 1997; Cerling *et al*., 1997). These views are widely cited, but both have been challenged in recent treatments. For example, a large proportion of the C_4 flora requires growth season precipitation to complete the life cycle, and C_4 plants do not appear to be any more drought-adapted than C_3 species from arid zones (Sage *et al*., 1999b; Ehleringer, 2004). Many C_4 species are also wetland plants with little drought tolerance, and a diverse flora of C_4 grasses occurs in the wet tropics (Jones, 1986; Maberly & Madsen, 2002). If the frequent occurrence of C_4 plants in arid regions is a sign that C_4 photosynthesis is an adaptation to aridity, then by the same logic C_4 photosynthesis would have to be considered an adaptation to moist conditions, and C_3 photosynthesis would be an adaptation to arid conditions, given the large number of C_3 species in arid regions. The challenge to low CO_2 as an environmental imperative for C_4 evolution arises from a disparity between the timing of C_4 expansion across the earth and the appearance of low atmospheric CO_2 (Pagani *et al*., 1999). The best estimates for CO_2 in ancient atmospheres indicate that CO_2 levels below the current value of 370 p.p.m. appeared by 25 million yr ago (Zachos *et al*., 2001). C_4-dominated ecosystems expanded across regions of mid- to low latitude 5 and 10 million yr ago, but no obvious shift in CO_2

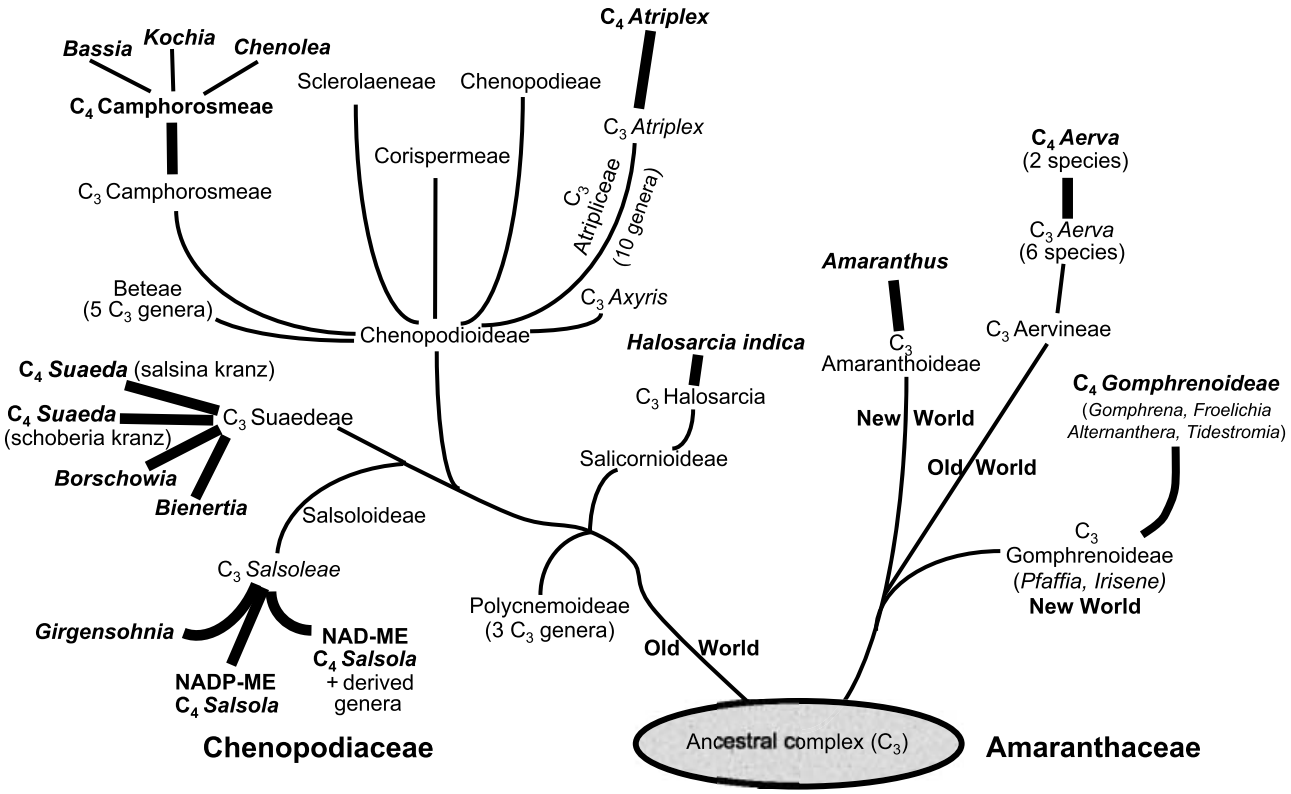

Fig. 8 Taxonomic tree showing C_4 lineages in the Chenopodiaceae and Amaranthaceae. C_4 lineages are indicated by bold lines and bold text. Adapted from Sage *et al*. (1999a), Kühn (1993), Townsend (1993) and Kadereit *et al*. (2004).

has been documented for this period (Latorre *et al*., 1997; Cerling, 1999; Zachos *et al*., 2001).

Instead of considering C_4 photosynthesis as a specific drought, salinity or low-CO_2 adaptation, it is better to think of it as an adaptation that compensates for high rates of photorespiration and carbon deficiency. In this context, any environmental factor that enhances photorespiration and reduces carbon balance could potentially select for traits leading to C_4 photosynthesis. Heat, drought, salinity and low CO_2 are the most obvious factors, but others, such as flooding, could also stimulate photorespiration in certain situations. The following section discusses how these factors stimulate photorespiration and inhibit carbon balance. This discussion sets the stage for a subsequent evaluation of how enhanced photorespiration could initiate the evolutionary sequence leading to C_4 photosynthesis.

Heat, drought and salinity High temperature is a major environmental requirement for C_4 evolution because it directly stimulates photorespiration and dark respiration in C_3 plants (Brooks & Farquhar, 1985; Sharkey, 1988). The availability of CO_2 as a substrate also declines at elevated temperature due to reduced solubility of CO_2 relative to O_2 (Jordan & Ogren, 1984). Aridity and salinity are important because they promote stomatal closure and thus reduce intercellular CO_2 level, again stimulating photorespiration and aggravating a CO_2 substrate deficiency (Guy *et al*., 1980; Schulze & Hall, 1982; Adam, 1990). Relative humidity is particularly low in hot, arid regions, which will further reduce stomatal conductance, particularly if the plant is drought stressed (Sage & Sharkey, 1987). Together, the combination of drought, increased salinity, low humidity and high temperature produces the greatest potential for photorespiration and CO_2 deficiency (Ehleringer & Monson, 1993), so it is not surprising that these environments are where C_4 photosynthesis would most frequently arise. Further evidence for dry and/or saline conditions supporting C_4 evolution comes from habitat observations of C_3–C_4 intermediate species, and the distribution of C_4 species at their extreme range limits. Many C_3–C_4 intermediates are from arid or saline zones, for example intermediate species of *Heliotropium* (Frohlich, 1978); *Salsola* (Voznesenskaya *et al*., 2001a), *Neurachne* (Poaceae, Monson & Moore, 1989); *Alternanthera* (Amaranthaceae, Monson & Moore, 1989); and a number of the *Flaveria* intermediates (Asteraceae, Powell, 1978). At the cold extremes of the C_4 range, the advantages of the C_4 pathway are nullified by low temperature. Here, the few remaining C_4 species are restricted to saline soils, dry soils, or microsites where bright sunshine can warm the leaf canopy (Long, 1983; Pyankov & Mokronosov, 1993; Sage & Sage, 2002).

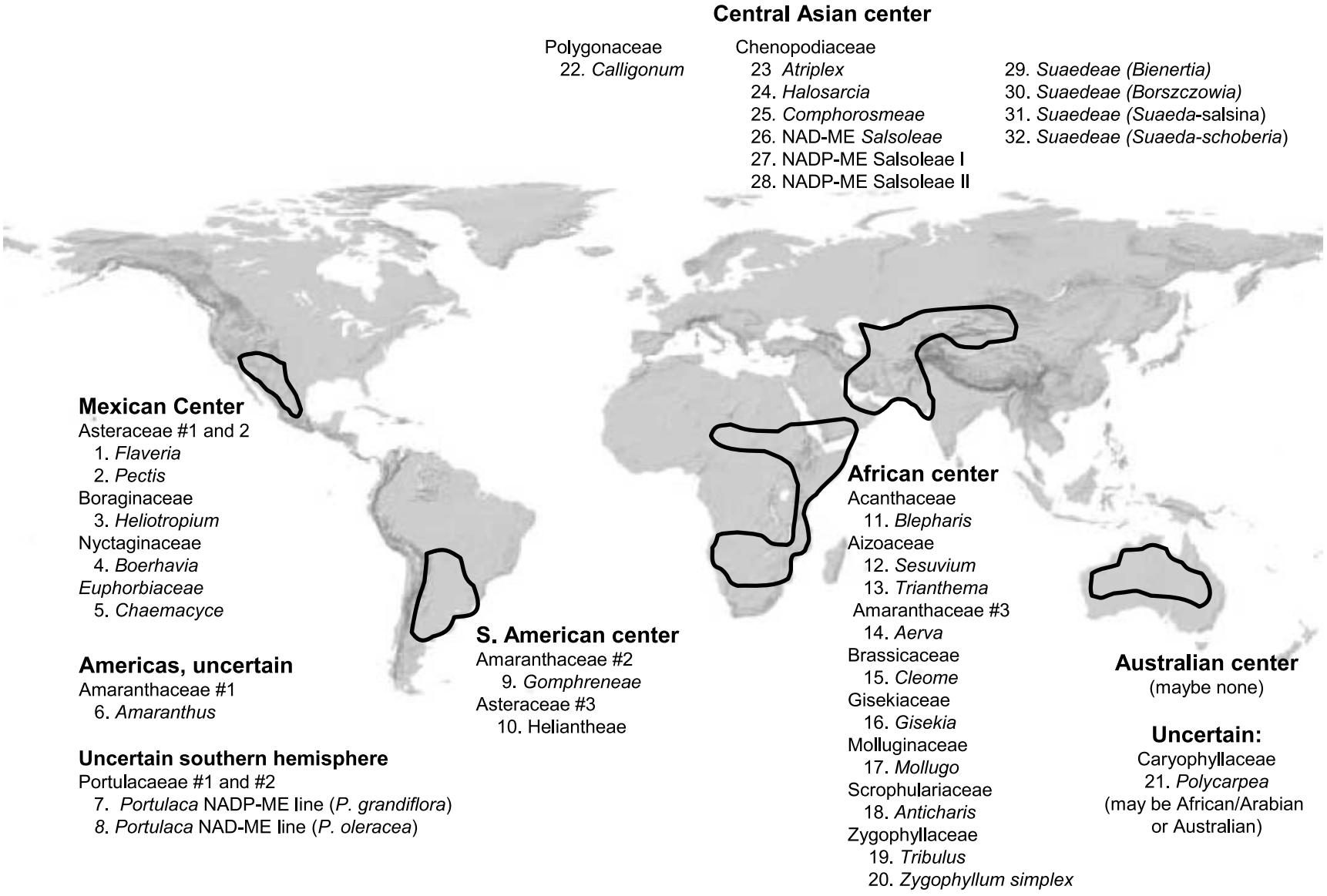

Fig. 9 Postulated regions of origin for C_4 photosynthesis in the dicot lineages of Table 1. Regions of origin were determined assuming C_4 photosynthesis originated where the diversity of species and photosynthetic pathway within a lineage are highest. Modified from Sage (2004).

In addition to their direct effects, heat, drought and salinity also favor C_4 evolution by altering ecosystem properties in a manner that enhances photorespiration. Vegetation cover tends to be low in arid and saline areas, such that the combination of warm climate and high insolation cause very high ground temperatures (> 50°C) which, in turn, heat the herbaceous layer (Archibold, 1995; Sage, 2004). Heat and drought also promote fire, a major disturbance favoring C_4 vegetation (Sage *et al.*, 1999b; Keeley & Rundel, 2003).

C_4 photosynthesis may have evolved in moist environments as well, which can be consistent with the carbon-balance hypothesis if environmental conditions are hot enough to promote photorespiration. The sedge lineages largely occur in low-latitude wetlands, indicating they may have evolved on flooded soils (e.g. *Eleocharis*; Takeda *et al.*, 1985; Ueno & Takeda, 1992), and the aquatic C_4 species certainly evolved in wet environments (Bowes *et al.*, 2002). In the case of the aquatic, single-celled C_4 species, warm shallow ponds typically become depleted in CO_2 during the day when photosynthetic activity from algae and macrophytes is high (Spencer *et al.*, 1994). Consequently, photorespiration can be very high.

Many of the C_3–C_4 intermediates also occur in moist, disturbed habitats such as riverbanks, roadsides and abandoned fields (Monson & Moore, 1989; Monson, 1989b). In Florida, for example, *Flaveria linearis* quickly proliferates on recently disturbed sites, but it generally does not hold these sites for more than a few years (Monson, 1989a). *Mollugo verticillata* (Molluginaceae) is scattered throughout eastern North America in highly disturbed areas, and C_3–C_4 intermediates of *Panicum* (= *Steinchisma*) grow on moist, open grasslands sites (Monson & Moore, 1989). These observations indicate that disturbance is also an important factor in C_4 evolution, particularly for lineages that may have arisen in wetter locations.

Low CO_2 In recent geological time, much lower CO_2 levels were the norm (Petit *et al.*, 1999). Between 100 and 12 000 yr ago, CO_2 levels ranged between 260 and 280 p.p.m., over 30% less than today's value. In the past 400 000 yr, atmospheric CO_2 was below 270 p.p.m. 96% of the time, and below 240 p.p.m. 67% of the time (Sage & Coleman, 2001). For about a fifth of this period, CO_2 was below 200 p.p.m. Although there is debate over when low-CO_2 conditions appeared in the past 100 million yr (Cerling *et al.*, 1997; Pagani *et al.*, 1999), there is evidence that they may have extended back to the Oligocene, some 25–30 million yr ago (Zachos *et al.*, 2001; Retallack, 2002). Because low CO_2 prevailed in recent geological time, discussions of C_4 evolution must consider selection pressures in atmospheres with less CO_2 than today.

In low CO_2, C_3 photosynthesis is impaired by the lack of CO_2 as a substrate in addition to enhanced photorespiration (Jordan & Ogren, 1984; von Caemmerer, 2000). As a result, water and nitrogen-use efficiencies are low, growth rates are low, competitive ability is reduced, recovery from disturbance is slow, and fecundity is low (Johnson *et al.*, 1993; Sage, 1995; Tissue *et al.*, 1995; Polley *et al.*, 1996; Ward, 2004). The inhibitory effects of heat, drought and salinity increase considerably in low CO_2, such that C_3 plants fail to reproduce and landscapes may become barren (Sage, 1995; Sage & Cowling, 1999). In *Phaseolus vulgaris*, wheat and tobacco, for example, growth at 200 p.p.m. CO_2 and moderate temperatures (26°C day and 19°C nights) was about half that of plants growing at current CO_2 levels, and the same growth temperature (Cowling & Sage, 1998; Sage & Cowling, 1999). Growth at elevated temperatures (36°C day/29°C night) and current CO_2 levels reduced plant growth by one-third to a half. Growth of plants at both elevated temperature and low CO_2 was > 85% less than growth in current CO_2 and moderate temperature, demonstrating strong additive effects of heat and CO_2 depletion. The plants were fully watered and fertilized, indicating that even in luxurious conditions, C_3 plants can fail in hot, low-CO_2 conditions (Sage & Cowling, 1999). Notably, none of the plants in the warm, low-CO_2 treatments flowered.

Conditions leading to the failure of C_3 vegetation are diagrammed in a conceptual model of the relationship between temperature and the CO_2 compensation point at different levels of organization in plants (Fig. 10). The CO_2 compensation point reflects the minimum CO_2 requirements for an autotrophic process to occur. For instantaneous gross and net photosynthesis, the CO_2 compensation point is well described and rises with temperature, largely reflecting a rise in photorespiration and mitochondrial respiration with rising temperature (Fig. 10 curves A and B; Kirschbaum & Farquhar, 1984; Brooks & Farquhar, 1985; Sage *et al.*, 1990). CO_2 compensation points rise with increasing levels of organization because mitochondrial respiration becomes a greater proportion of the overall carbon budget as the scale increases. For leaves over a 24 h period, incorporation of respiration in photosynthetic and nonphotosynthetic cells further increases the CO_2 compensation point (Fig. 10 curve C). For whole plants over 24 h, the carbon requirements of stems and roots increase the CO_2 compensation point (Fig. 10 curve D). Respiration costs over 24 h are substantial, accounting for 20–50% of daily carbon intake (Lambers, 1985; van der Werf *et al.*, 1992). Over the life span of an organ or individual plant, carbon costs associated with growth have to be met, so the life span CO_2 compensation point is greater than at lower levels of organization (Fig. 10 curve E). The ultimate measure of carbon balance from an evolutionary standpoint is the life-cycle CO_2 compensation point, which reflects carbon costs of flowers, fruits and seeds and thus is greater than the CO_2 compensation point of the whole plant over the growing season (Fig. 10 curve F). Drought or salinity stresses further increase CO_2 compensation points, because lower stomatal conductance and photosynthetic capacity reduce carbon income, allowing respiration to consume proportionally more of carbon acquired by the plant (Fig. 10 curve G).

Conceivably, life-cycle CO_2 compensation points at warmer temperatures could exceed the CO_2 level in the atmosphere,

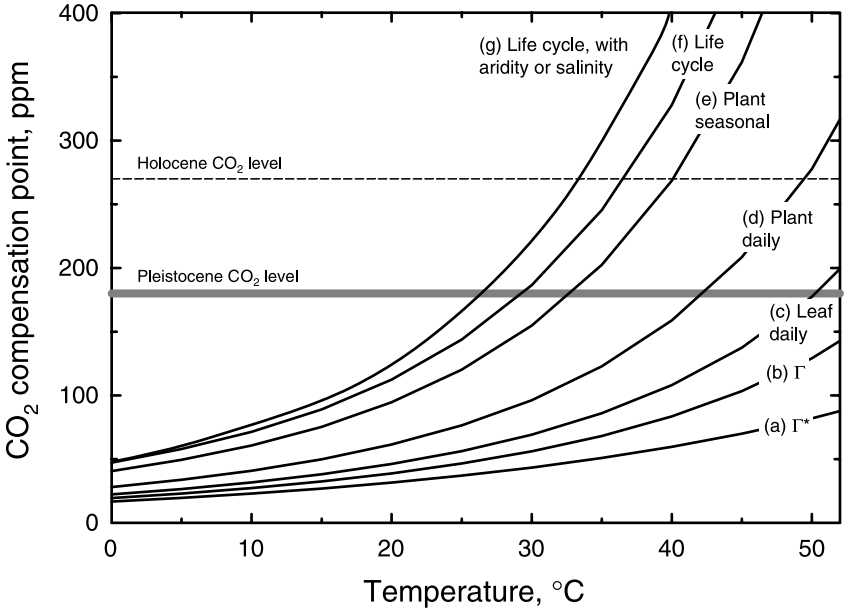

Fig. 10 Potential relationships between temperature and the CO_2 compensation point of plants determined over different spatial and temporal scales. CO_2 compensation points for the rate of Rubisco carboxylation (Γ*) and instantaneous net CO_2 assimilation rate (Γ) are based on measured data (Brooks & Farquhar, 1985; Sage *et al*., 1990). CO_2 compensation points at greater levels of complexity and time are educated guesses to demonstrate the potential effect. The grey line indicates atmospheric CO_2 levels corresponding to the late-Pleistocene; dashed lines are preindustrial Holocene levels of CO_2 that predominated over the past 10 000 yr. Adapted from Sage (2004). Portions of a curve above CO_2 lines indicate temperatures where a plant would be unable to meet its CO_2 requirement at a given CO_2 level.

particularly during past low-CO_2 events (Fig. 10, compare where curves F and G rise above the lines indicating Pleistocene and Holocene CO_2 levels). This would occur first in regions where drought and salinity interact with heat to enhance photorespiration, but could also occur in wet areas if the temperature is high and CO_2 levels sufficiently low. Where CO_2 supply is insufficient to meet minimum carbon requirements for the life cycle, landscapes would be unable to support C_3 vegetation and could become barren as a result. Genotypes expressing carbon conservation traits that lower CO_2 compensation points could potentially colonize these barren sites. They could then be subject to further selection pressure that could, in time, produce increasingly sophisticated modes of carbon conservation (Sage, 2004). One of these was probably C_4 photosynthesis.

2. Low CO_2 and the photorespiratory bridge to C_4 photosynthesis

Most discussions addressing the contributions of low CO_2 to C_4 evolution have emphasized the inhibition of carbon balance by elevated photorespiration. However, high rates of photorespiration also provide a metabolic resource that can be manipulated by natural selection to create a weak carbon conservation mechanism. In so doing, the sequence of events leading to C_4 evolution may be initiated (Monson & Moore, 1989; Rawsthorne, 1992; Bauwe & Kolukisaoglu, 2003). For this reason, low CO_2 is considered necessary to establish the bridge spanning the evolutionary trough separating C_3 and C_4 photosynthesis.

Photorespiratory metabolites are a carbon source that can be exploited to improve the efficiency of Rubisco in C_3 leaves (Hunt *et al*., 1987; von Caemmerer, 1989; Rawsthorne, 1992). If glycine decarboxylase (GDC) is localized into an interior compartment, photorespiratory metabolites such as glycine would have to be shuttled in from the surrounding mesophyll for decarboxylation (Fig. 11). The CO_2 released in photorespiration could then be trapped and used to enhance the activity of any Rubisco present in the interior tissue (von Caemmerer, 1989, 2000). By localizing GDC into the bundle sheath, therefore, plants can exploit photorespiration to create a weak CO_2-concentrating mechanism and enhance photosynthesis in low-CO_2 atmospheres (von Caemmerer, 1989; Rawsthorne, 1992). Photorespiratory CO_2 pumps occur in some two dozen species in *Alternanthera* (Amaranthaceae), *Panicum* and *Neurachne* (Poaceae), *Parthenium* (Asteraceae), *Moricandia* (Brassicaceae) and *Flaveria* (Asteraceae) (Monson, 1999). In *Moricandia arvensis* the CO_2 compensation point of photosynthesis is reduced 50–80% relative to normal C_3 plants, and photosynthesis at current CO_2 levels increases about 20% (Hunt *et al*., 1987; Hylton *et al*., 1988). Similarly, *Alternanthera*, *Parthenium* and *Panicum* species shuttling glycine exhibit low-CO_2 compensation points and high water and nitrogen-use efficiencies (Hylton *et al*., 1988; Brown & Hattersley, 1989; Monson & Moore, 1989; Morgan *et al*., 1993). The photorespiratory CO_2 pump is a stable feature in its own right, as evidenced by the lack of C_4 photosynthesis in *Moricandia* and other closely related genera of the Brassicaceae.

While not necessarily leading to C_4 photosynthesis, glycine shuttling is probably an important, if not essential, step in C_4 evolution. A common feature in all C_3–C_4 intermediates is the loss of GDC activity in the mesophyll and its enhancement in bundle sheath cells (Hylton *et al*., 1988; Rawsthorne, 1992; Morgan *et al*., 1993; Monson & Rawsthorne, 2000). Relative to C_3 species, the intermediates also exhibit close vein spacing, enlarged bundle sheath cells, increased frequency of

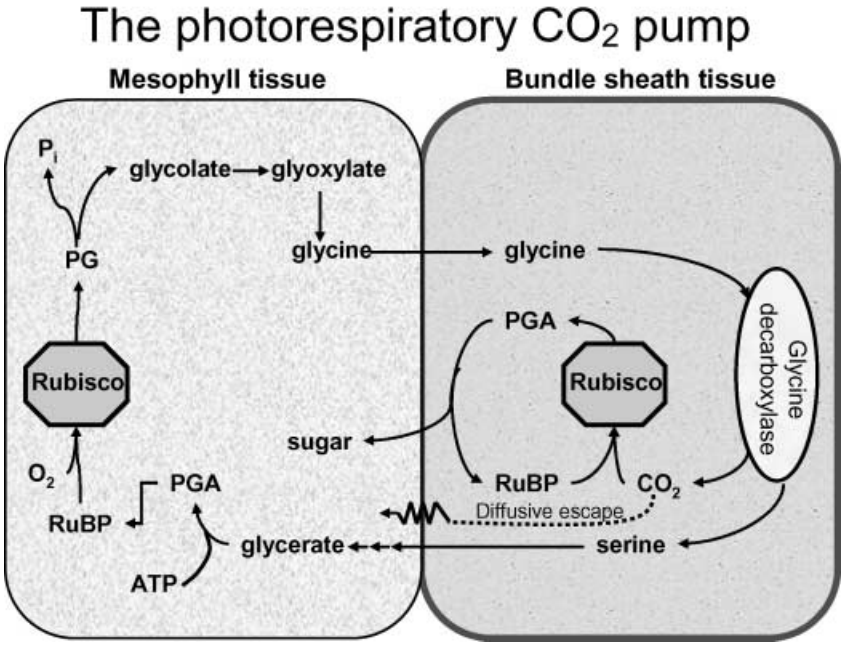

Fig. 11 The photorespiratory CO_2 pump. When glycine decarboxylase is localized in the bundle sheath tissue, the existing photorespiratory metabolism has to be redirected to shuttle glycine into the bundle sheath cells. Efflux of CO_2 released from glycine decarboxylation can then be slowed by high resistance in the bundle sheath wall, thereby allowing Rubisco in the bundle sheath to refix the CO_2. Adapted from Sage (2001) by permission.

plasmodesmata between mesophyll and bundle sheath cells, and increased number of organelles in the bundle sheath cells (Brown *et al.*, 1983; Brown & Hattersley, 1989; Monson & Moore, 1989; Rawsthorne, 1992). These developments facilitated efficient function of the glycine shuttle by reducing diffusion distances, increasing intercellular transport, and enhancing metabolic capacity in the bundle sheath. In doing so, they established the anatomical and ultrastructural framework required for the subsequent evolution of C_4 metabolism.

The importance of low CO_2 is further highlighted by theoretical assessments showing the effect of a glycine shuttle on C_3 photosynthetic efficiency (von Caemmerer, 1989, 1992, 2000). At current levels of CO_2, oxygenation is modeled to be 14% of carboxylation at 25°C, increasing to 23% at 40°C. At the low-CO_2 levels of the late Pleistocene, oxygenation relative to carboxylation rises to 25% at 25°C and 45% at 40°C. Assuming all GDC activity is localized in the bundle sheath tissue, net CO_2 assimilation is enhanced 8% at current levels of CO_2, but up to 40% at Pleistocene levels of CO_2 (von Caemmerer, 2000). In the absence of a glycine shuttle, the CO_2 compensation point of C_3 photosynthesis at 25°C is near 45 p.p.m.; with a glycine shuttle and 20% of the Rubisco in the bundle sheath, it is below 10 p.p.m. (von Caemmerer, 1989).

3. Evolutionary pathways to C_4 photosynthesis

Over the past 25 yr many groups have examined C_3–C_4 intermediates in order to identify the important phases of C_4 evolution, and numerous models of the evolutionary sequence have been proposed (e.g. Edwards & Ku, 1987; Brown & Hattersley, 1989; Rawsthorne, 1992; Monson, 1999). Based on this work, I present a summary model for C_4 evolution that recognizes seven significant phases (Fig. 12). For the sake of clarity, this model treats each phase as a distinct step that evolving species must proceed through in sequence. In reality there is extensive overlap between steps, and certain developments assigned to one stage may actually appear earlier or later in a given evolutionary lineage. To reflect this, Fig. 12 is presented as a gradation between full C_3 (dark) and C_4 (light) conditions.

As with most complex traits, the C_4 pathway appeared not at once, but in a series of incremental steps. Evolution was not directed towards C_4 photosynthesis, and each step had to be stable in its own right, either by improving fitness or at a minimum by having little negative effect on survival of the genotype. While lacking directionality, specific evolutionary events had to occur in a certain order, in that some steps were necessary to establish the conditions for subsequent developments. The model below is designed to reflect the ordered nature of C_4 evolution, beginning with a proposal that numerous preconditions had to be met if an evolutionary lineage were to even begin evolving C_4 characteristics.

Phase 1: general preconditioning The multiple origin of the C_4 pathway in some angiosperm families indicates that certain taxa developed characteristics that predisposed them to evolve C_4 photosynthesis. Phase 1 recognizes the probable existence of specific traits or preconditions that are needed if the C_4 evolutionary sequence is to commence. Specific preconditions have not been demonstrated experimentally, although speculations have identified potential candidates. The most important of these may be an ability to create and maintain large numbers of duplicated genes (Monson, 2003).

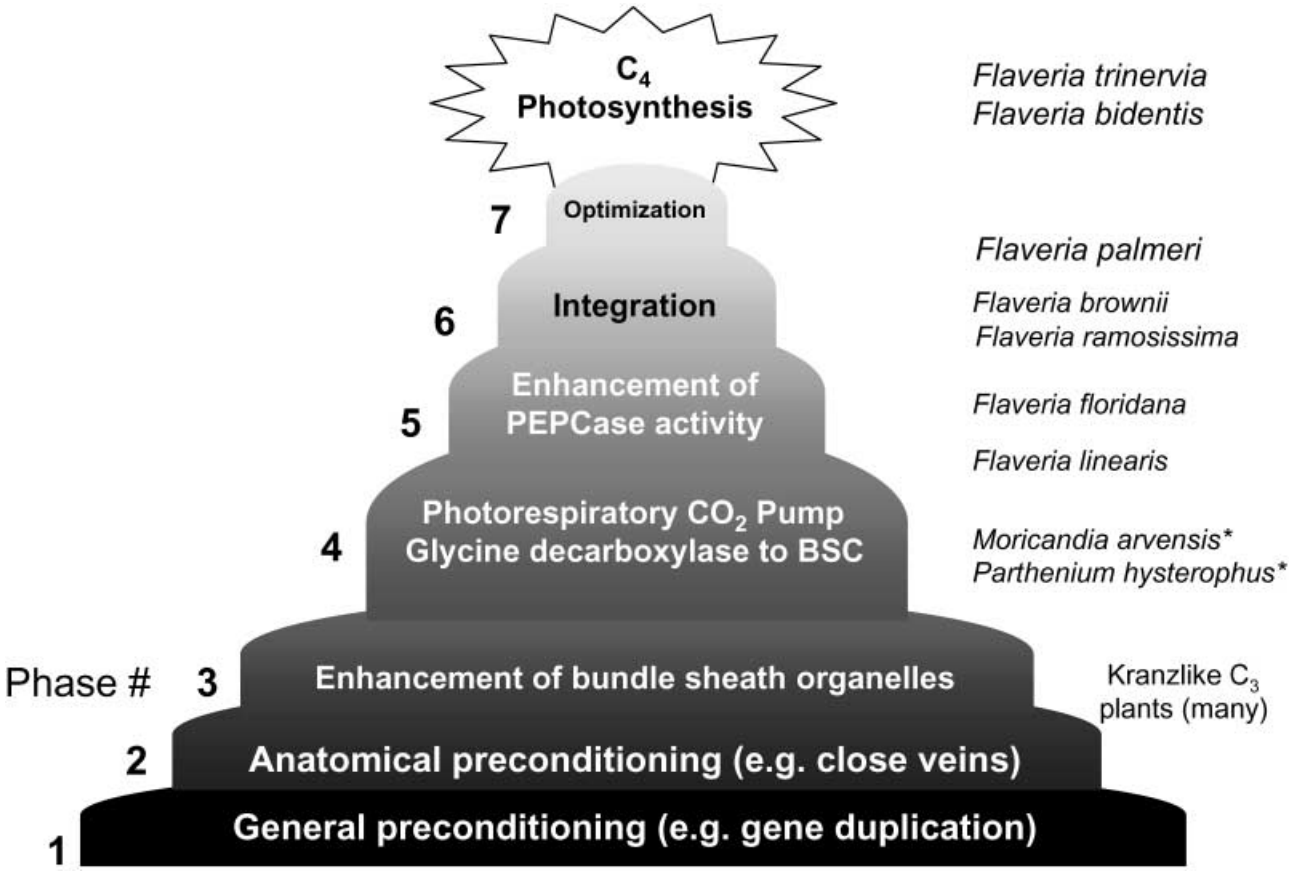

Fig. 12 Ascending the pyramid of C_4 photosynthesis: a simplistic model of the main phases of C_4 evolution. Species that mainly shuttle glycine (*) and C_3–C_4 intermediates are listed beside the phases they represent. Developed from Monson (1999).

Gene duplication creates multiple copies of a gene, allowing for modification of the copies without losing the original function of the transcribed protein (Lynch & Conery, 2000). Loss of function in a gene is usually deleterious without redundancy. A requirement for an abundance of duplicated genes would also restrict the type of species that could potentially evolve the C_4 pathway. Gene duplications occur through sexual recombination, and thus are more likely to accumulate in short-lived annuals and perennials where sexual reproduction occurs many times per decade. Short-lived plants will also experience intense selection pressure on a yearly basis, so duplicated genes and novel mutations will be screened frequently for adaptive function (Monson, 2003). Once selection acts on duplicated genes, reproductive barriers could appear rapidly and genetically isolate populations (Lynch & Conery, 2000). In the absence of reproductive barriers, gene flow from source populations could conceivably swamp the appearance of C_4 genes in populations evolving C_4-like traits.

Phase 2: anatomical preconditioning To evolve an effective CO_2 concentration mechanism, the distance between mesophyll and bundle sheath cells has to decline to allow for rapid diffusion of metabolites (Raghavendra, 1980; Ehleringer *et al.*, 1997). This is typically accomplished by reducing interveinal distance and/or enhancing the size of the bundle sheath layer. In C_4 plants, veins are typically separated by 60–150 μm and one to four mesophyll cells, while in C_3 plants interveinal distance is generally > 200 μm, with more than five mesophyll cells between the veins (Dengler *et al.*, 1993; Ogle, 2003). Reducing interveinal distance and increasing bundle sheath size may initially have little to do with photosynthetic metabolism; rather, they may improve structural integrity in windy locations or enhance the water status of the leaf in hot environments. Reducing intervein distance reduces evaporative surface area relative to conduit size, while increasing bundle sheath volume can enhance water storage in a leaf, thereby protecting against surges in transpiration that follow sunflecks or wind gusts (Sage, 2001). C_3 species from low latitudes often have closely spaced veins and larger bundle sheaths, and aridity and wind both enhance vein density, which is inversely correlated with vein spacing (Shields, 1950; Uhl & Mosbrugger, 1999; Roth-Nebelsick *et al.*, 2001). Interveinal distance may be easier to reduce in species with parallel venation (grasses) than in species with reticulate venation (dicots), which may explain in part why C_4 photosynthesis is prolific in the grass family (Ehleringer *et al.*, 1997).

Phase 3: increase in bundle sheath organelles In typical C_3 plants the bundle sheath cells have few chloroplasts and little photosynthetic activity (Metcalfe & Chalk, 1979). To create the necessary metabolic sinks for glycine metabolism and eventual metabolism of C_4 acids, the number of chloroplasts and mitochondria in the bundle sheath must increase. This could initially occur simply to maintain photosynthetic capacity in leaves with enlarged bundle sheath cells. As vein spacing declines and bundle sheath size increases, the bundle sheath cells become a significant fraction of the leaf area. Without increases in chloroplast number in the bundle sheath, light absorbance would fall as interveinal distance declines. Once chloroplast numbers in the bundle become a significant fraction of total number of chloroplasts in the leaves, the capacity of the bundle sheath cells to process glycine from the mesophyll could be large enough to support the subsequent development of a photorespiratory CO_2 pump (Brown & Hattersley, 1989; Rawsthorne, 1992).

As the glycine shuttle is enhanced, a further increase in organelle number could follow, potentially allowing for greater growth and fecundity in high photorespiratory environments. With each incremental rise in bundle sheath organelle content, increased efficiency is apparent, as evidenced by falling CO_2 compensation points (Fig. 13; Brown & Hattersley, 1989).

Phase 4: glycine shuttles and photorespiratory CO_2 pumps Once the anatomical preconditions are in place, the adjustments needed to establish a glycine shuttle might readily occur. Initially this may involve duplication of a gene coding for GDC, with the distinct copies eventually being expressed on separate promoters in the mesophyll and bundle sheath tissues (Monson, 1999). A loss of function mutation in the mesophyll GDC gene could then establish an imbalance in GCD function, such that glycine would have to move to the bundle sheath to prevent lethal accumulation of photorespiratory products. This apparently happened in *M. arvensis*, where the P-protein of the mesophyll GDC became nonfunctional (Morgan *et al.*, 1993). Following a mutation in mesophyll GDC, the resulting build-up of photorespiratory metabolites should promote subsequent selection for efficiency in the glycine shuttle. Genotypes with a low capacity to process glycine in the bundle sheath would be harmed by high levels of photorespiratory intermediates. By contrast, genotypes that efficiently transport and metabolize glycine in the bundle sheath would survive to influence future generations.

Phase 5: enhancement of mesophyll PEPCase activity Following the establishment of a glycine shuttle, CO_2 levels in the bundle sheath increase substantially, creating a large gradient for CO_2 efflux (von Caemmerer, 2000). To scavenge some of the CO_2 escaping from the bundle sheath, PEPCase activity could rise in the mesophyll, and the resulting C_4 acids could be directed back to the bundle sheath for refixation (Monson, 1999). As PEPCase activity increases further, it would also fix significant amounts of CO_2 diffusing in from the intercellular spaces, creating the potential for a true C_4-type CO_2 pump. PEPCase increase is well documented in the *Flaveria* intermediates with activity increasing about 40 times from C_3 to full C_4 species (Monson & Rawsthorne, 2000; Svensson *et al.*, 2003). Significant increases occur in the C_3-like intermediates *F. linearis* (five times over C_3 values) and *F. ramosissima* (seven times greater than C_3 values) and the C_4-like intermediate *F. brownii* (20 times greater than C_3 values) (Monson & Moore, 1989).

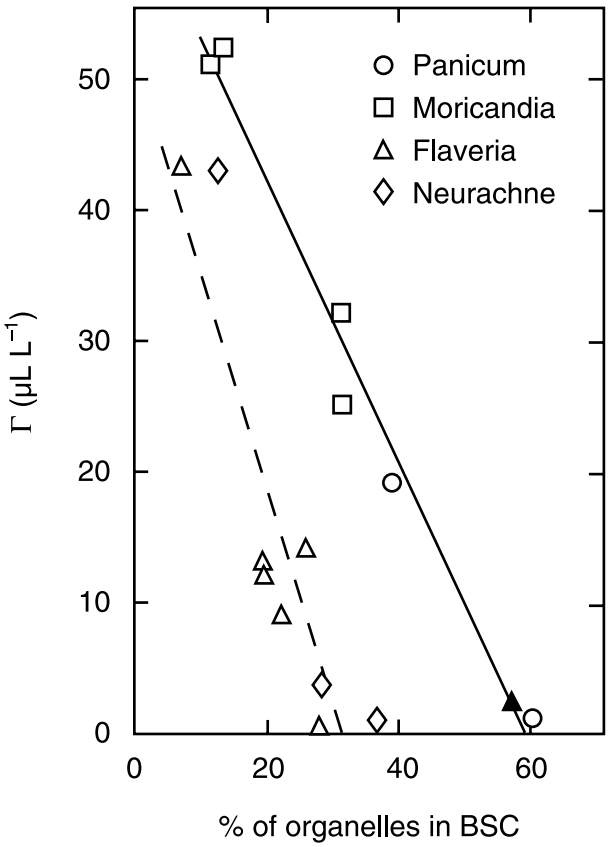

Fig. 13 Relationship between proportion of organelles that occur in the bundle sheath of species expressing C_3–C_4 intermediacy and the CO_2 compensation point of net CO_2 assimilation. The *Flaveria* response is offset from that of the other species because *Flaveria* operates a limited C_4 cycle in addition to a glycine shuttle. The other species primarily operate a glycine shuttle. From Brown & Hattersley (1989), reprinted by permission.

As PEPCase activity is increased, the other phases of the C_4 cycle should also increase in order to sustain PEP regeneration. Increasing activity of the decarboxylating enzymes may be relative simple, because NADP-ME and NAD-ME activities are already enhanced in the vascular tissue region of C_3 plants in order to metabolize organic acids leaving the vascular tissue. In tobacco, for example, NADP-ME activity and NAD-ME activity are ninefold to 13-fold greater in veins than mesophyll cells (Hibberd & Quick, 2002). These enzymes may already have promoters specific to procambial tissue, such

that further enhancing their activity in response to greater PEPCase expression may require only increased transcription intensity, rather than redirection of expression which is more involved (Hibberd & Quick, 2002).

In NADP-ME and NAD-ME subtypes, PPDK activity also has to be high to provide the necessary PEP for C_4 species. This step does not appear to occur in parallel with increasing PEPCase activity, because in C_3-like *Flaveria* intermediates, PEP regeneration occurs by increasing the activity of 3-PGA mutase and enolase, which convert PGA to PEP (Monson & Moore, 1989). Only in the later stages of C_4 evolution, after PEPCase dominates mesophyll carboxylation, does PPDK assume the primary role in PEP regeneration (Monson & Moore, 1989).

Phase 6: integration of C_3 and C_4 cycles As the activity of the C_4 cycle climbs, it increasingly competes with Rubisco and the C_3 cycle in the mesophyll for CO_2 and ATP. To avoid this competition, and to fully integrate the C_3 and C_4 cycles, the expression pattern of most enzymes in the photosynthetic apparatus has to be reorganized. The extent of reorganization has recently been documented in the C_4 grass *Sorghum bicolor* using a differential screening approach to identify genes that are expressed in either mesophyll or bundle sheath cells (Wyrich *et al.*, 1998). Twenty-five cDNAs exhibited mesophyll-specific expression, and eight showed bundle sheath-specific expression. The mesophyll-specific expression included the main C_4-cycle enzymes (PEPCase, PPDK), carbonic anhydrase, photosystem II proteins, NADP-oxidoreductase, and ferridoxin. The bundle sheath-specific enzymes include Rubisco, NADP-ME, Rubisco activase, and numerous Calvin cycle enzymes. In the case of Rubisco, localization to the bundle sheath occurs later in the integration sequence, at least in *Flaveria* (Monson & Rawsthorne, 2000). A significant reduction of mesophyll Rubisco activity occurs in the C_4-like *F. brownii*, but not in the other, less C_4-like intermediates (Bauwe, 1984; Reed & Chollet, 1985; Cheng *et al.*, 1988).

Based on *Flaveria* studies, integration becomes necessary above the point where leaf PEPCase activity equals Rubisco activity (Monson *et al.*, 1988; Monson & Rawsthorne, 2000). Below this point, PEPCase activity appears primarily to support the glycine shuttle, as indicated by carbon isotope ratios that are typical for C_3 species (Monson *et al.*, 1988; Monson & Rawsthorne, 2000). Above 50% C_4-cycle contribution, carbon isotope ratios increase towards C_4 values, indicating increasing integration of C_3 and C_4 cycles (Monson & Rawsthorne, 2000). Once fully integrated, the C_4 cycle activity efficiently concentrates CO_2 into the bundle sheath, and carbon isotope ratios exhibit normal C_4 values.

Of particular note in the integration process is the new role assumed by carbonic anhydrase (CA) in the C_4 leaf. In C_3 leaves, a chloroplast form of CA assists in the diffusion of CO_2 into the stroma (Coleman, 2000). In C_4 leaves, cytosolic CA activity increases in the mesophyll, while the activity of the chloroplast form is negligible, particularly in the bundle sheath (Hatch & Burnell, 1990; Ludwig *et al.*, 1998). The mesophyll form of the C_4 CA converts CO_2 to bicarbonate in order to support high PEPCase activity. Without high CA activity in the mesophyll, PEPCase quickly drains the bicarbonate pool and photosynthesis slows by 80 – 90% (Hatch & Burnell, 1990; Coleman, 2000). Very low CA activity in the bundle sheath chloroplasts is also important for efficient C_4 photosynthesis. If CA activity was high in the bundle sheath, the CO_2 released from C_4 acids would be converted to bicarbonate, which would then diffuse out of the cell without being consumed by Rubisco (Ludwig *et al.*, 1998).

Phase 7: optimization and whole-plant coordination As the C_4 pathway approaches full functionality, the concentration of substrates and effector metabolites in the photosynthetic cells changes significantly. To optimize photosynthetic efficiency, kinetic properties and regulatory set-points of many enzymes have to be adjusted to compensate for changes in the metabolic environment (Leegood & Walker, 1999). For example, PEPCase is inhibited by malate, but malate levels in the mesophyll have to rise substantially in order to establish a sufficiently large gradient to permit rapid malate diffusion into the bundle sheath. In response, C_4 PEPCase has a lower sensitivity to malate, while sensitivity to the activator glucose-6-phosphate is enhanced (Bläsing *et al.*, 2000; Svensson *et al.*, 2003). C_4 PEPCase also has a higher affinity for bicarbonate relative to the C_3 form of the enzyme, and a lower affinity for PEP (Chollet *et al.*, 1996). PEP levels are high due to enhanced PPDK activity, so the lower affinity for PEP may not be disadvantageous (Svensson *et al.*, 2003). The changes in PEPCase are not present in the C_3–C_4 intermediates, but only in fully developed C_4 species, indicating that optimization of PEPCase occurs in the final stages of C_4 evolution.

In the case of NADP-ME, the C_4 isoforms have higher specific activity and a lower K_m for malate then the ancestral C_3 form (Drincovich *et al.*, 2001). The intermediate *Flaveria floridana* exhibits specific activities and K_m values of NADP-ME that are between values of C_3 and C_4 forms of the enzyme (Casati *et al.*, 1999), indicating that optimization of NADP-ME regulation overlaps extensively with earlier phases (Fig. 12).

In C_3 plants, Rubisco has a relatively low catalytic capacity, which is a consequence of its high relative specificity (Andrews & Lorimer, 1987; Hudson *et al.*, 1990). Because C_4 Rubisco encounters high CO_2 in C_4 bundle sheaths, oxygenase activity is minor and hence Rubisco can evolve into a higher k_{cat} form with no negative consequences (Seemann *et al.*, 1984). This has occurred in many C_4 grasses and some C_4 dicots, as demonstrated by higher values of k_{cat} and K_m for CO_2 (Yeoh *et al.*, 1981; Seemann *et al.*, 1984; Sage & Seemann, 1993; von Caemmerer & Quick, 2000; Sage, 2002a). In maize, for example, both k_{cat} and K_m for CO_2 of Rubisco are nearly double those of C_3 grasses (Seemann *et al.*, 1984; von Caemmerer & Quick, 2000). Rubisco from the C_3–C_4 *Flaveria* intermediates

exhibits C_3 kinetic parameters (Wessinger *et al.*, 1989), showing that alteration of Rubisco kinetics occurs late in the evolutionary sequence, probably after a full C_4 cycle has evolved. Consistently, not all C_4 plants alter Rubisco properties. *Flaveria bidentis* and *F. vaginata* (both C_4) have a high k_{cat} of Rubisco, while the C_4 species *Flaveria trinervia* has a C_3-like k_{cat} value (Wessinger *et al.*, 1989; Hudson *et al.*, 1990).

C_4 plants have greater water-use efficiency (WUE) than C_3 plants, allowing for two important developments at the whole-plant level. First, stomatal sensitivity to CO_2 and light are increased, enhancing the ability of stomata to respond to environmental variation at the relatively low conductances exhibited by C_4 plants (Schulze & Hall, 1982; Huxman & Monson, 2003). *Flaveria* C_3–C_4 intermediates exhibit stomatal responses to CO_2 that are similar to C_3 responses, indicating that stomatal set points shift late in the evolutionary process (Huxman & Monson, 2003). Second, superior WUE relaxes hydraulic demands on the conducting pathway in the xylem, allowing for alteration of xylem structure and patterns of biomass allocation to exploit the environmental conditions more effectively. On average, C_4 plants have a leaf specific conductivity (hydraulic conductivity per leaf area) that is one-third that of C_3 species from similar habitats or taxonomic groups (Kocacinar & Sage, 2003). C_4 plants from resource-rich areas achieve a lower leaf specific conductivity by increasing leaf area per unit of conducting tissue. This enhances canopy size and growth potential in competitive environments. C_4 plants from arid regions produce safer xylem with greater resistance to cavitation and, as a result, may be better able to resist extreme drought stress (Kocacinar & Sage, 2003).

In summary, phase 7 represents selection for traits that allow plants to exploit the productive potential of the C_4 pathway to the maximum. As plants enter this phase, they may have a functional C_4 pathway that is effective in stressed environments where competition is low. However, inefficiencies within the photosynthetic biochemistry, and limited coordination between the C_4 biochemistry and stomata, should limit overall performance. At the end of this phase, C_4 plants are proposed to be finely tuned photosynthetic machines where the leaf physiology is well integrated into whole plant function, leading to large gains in productive potential and competitive ability. Consistently, grasses from the older C_4 lineages dominate significant portions of the Earth's surface and exhibit record levels of primary productivity (Kellogg, 1999; Long, 1999). Younger C_4 lineages in the dicots largely occur in uncompetitive settings either due to high disturbance, chronic aridity or elevated salinity (Ehleringer *et al.*, 1997; Pyankov *et al.*, 2001b).

VII. Molecular evolution of C_4 photosynthesis

The predominant mechanisms in the evolution of C_4 genes are proposed to be gene duplication followed by nonfunctionalization and neofunctionalization (Marshall *et al.*, 1996; Monson, 1999, 2003), and alteration of *cis*-regulatory elements in single copy genes to change expression patterns (Rosche & Westhoff, 1995). Major targets for non- and neofunctionalization are the promoter and enhancer region of genes to allow for altered expression and compartmentalization, and the coding region to alter regulatory and catalytic properties. Both non- and neofunctionalization can come about through mutations, crossover events, and insertions of mobile elements (Kloeckener-Gruissem & Freeling, 1995; Lynch & Conery, 2000). Mutations in the coding region change or eliminate protein function, while mutations in the promoter region typically cause loss of expression. For example, mutation in the mesophyll GDC gene causes loss of mesophyll function in *M. arvensis*, setting up a need to shuttle glycine to the bundle sheath (Morgan *et al.*, 1993). Likewise, mutation in the promoter region of chloroplastic CA in mesophyll cells eliminated the C_3 function of CA in *Flaveria* (Ludwig & Burnell, 1995; Monson, 2003). Movement of DNA via transposable elements and crossover events could be particularly important in the evolution of *cis*-regulatory elements in promotor regions (Doebley & Lukens, 1998). Cell-specific promotors are widespread in plant genomes, such that it is easier to envision changes in tissue-specific expression arising from insertion of an existing promoter or enhancing sequences into a *cis*-regulatory element, rather than evolution of novel regulatory elements by mutation (Kirchhamer *et al.*, 1996). Insertion of functional promoters or enhancers into single copies of genes could also change expression without gene duplication (Rosche & Westhoff, 1995).

The model system for studying C_4 gene evolution has been the PEPCase gene family in *Flaveria*, with more recent work examining PEPCase genes in *Alternanthera* (Bläsing *et al.*, 2000, 2002; Svensson *et al.*, 2003). Three PEPCase genes are present in *Flaveria* (*ppcA*, *ppcB*, *ppcC*) coding for the range of functions observed in C_3 plants. The C_4 enzyme is a variation of *ppcA*, and is proposed to have arisen by gene duplication of *ppcB*, an ancestral gene for the existing C_3 and C_4 PEPCase genes in *Flaveria* (Bläsing *et al.*, 2002). In the C_4 lineage, three major alterations changed *ppcA* into the C_4 gene. First, a 2 kb *cis*-regulatory element in the promoter of *ppcA* was altered to produce mesophyll specificity and increase PEPCase transcription (Stockhaus *et al.*, 1997). The other significant changes occurred in the coding region of *ppcA* (Svensson *et al.*, 2003). At position 774 of the C_4 *ppcA*, a serine has been substituted for an alanine present at this position in C_3 *ppcA*. This substitution is common to all C_4 PEPCase genes examined, indicating that it is critical to C_4 photosynthesis. It occurs late in the evolutionary sequence, as none of the C_3–C_4 intermediates in *Flaveria* or *Alternanthera* exhibit the serine substitution. At positions 296–427 (region 2 in *ppcA*) numerous substitutions are present, altering glucose-6-phosphate sensitivity. Together, the changes in region 2 and at position 774 interact to alter the malate sensitivity and the k_m for PEP (Svensson *et al.*, 2003).

NADP-ME in C_3 *Flaveria* plants is present in a cytosolic form (*CytMe1*, a 72 kDa protein) and two chloroplastic forms (*ChlMe1*, which codes for a 62 kDa product and *ChlMe2*, which codes for a 64 kDa protein; Marshall *et al.*, 1996; Drincovich *et al.*, 1998; Lai *et al.*, 2002). The 72 kDa form is the predominant form in C_3 plants and is expressed at low levels throughout the plant (Marshall *et al.*, 1996; Drincovich *et al.*, 1998). All three isoforms are expressed in the more C_3-like C_3–C_4 intermediates, while in C_4-like intermediates the 64 kDa isoform begins to dominate expression. In leaves of C_4 *Flaveria* the 62 kDa isoform is required for complete C_4 functioning, and its expression is greater than in the other forms (Drincovich *et al.*, 1998). This isoform evolved from ancestral NADP-ME by duplication followed by promoter modifications that enhanced expression and conferred bundle sheath specificity (Marshall *et al.*, 1996, 1997; Ali & Taylor, 2001; Lai *et al.*, 2002).

The evolution of the PPDK gene presents an interesting alternative to the evolution of *ppcA*. In C_4 plants PPDK is not significantly changed from the C_3 form, because kinetic properties and the coding sequences for the functional proteins are the same (Rosche & Westhoff, 1995; Miyao, 2003). Rather, the major changes occur in the promoter region (Matsouka & Numazawa, 1991). In maize, an ancestral cytosolic *Pdk* gene with one promoter is proposed to have been duplicated (Miyao, 2003). One of the products (Pdk1) gained a second promoter and a chloroplast transit peptide. Light- and tissue-responsive elements were added next, allowing the second promoter to restrict transcription to illuminated green leaves. This gene codes for the chloroplast PPDK of C_3 grasses. In the evolution of the C_4 Pdk1 in maize, the second promoter acquired enhancer and tissue-specific elements that directed high expression of PPDK to the chloroplasts of the mesophyll cells. In C_3 and C_4 *Flaveria*, by contrast, only one PPDK gene with two promoters is present (Rosche & Westhoff, 1995). The first promoter initiates transcription of a cytosolic form of PPDK; the second resides upstream of a chloroplast transit sequence and initiates transcription of a chloroplast form. Because *Flaveria* has only one PPDK gene, it is thought the C_4 form arose via insertion of mesophyll-specific enhancing elements into the second promoter, rather than by gene duplication (Rosche & Westhoff, 1995).

The localization of Rubisco to the bundle sheath is controlled by a bundle sheath-specific promoter on the small subunit gene of Rubisco, *rbcS*. Maize has *cis*-acting elements in the *rbcS* promoter that are bundle sheath-specific, while rice lacks these elements and transcribes *rbcS* in both mesophyll and bundle sheath tissues (Sheen, 1999; Nomura *et al.*, 2000). Addition of a maize promoter sequence to the rice promoter silenced mesophyll expression of *rbcS*, indicating that addition of this element may have occurred during C_4 evolution (Schäffner & Sheen, 1991). In terms of the kinetic properties, the shift in K_m of Rubisco observed in the C_3 to C_4 transition is brought about by subtle changes in the coding region of *rbcL*, possibly by changing active site geometry (Hudson *et al.*, 1990). In *Flaveria*, three codon changes occurred during the evolution of the C_4-type *rbcL* (Hudson *et al.*, 1990).

VIII. When did C_4 photosynthesis evolve?

The appearance of C_4 photosynthesis in geological time has been assessed in three ways. First, screening for C_4 isotopic signatures in fossil soils, or fossilized plant and animal material, potentially reveals the presence of C_4 plants on the landscape. C_4 plants have 8 to 15 more carbon 13 molecules per hundred thousand carbon 12 molecules than do C_3 plants (Cerling, 1999). If there has been no change in the carbon isotope ratio during metabolism by animals or fossilization, anything built from carbon should reflect the carbon isotope ratio of the source plant. Carbon isotope ratios of fossil soils, animal tooth enamel, and fossil eggshells show a dramatic shift from C_3- to C_4-like values between 8 and 5 million yr ago in Africa, South America, China, North America and Pakistan, demonstrating widespread expansion of C_4-dominated biomes during this period (Cerling *et al.*, 1997; Latorre *et al.*, 1997; Cerling, 1999). Modest shifts in isotopic ratios from soils and herbivores in East Africa and North America at 14–20 million yr ago have also been reported (Kingston *et al.*, 1994; Morgan *et al.*, 1994; Fox & Koch, 2003), indicating that C_4 plants may have been common on mid-Miocene landscapes.

Second, fossils of leaves exhibiting Kranz anatomy indicate past presence of C_4 photosynthesis in specific species. Ideally, unaltered carbon can also be extracted from the sample to allow for an isotopic confirmation. By these two criteria, the oldest undisputed C_4 fossils are from 12.5 million yr old grass leaves that grew in California (Tidwell & Nambudiri, 1989; Cerling, 1999). These resemble Panicoid grasses, have C_4 carbon isotope signatures, and exhibit clear Kranz anatomy. The oldest suspected fossilized C_4 leaves are 14.5 million yr old, from Kenya. These match the cuticular morphology of the grass blades of extant members of the Chloridoideae, which are now almost completely C_4. Because the samples were altered during fossilization, Kranz tissues are not available and isotopic ratios are unreliable (Dugas & Retallack, 1993). Therefore the presence of C_4 photosynthesis in these samples cannot be definitively confirmed.

Uncommon plants are rarely found in fossil floras, and they are too infrequent to alter isotopic ratios in herbivores or soil detritus. Hence the presence of C_4 fossils or isotopic signatures reflects when C_4 plants were common on a landscape, rather than when C_4 photosynthesis first evolved. To assess the earliest origin of C_4 photosynthesis within a lineage, it is necessary to compare gene sequences in extant taxa, and to use a molecular clock approach to estimate the divergence time. This technique has been used in the grasses and Chenopods, but not in other taxa. In grasses, molecular clock analyses indicate that the earliest C_4 plants arose at least 20–30 million yr

ago (Kellogg, 1999; GPWG, 2001). Sequence variation in the genes of malate dehydrogenase and starch synthase indicate that maize and sorghum diverged 17 million yr ago, while analysis of alcohol dehydrogenase sequences indicate *Pennisetum* and maize diverged at least 25 million yr ago (Gaut & Doebley, 1997). *Danthoniopsis* is estimated to have diverged from other C_4 panicoid grasses by about 16 million yr ago (Kellogg & Russo in GPWG, 2001). Because these grasses are all C_4, this indicates the origin of C_4 photosynthesis had to occur before their estimated divergence (Kellogg, 1999). In the Chenopodiaceae, recent work with *rbcL* indicates C_4 members of the Salsoleae diverged 14–21 million yr ago, while C_4 *Atriplex* species diverged from C_3 *Atriplex* 8–11.5 million yr ago (Kadereit *et al.*, 2004).

Numerous groups have suggested C_4 photosynthesis evolved much earlier in geological time. The Carboniferous period (280–340 million yr ago) has been suggested as a possible time of C_4 photosynthesis based on atmospheric conditions that were present, and on suggestive isotope signatures in Carboniferous deposits (Wright & Vanstone, 1991). No fossil evidence for C_4 photosynthesis exists from the Carboniferous, and the validity of the isotopic data has been questioned (Cerling, 1999). Assuming C_4 photosynthesis was present in the Carboniferous, fossils would probably be lacking if the C_4 flora was herbaceous and frequented arid lands: fossilization is poor in arid regions. Alternatively, the preconditions for C_4 photosynthesis may not have been present in the primitive plants that existed at this time. Herbaceous plants of the Carboniferous were primarily ferns and their allies, species that require free water to reproduce and that show no evidence for C_4 photosynthesis today. Transitory increases in the carbon isotope record corresponding to the mid-Cretaceous (92 million yr ago) have also been suggested to signal an appearance of C_4 plants in the fossil record (Kuypers *et al.*, 1999). This proposal is difficult to accept at present because changes in the isotopic ratios in the atmosphere have not been ruled out, and the size of the isotopic shift would indicate a sudden, massive expansion of C_4 biomass in a mid-Cretaceous flora that shows no independent evidence of containing C_4 species.

While much of the focus has centered on the earliest appearance of C_4 photosynthesis, there have been many subsequent origination events, and the timing of these should be examined as well. Except for the Salsolae and Atriplicae tribes of the Chenopodiaceae, there has been little detailed examination of when C_4 photosynthesis may have appeared in nongrasses. Low diversity of species and genera suggest that many of the C_4 dicot lineages are very young, perhaps as late as the Pleistocene epoch (Ehleringer *et al.*, 1997). Using generic diversity as a rough guide, C_4 photosynthesis probably appeared most recently in *Blepharis* (Acanthaceae), *Flaveria* (Asteraceae), *Polycarpaea* (Caryophylaceae), *Mollugo* (Molluginaceae), *Anticharis* (Scrophulariaceae) and *Zygophyllum* (Zygophyllaceae). In support of this possibility, variation between PEPCase isoforms is less in *Flaveria* than in the C_4 grass sorghum, which occurs in one of the older C_4 lineages. *Flaveria bidentis* exhibits over 90% sequence homology between the C_4 and C_3 PEPCase isoforms, while in sorghum the overlap is 70% (Svensson *et al.*, 2003).

IX. The rise of C_4 photosynthesis in relation to climate and CO_2

The period over which C_4 photosynthesis is postulated to have arisen in the grasses, and later in other groups, is characterized by progressive climate deterioration and falling atmospheric CO_2 levels. Atmospheric models estimate that CO_2 levels were three- to fivefold greater than today during the mid-Cretaceous, and gradually declined to below current levels by Miocene/Pliocene times (5–15 million yr ago) before reaching a low point in the later Pleistocene (Berner & Kothavala, 2001; Royer *et al.*, 2001). Proxy estimates of atmospheric CO_2 using isotopic signatures in alkenones produced by algae, boron isotopic signatures and stomatal indexes generally support the modeled predictions, in that CO_2 levels were high during the Cretaceous and fell in the Mid-Tertiary (Fig. 14; Zachos *et al.*, 2001; Pagani, 2002; Retallack, 2002). The alkenone and boron isotope approach estimates that CO_2 levels had fallen below current levels by 25 million yr ago, but exactly when this happened is unclear due to a gap in the record from 40 to 25 million yr ago (Fig. 14). Increases in the stomatal indexes of fossil leaves indicate that the reduction in CO_2 coincided with the Eocene–Oligocene boundary (Fig. 14; Retallack, 2002).

The oxygen isotope record from deep sea cores provides a detailed view of climate conditions throughout the past 70 million yr. Oxygen isotope ratios are a robust index of mean temperatures of the atmosphere and ocean, and they clearly delineate major climate events in geological time (Zachos *et al.*, 2001). Over the past 50 million yr the O_2 isotope record shows a general cooling of the climate, with major dips in global temperature 33 million yr ago and in the past 10 million yr (Fig. 14). During episodes of global cooling, the atmosphere at low latitudes becomes drier and seasonality of precipitation increases (Prothero, 1994; Farrera *et al.*, 1999). This is important for C_4 evolution because arid and seasonal climate zones expand with each cooling phase. Cooling itself does not favor C_4 photosynthesis, but global cooling is largely a high-latitude phenomenon; tropical areas remain warm, and could even experience greater surface temperatures if aridification reduces vegetation cover and exposes bare ground (Farrera *et al.*, 1999). As indicated in Fig. 14, the postulated combination of aridity and low CO_2 that favors C_4 evolution appears to arise first during the Oligocene epoch, between 24 and 33 million yr ago.

The Oligocene begins with marked climatic deterioration, as shown by a sudden rise in the $\delta^{18}O$ ratio (Fig. 14; Prothero, 1994; Zachos *et al.*, 2001). During this time, forests decline across the globe and there is a large diversification of herbaceous angiosperms, annual plant species and drought-adapted features in plants (Leopold *et al.*, 1992; Wolfe, 1997; Retallack,

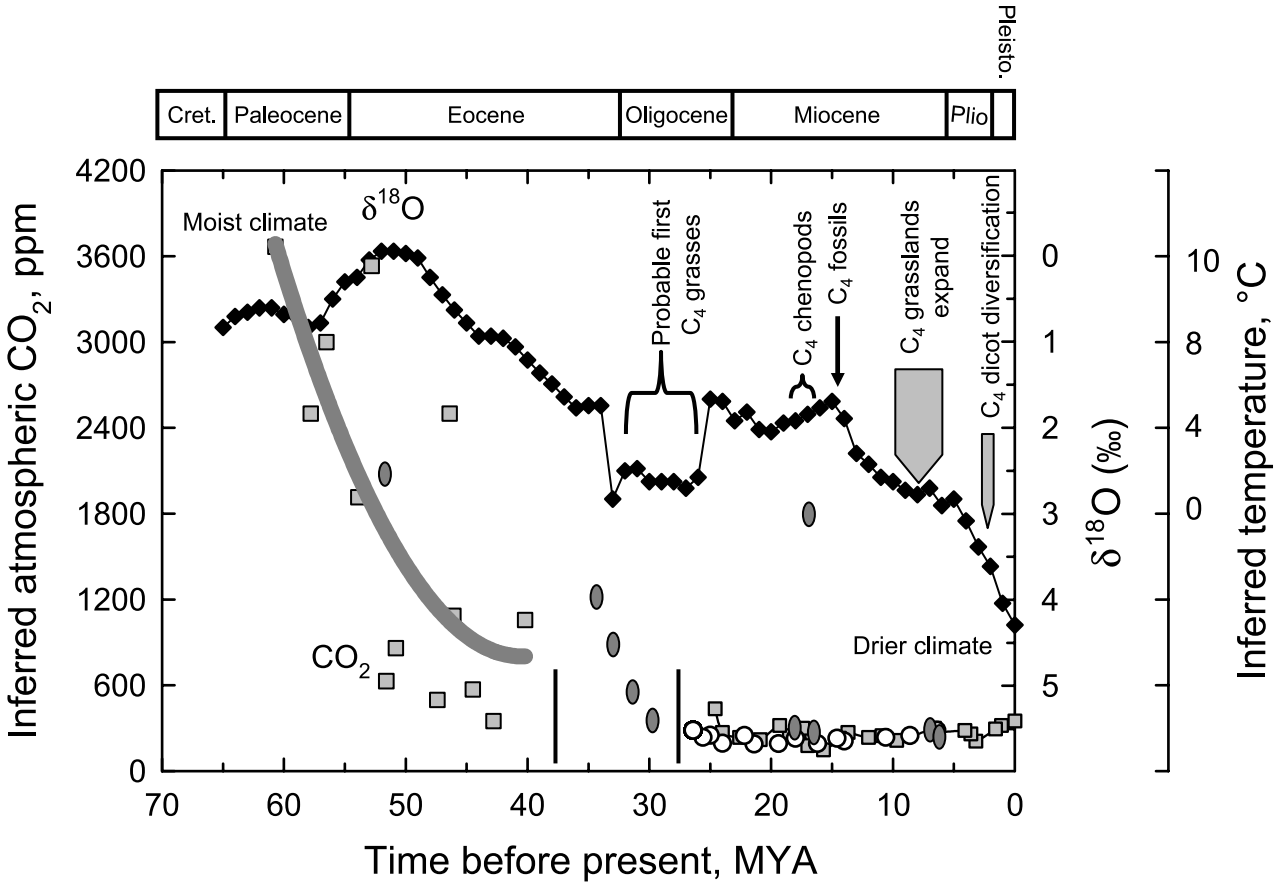

Fig. 14 Changes in deep-sea core $\delta^{18}O$ ratios and atmospheric CO_2 since the beginning of the Tertiary period 65 million yr ago. Atmospheric CO_2 estimates are by proxy using either boron isotopes (open circles, Pearson & Palmer, 2000); carbon isotope ratios in alkenone fragments (grey squares, Pagani *et al*., 1999); or stomatal indexes (filled ovals, Retallack, 2002). The grey curve is the best fit regression to the CO_2 data older than 40 million yr. $\delta^{18}O$ data (black diamonds) indicate mean global temperatures, and inferred temperatures corresponding to the $\delta^{18}O$ data are shown on the right axis. Key developments in the history of C_4 plants are also shown. Adapted from Zachos *et al*. (2001), by permission.

2002). Many of the families that eventually evolved C_4 photosynthesis diversified during this time, or first appear in the fossil record (Collinson *et al.*, 1993; Sage, 2001). Grasses and chenopods first appeared 60–70 million yr ago, but become common during the Oligocene, while six families that eventually evolve C_4 plants (Asteraceae, Boraginaceae, Carophyllaceae, Cyperaceae, Euphorbiaceae and Zygophyllaceae) appear in the fossil record at about the time of the Oligocene climate deterioration (Collinson, 1993).

Taken together, the data from molecular phylogenies, isotopes, fossils, and the history of the angiosperms produce a scenario where climate and atmospheric deterioration in the Oligocene favor the rise of functional types that met the general preconditions for C_4 evolution. Molecular phylogenies indicate that grasses were the first C_4 plants, arising about 24–34 million yr ago. Chenopods were probably the first C_4 dicots, appearing 15–20 million yr ago. By 12–14 million yr ago, C_4 grasses were abundant enough to leave detectable fossil and isotopic signatures. By the end of the Miocene, C_4-dominated grasslands expanded across many of the low-latitude regions of the globe, and temperate C_4 grasslands were present by 5 million yr ago (Cerling *et al.*, 1997). Grassland expansion in India and Pakistan was probably promoted by establishment of the South Asian monsoon system, while increasing seasonality of precipitation is suggested to have promoted the expansion of C_4 grasslands in the Americas, China and Africa (Pagani *et al.*, 1999). A further reduction of CO_2 is also proposed to have favored the rise of C_4 grasslands during the late-Miocene (Cerling *et al.*, 1997; Cerling, 1999). A reduction in CO_2 during the late-Miocene is not consistent with the proxy data for atmospheric CO_2 based on boron isotopes or alkenones from algae (Pagani, 2002), but it is supported by stomatal indexes of fossil oak leaves which indicate a late-Miocene reduction from 350 to 280 p.p.m. (Retallack, 1997).

A final burst of C_4 evolution came recently in geological time, most likely during the Pleistocene when climatic and atmospheric CO_2 conditions reached their low point. As indicated by the steep $\delta^{18}O$ rise in the past 5 million yr (Fig. 14), climate and atmospheric conditions progressively deteriorated

from the late-Miocene until the current pattern of glacial–interglacial oscillations was established 1 million yr ago. In the current pattern, interglacial periods last about 10 000 yr, while glacial periods develop over a 100 000 yr period. CO_2 levels generally vary between 260 and 300 p.p.m. during interglacials, and between 240 and 180 p.p.m. during glacial episodes (Petit *et al.*, 1999). Although high latitudes were cold during glacial episodes, low latitudes remained warm but tended to be more arid (Farrera *et al.*, 1999). The combination of warmth, aridity and very low CO_2 favored further origins of C_4 photosynthesis, which is consistent with the postulated rise of many C_4 dicot lineages in the Pleistocene epoch (Ehleringer *et al.*, 1997). The success of C_4 species in the low-CO_2 atmospheres of recent geological time is supported by growth and competition experiments with existing species (Sage, 1995; Tissue *et al.*, 1995; Polley *et al.*, 1996), model assessments (Cerling *et al.*, 1997; Collatz *et al.*, 1998), and paleoecological studies showing expansion of C_4 biomass in tropical environments during low-CO_2 episodes (Cerling *et al.*, 1997; Cerling, 1999; Huang *et al.*, 2001; Boom *et al.*, 2002).

X. Final thoughts: the future evolution of C_4 photosynthesis

The occurrence of very low CO_2 in recent geological time, and the associated rise of C_4 dicots, represents a novel combination that could radically change the nature of the biosphere in millennia to come. Low-CO_2 atmospheres such as occurred in the late-Pleistocene were unique in that they promoted a wide range of new C_4 lineages among the dicots, including woody life forms (Sage, 2001). Assuming low-CO_2 conditions return with the next ice age, the advantage of the C_4 pathway in low-CO_2 conditions may allow woody C_4 species to develop into trees capable of forming dense canopies. One canopy-forming C_4 tree (*Chamaesyce olowaluana*) is already present in Hawaii (Carr, 2003), demonstrating that there are no inherent obstacles to the evolution of C_4 forests. Once canopy-forming C_4 trees become common, then C_4 forests become a possibility. Forests are the major terrestrial carbon sink and exert an important control over atmospheric CO_2 levels (Berner & Kothavala, 2001). At low CO_2, the sink strength of C_3 forests declines due to less photosynthetic potential, and this slows the removal of CO_2 from the atmosphere during glacial extremes. C_4 forests could maintain the carbon sink at low CO_2, and thus could contribute to a lower steady-state CO_2 level in the atmosphere than may occur with C_3 forests. Because the CO_2 minimum of the ice ages may not be far above the life-cycle CO_2 compensation point of C_3 plants, any further reduction in CO_2 could threaten their existence. By providing such a reduction during future glacial episodes, C_4 trees could eliminate much of the C_3 flora and radically change the nature of the biosphere.

The immediate complication with this scenario is human manipulation of the biosphere. In particular, increases in atmospheric CO_2 could halt the rise of new C_4 life forms and may lead to the reduction of existing ones (Edwards *et al.*, 2001). However, certain C_4 species are favored by other global change variables such as climate warming and deforestation (Sage & Kubien, 2003). Hence, while many C_4 species may be at risk, C_4 photosynthesis as a functional type should not be threatened by CO_2 rise in the near term (Sage *et al.*, 1999b).

Humanity creates another avenue for the rise of novel C_4 species, namely the engineering of C_4 photosynthesis into C_3 crops (Sheehy *et al.*, 2000; Miyao, 2003). Initial work has focused on inserting genes for C_4 enzymes into rice. While improvements in yield have been noted, it does not result from the engagement of a C_4 photosynthetic cycle (Matsouka *et al.*, 2001). Current discussions consider whether it is worth trying to set up single-celled C_4 photosynthesis, as occurs in *Borszczowia* (Leegood, 2002; von Caemmerer & Furbank, 2003). Single-celled C_4 photosynthesis probably will not work in a C_3 crop because the single-cell system is inefficient and seems to be adaptive only in extreme environments. It is far better to redirect the C_3 leaf to form Kranz tissues, but the developmental changes are far more complex than simple reallocation of enzyme activity. Research on the natural pathways for C_4 evolution may provide important insights for overcoming the developmental barriers to C_4 photosynthesis; however, the complexity of the developmental changes may require more examples from the natural world than just the *Flaveria* model. With the realization that there are dozens of independent C_4 lineages, and that many of them are recent and perhaps rich with intermediates, we have at our disposal many new systems with which to unravel the secrets of C_4 evolution.

Acknowledgements

I thank Katherine Sage, Tania Johanssen, Danielle Way and Riyad Muhaidat for their assistance in the preparation of this manuscript. I also thank Gundrun Kadereit, Russ Monson, Peter Westhoff and Helmut Freitag for providing advance copies of their work. This paper was prepared with assistance from NSERC grant OGP0154273.

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