The Origins of C_4 Grasslands: Integrating Evolutionary and Ecosystem Science

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The evolution of grasses using C_4 photosynthesis and their sudden rise to ecological dominance 3 to 8 million years ago is among the most dramatic examples of biome assembly in the geological record. A growing body of work suggests that the patterns and drivers of C_4 grassland expansion were considerably more complex than originally assumed. Previous research has benefited substantially from dialog between geologists and ecologists, but current research must now integrate fully with phylogenetics. A synthesis of grass evolutionary biology with grassland ecosystem science will further our knowledge of the evolution of traits that promote dominance in grassland systems and will provide a new context in which to evaluate the relative importance of C_4 photosynthesis in transforming ecosystems across large regions of Earth.

Photosynthesis is the fundamental biological process that transforms solar energy into the chemical fuel for life by generating sugars from water and CO_2. The ancestral pathway (C_3 photosynthesis) evolved in a CO_2-rich atmosphere more than 2800 million years ago (Ma), but depletion of atmospheric CO_2 about 30 Ma has reduced the efficiency and rate of carbon uptake in many terrestrial plants, especially under high temperatures and water deficits (*1*). This limitation has been alleviated through the convergent evolution of C_4 photosynthesis in more than 45 independent flowering plant lineages (*1*). C_4 photosynthesis is a coordinated system of anatomical and physiological traits that concentrate CO_2 around the C_3 photosynthetic machinery, through the use of a solar-powered biochemical cycle. The emergence of ecosystems dominated by C_4 species has transformed the biosphere; although comprising only 3% of vascular plant species (*1*), they account for some 25% of terrestrial photosynthesis (*2*).

Sixty percent of C_4 species are grasses, dominating warm-climate grasslands and savannas (Fig. 1A), where their high rates of foliage production sustain Earth's highest levels of herbivore consumption (*3*). Stable carbon isotopic data ($\delta^{13}C$) collected over the past 20 years document a worldwide expansion of C_4 grasslands through the displacement of C_3 vegetation during the Late Miocene and Pliocene (3 to 8 Ma) (*4*). This was a dramatic event of biome evolution in Earth's history, outpacing the rise to dominance of flowering plants during the Cretaceous by one order of magnitude (*5*), but its drivers are still debated.

The last decade has seen much progress in our understanding of C_4 grass ecophysiology, C_4 grassland ecosystem ecology and geologic history, and the evolutionary history of the C_4 pathway within the grass lineage. However, these independent strands of research are not well integrated. Here, we outline a framework that explicitly links the evolutionary biology of grasses with the ecology and history of grasslands. We review the current state of knowledge in the field and argue that a shift in emphasis from photosynthetic pathway to broader assemblages of plant traits may be essential for understanding the rise of C_4 grasslands. This boils down to one question: Just how responsible is C_4 photosynthesis for the distribution of C_4 grasslands?

Crossing Environmental Thresholds

Today's C_4 grasses are mostly confined to low latitudes and altitudes, whereas C_3 species dominate at higher latitudes and elevations (Fig. 1A). These patterns correlate best with temperature, with several classic studies (*6*) showing the relationship on every continent. Explanations of these gradients have traditionally focused on fundamental physiological differences between C_3 and C_4 photosynthesis. At high temperatures and low atmospheric CO_2, the key C_3 photosynthetic enzyme rubisco fails to completely distinguish CO_2 and O_2. The process of O_2 uptake leads to photorespiration in C_3 plants, resulting in net losses of $\leq$40% of photosynthetic carbon in today's low-CO_2 atmosphere (*1*). C_4 photosynthesis suppresses photorespiration by concentrating CO_2 internally, but this comes with an energetic cost, which exceeds the photorespiratory costs of C_3 photosynthesis at

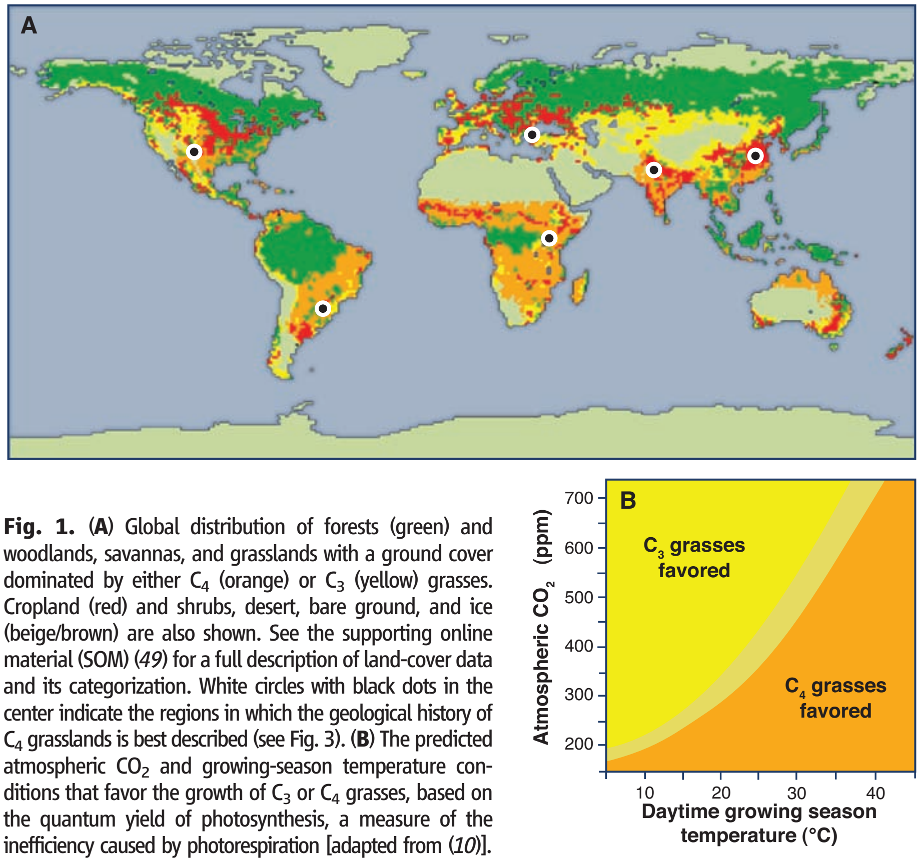

Fig. 1. (**A**) Global distribution of forests (green) and woodlands, savannas, and grasslands with a ground cover dominated by either C_4 (orange) or C_3 (yellow) grasses. Cropland (red) and shrubs, desert, bare ground, and ice (beige/brown) are also shown. See the supporting online material (SOM) (*49*) for a full description of land-cover data and its categorization. White circles with black dots in the center indicate the regions in which the geological history of C_4 grasslands is best described (see Fig. 3). (**B**) The predicted atmospheric CO_2 and growing-season temperature conditions that favor the growth of C_3 or C_4 grasses, based on the quantum yield of photosynthesis, a measure of the inefficiency caused by photorespiration [adapted from (*10*)].

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high CO_2 and low temperatures (*7*, *8*). All else being equal, C_4 grasses will therefore outperform C_3 grasses below a critical threshold in CO_2, the level of which depends on growing-season temperature (Fig. 1B) (*7*, *8*). By saturating rubisco with CO_2, the C_4 pathway also allows the enzyme to achieve maximum catalytic rates under high-light conditions (*9*); conversely, the overall C_4 advantage is often lost in shaded forest understories, where cool conditions improve the quantum efficiency in C_3 species (*7*).

This functional model forms the central basis for understanding the current distribution of C_4 grasses and grasslands (Fig. 1A) and the general absence of C_4 grasses from forest understory habitats (*10*). It explains glacial-interglacial cycles of C_4 grassland expansion and contraction (*10*) and underpins forecasts of future impacts of global change on Earth's C_3-C_4 balance (*11*). The extension of this model to the geological past generates the hypothesis that declining atmospheric CO_2 drove the displacement of C_3 plants by C_4 grasses (*4*, *8*, *10*). Because lower temperatures reduce the crucial CO_2 threshold for a C_4 photosynthetic advantage (Fig. 1B), C_4 grasslands should have appeared first in the tropics at 350 to 550 parts per million (ppm) CO_2 and then spread to higher latitudes as CO_2 declined further (*10*).

Reconstructing a Botanical Revolution

It is probable that tectonic events ultimately drove a major decline in CO_2 during the Early Oligocene (*12*, *13*), but the subsequent history of CO_2 is less certain. Most CO_2 proxy records and model calculations indicate CO_2 levels substantially lower than 550 ppm for the past 28 million years (My) (Fig. 2), thus also indicating an uncoupling of C_4 grassland expansion from atmospheric CO_2 during the Miocene (*12*). In contrast, two recent studies show CO_2 variation close to the C_4 crossover threshold during the Middle or Late Miocene (Fig. 2) (*14*, *15*). The assumptions, uncertainties, and imprecision inherent to each CO_2 proxy, as well as the range of uncertainty in the CO_2 crossover threshold itself, make these alternatives difficult to evaluate. Nevertheless, CO_2 does seem to have fallen below the upper bound of the threshold 20 My before the origin of C_4 grasslands, provoking a reappraisal of the ecological context and environmental drivers of C_4 grassland expansion.

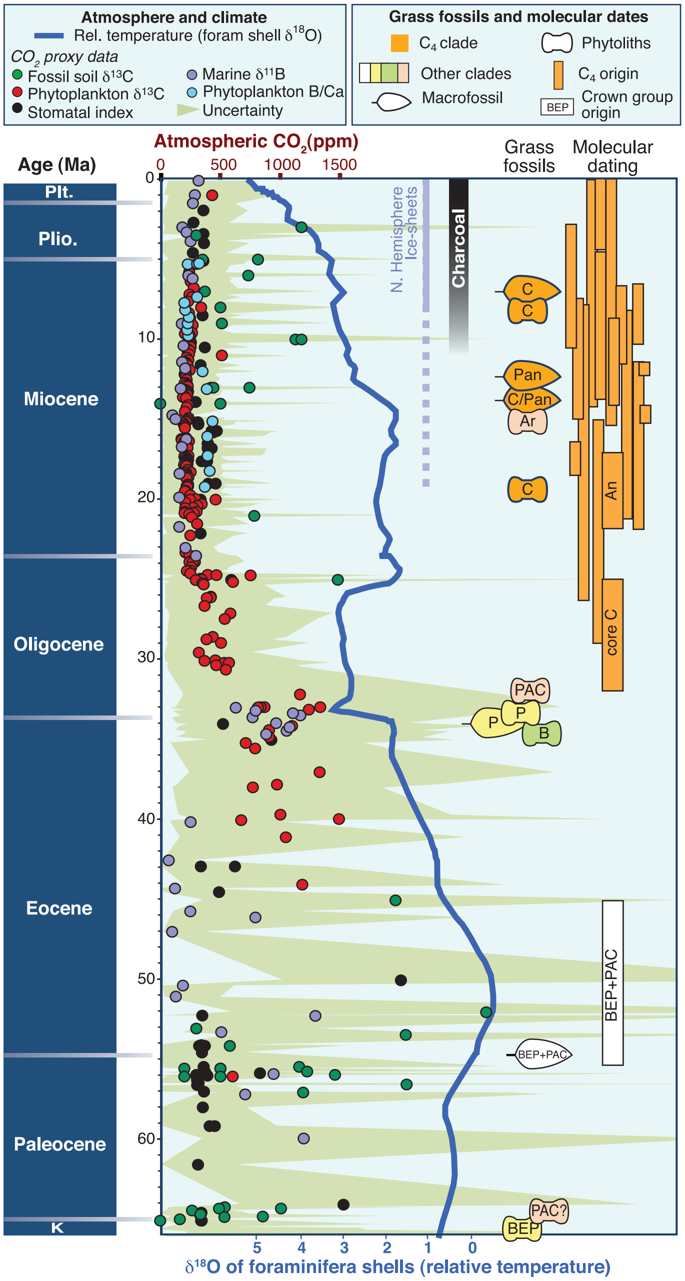

Fig. 2. Cenozoic record of CO_2 and temperature change, including evidence for Arctic glaciation, fossil and molecular dating evidence for grass evolution, and charcoal records. See the SOM (*49*) for data sources and methods. Plt., Pleistocene; Plio., Pliocene; K, Cretaceous; C, Chloridoideae; Pan, Panicoideae; Ar, Arundinoideae; An, Andropogoneae; B, Bambusoideae; P, Pooideae; BEP, Bambusoideae-Ehrhartoideae-Pooideae; PAC, PACMAD.

Recent phylogenetic reconstructions show that C_4 photosynthesis has evolved multiple times in grasses (*16*, *17*). Time-calibration of these phylogenies using fossilized grass pollen and inflorescences places the earliest probable origin in the Early Oligocene (~30 to 32 Ma) (Fig. 2) and suggests that subsequent origins arose in clusters (for example, in the Middle Miocene). This timing has led researchers to hypothesize that the Early Oligocene drop in CO_2 triggered evolution of the C_4 pathway (*16*, *17*). However, the proposal is challenged by the discovery of Late Cretaceous microscopic plant silica (phytoliths) diagnostic of grasses (Fig. 2), suggesting that this lineage may be much older than previously thought (*18*). A recalibration with these fossils would date the earliest C_4 grasses to the Middle Eocene (*17*), a time of warm equable climates and probably of high CO_2 (Fig. 2). Even more controversial are $\delta^{13}C$ records from leaf-wax molecules (*n*-alkanes) in marine sediments, indicating that C_4 photosynthesis existed in Cretaceous land plants (*19*), albeit not necessarily in grasses.

New paleontological evidence also reveals crucial information about the Miocene environments that preceded C_4 grasslands. Rather than being forested, as initially thought (*20*), it now appears that landscapes were relatively open. The evolution of ungulate grazers or mixed feeders (feeding on grasses and broad-leaved plants) and pollen data (*21*) supplemented by new, phytolith-based reconstructions of vegetation (*22*) document the emergence of savannas or woodlands with predominantly C_3 grasses in the Early-Middle Miocene (11 to 24 Ma), several million years before C_4 grasslands spread (Fig. 3). This vegetation shift is evident in all of the studied cases, although its timing and pace seem to have varied among regions (Fig. 3).

C_4 grasses occurred in the landscape soon after this transition. Phytoliths show that C_4 Chloridoideae species were represented in North American grassland communities 19 Ma (Fig. 2). Similarly, $\delta^{13}C$ records from fossil soils suggest that C_4 grasses contributed 20 to 40% of local vegetation in several regions for many million years before C_4 species completely dominated communities (Fig. 3) (*23*, *24*). Spatially detailed sedimentological and isotopic reconstructions of the paleolandscape (*25*, *26*) indicate substantial heterogeneity in vegetation structure, with tree-grass mosaics before and during the C_3-C_4 shift. C_4 grasses seemed to have first invaded drier parts of floodplains, whereas C_3 plants preferred moister habitats in topographic lows (*25*, *26*).

The explosive, broadly synchronous Late Miocene–to–Pliocene spread of C_4 grasses, originally diagnosed by the $\delta^{13}C$ of fossil soil carbonates (*20*), has since been abundantly documented by $\delta^{13}C$ records of ungulate teeth (*4*, *27*) and *n*-alkanes in soils and marine sediments (*24*, *28*) across many low- to mid-latitude regions (Fig. 3). However, with more C_4 proxy data available for each region, it has become clear that the $\delta^{13}C$

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records of tooth enamel and soil carbonate record different aspects of C_4 expansion. Evaluating both proxies concurrently allows us to distinguish overall vegetation change, recorded by soil $\delta^{13}C$, from changes in herbivore food source, mirrored in tooth enamel $\delta^{13}C$. Specifically, data from North America, Siwaliks (Himalayan foreland in Pakistan), Argentina, and Kenya show that particular mammals started feeding largely on C_4 grasses ≥1 My before these grasses became abundant (>50%) in the ecosystem (Fig. 3) (*29*). The rapid adoption of C_4 grazing in these herbivores may mark the emergence of C_4 dietary specialists in faunal communities or else expansion of C_4 grasses at the expense of C_3 grasses, but not necessarily at the expense of C_3 woody vegetation.

In contrast, the overall C_3-C_4 transition in vegetation (inferred from soil carbonate $\delta^{13}C$) seems to have typically been much slower, with little evidence of the latitudinal gradient in C_4 grassland appearance expected from the crossover threshold model. It occurred ~3 My earlier in the Siwaliks and China, compared with temperate North America and tropical Kenya (Fig. 3). The Eastern Mediterranean, North America, and China were located at roughly equivalent paleolatitudes, yet only the latter two became C_4-dominated in the Late Miocene, and at slightly different times and rates. The Eastern Mediterranean remained dominated by C_3 plants throughout the Neogene, although the summer droughts that are now thought to exclude C_4 grasses from this region originated only 3 Ma (*30*).

The wealth of high-resolution data now available point to a complex Late Miocene–to–Pliocene ecological transition, where the roughly correlated C_3-C_4 shifts across continents differ in many details. The decoupling in time of the transition from forest to C_3 grassland and the later expansion of C_4 grasses indicate that different drivers were probably involved. Similarly, the lag between the origins of C_4 grasses and their subsequent rise to dominance suggest separate triggers. These observations necessitate a reassessment of the factors driving C_4 grassland origins and suggest that the problem is best framed as two related questions: (i) What drove the forest-to-grassland transition? (ii) Why did these grasslands later become C_4-dominated in warm-climate regions?

What Drove the Forest-to-Grassland Transition?

The abundance of woody vegetation (i.e., trees and shrubs) versus grasses in modern grasslands is maintained by two primary mechanisms. First, C_3 and C_4 grasses may tolerate soil factors or climatic extremes that limit the establishment or survival of woody plants. Second, grasslands are sustained by fires and herbivory, which limit the recruitment and growth of woody species (*31*). Improved understanding of these different drivers and their interactions has inspired novel hypotheses about the origins of C_4 grasslands and has revived some old ones. All invoke regional or local factors and focus on increased aridity and/or shifts in disturbance regime. Stable oxygen isotope ratios ($\delta^{18}O$), sedimentology, and floral records point to the development of seasonal climates with warm-season precipitation in South Asia (*20*, *32*). The onset of a dry season causing intensified fire cycles in this region is therefore favored as a driver of the forest-to-grassland transition and is supported by charcoal records indicating increased occurrence of fire on several continents (*29*, *33*). Based on $\delta^{18}O$ and leaf-wax hydrogen isotope ratios (δD), an overall increase in aridity (as opposed to seasonality) has been proposed for both South Asia and East Africa (*28*, *34*, *35*), with no explicit role for disturbance. In a third scenario using fossil-soil data from South Asia, East Africa, and North America, dry-adapted C_4 grasses evolved herbivore resistance and/or traits leading to fuel accumulation, allowing them to expand their ecological niche into more mesic habitats, at the expense of trees (*36*, *37*).

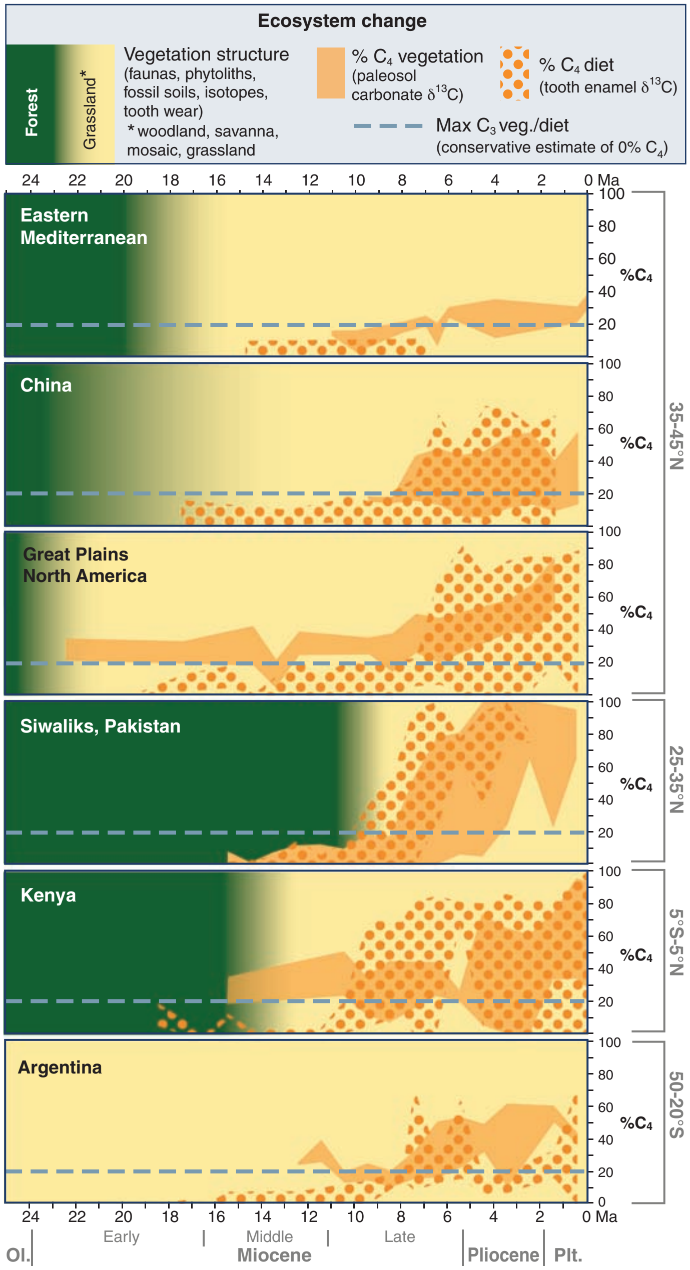

Fig. 3. Neogene record of changes in vegetation structure (from forest vegetation to an environment with substantial amount of grass cover), and of photosynthetic pathway (C_3 versus C_4), in vegetation and in diet of ungulates from various regions (*49*). Ol., Oligocene.

A major problem with these Late Miocene and Pliocene scenarios is that they rely on mechanisms that today maintain the grass-to-tree/shrub balance in C_4-dominated ecosystems, whereas the fossil record shows that the (much earlier) forest-to-grassland transition involved mainly C_3 grass species (Fig. 3). Whether similar processes drove the spread of C_3 grasslands is an important question that remains to be tackled (*22*). Nevertheless, modern ecology illustrates that the abundance of grass versus woody vegetation is controlled by complex sets of factors playing out over different spatial scales. At the regional scale, grasslands are sustained by climate/soil/disturbance interactions, mediated by the traits of the grass flora and herbivore fauna. Locally, topographic effects generate microhabitat variation that supports a mosaic of distinct grass communities with very different trait combinations (*38*, *39*). The general message is clear: Traits that are, at best, only indirectly related to C_4 photosynthesis currently play important roles in allowing grasses to dominate vast areas of natural grassland.

Why Did Grasslands Become C_4-Dominated?

Questions of this scale have historically fallen into the realm of ecosystem ecologists and plant physiologists and have rarely received attention from the field of evolutionary biology (*40*). Our understanding of grass phylogenetics and patterns of C_4 evolution in grasses has improved considerably over the past decade (*16*, *17*, *41*). Though obviously im-

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portant for understanding C_4 origins, phylogeny should play an equally large role in evaluating ecological and physiological consequences of the pathway.

Most previous studies comparing C_3 and C_4 grasses have chosen taxa that span the deepest divergences within the grass lineage, comparing members of the Pooideae, a strictly C_3 lineage, with C_4 members of "PACMAD" (an acronym for Panicoideae, Aristidoideae, Chloridoideae, Micrairioideae, Arundinoideae, and Danthonioideae lineages), a large clade of C_3 and C_4 grasses containing ~18 independent origins of the C_4 pathway (Fig. 4). However, Pooideae and PACMAD last shared a common ancestor >50 Ma, allowing both lineages to evolve many differences that will confound any potential C_3-C_4 signal (*40*). Recent work indicates that PACMAD species tend to be warm-adapted whether or not they are C_4, suggesting that the evolution of cold tolerance in Pooideae may be as important as C_3-C_4 differences in establishing the ecological sorting of grass species along temperature gradients (*42*). Therefore, isolating the effects of C_4 photosynthesis on any aspect of grass biology (from biochemistry to ecology) requires comparisons between closely related C_3 and C_4 taxa within the PACMAD clade. For example, studies show that responses to climatic extremes of cold and drought are as important as differing photosynthetic performance in determining the ecological characteristics of C_3 and C_4 subspecies of the grass *Alloteropsis semialata* (*43*).

Similarly, grouping the multiple, independently derived C_4 lineages within PACMAD into a single C_4 functional type probably masks underlying variation in other traits that could be important at the community and/or ecosystem level. Key differences among C_4 plants have been acknowledged for many years because of the presence of C_4 photosynthetic subtypes distinguished by variation in anatomy, biochemistry, and physiology (*44*–*46*). The recurrent evolution of the C_4 pathway in grasses presents another opportunity for variation, as each origin arose within a unique internal (and probably external) environment. Researchers have already documented that independent C_4 lineages exhibit different growth responses to elevated CO_2 (*47*) and that previously recognized sorting of subtypes along climate gradients is better explained by the sorting of different C_4 lineages (*48*).

The above studies indicate that seemingly self-evident relationships between C_4 photosynthesis and physiological properties or ecological tolerances become decidedly less certain when viewed within a phylogenetic context. Phylogenies emphasize the rich organismal diversity that is contained within the C_4 grass functional type and also provide the perfect framework for evaluating how different grassland systems have been assembled from these independent lineages. The major challenge in this area is to understand how the C_4 pathway has been functionally integrated into each of these diverse organismal backgrounds, and in turn, how the resulting ecological characteristics of these lineages influence such large-scale processes as biome development and ecosystem function.

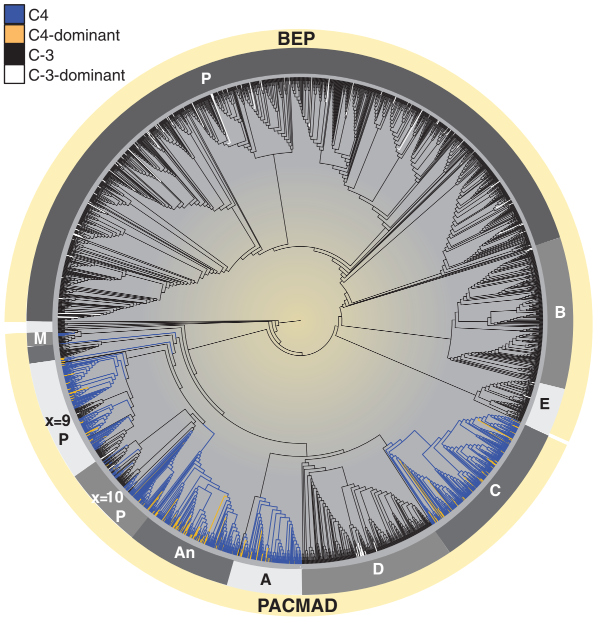

Fig. 4. The evolution of photosynthetic pathway and grassland dominants in Poaceae (the grass family). A 2684-taxon tree of Poaceae and outgroups, constructed from molecular-sequence data (*49*). Evolutionary patterns of photosynthetic-pathway variation and grassland dominance (*49*) are indicated by branch colors. The origins of both C_4 photosynthesis and dominance were more clustered on the phylogeny than expected by chance ($P < 0.05$), which was inferred by comparing the total of number of changes in each trait to a null distribution generated by re-shuffling tip values across the phylogeny 1000 times. This clustering indicates that some lineages are more prone than others to evolving dominant grassland species, and focusing comparative studies in these regions of the phylogenetic tree may uncover traits that played a key role in the origins of grassland ecosystems. Letters indicate major clades. E, Ehrhartoideae; M, Micrairioideae; A, Aristidoideae; D, Danthonioideae; x = 10P, Paniceae (base chromosome number $x = 10$); x = 9P, Paniceae (base chromosome number $x = 9$).

Most Grass Species Do Not Dominate Grasslands

The complexity of many ecosystems makes it difficult to understand how individual species influence ecosystem function. However, just a handful of species make up the majority of the standing biomass in most grasslands, and species richness is largely contributed by taxa occurring in very low densities. Furthermore, a minority of grasses (~600 out of ~11,000 species) is documented as being ecologically dominant in grasslands (*49*). These dominant species are phylogenetically clustered, suggesting that certain clades of grasses are more prone than others to evolve traits that promote ecological dominance (Fig. 4). The pattern implies that important characteristics other than the C_4 pathway enable these particular species to become abundant in grasslands. If we assume that paleograsslands had a similar diversity structure as their modern analogs, then understanding the Miocene expansion of C_4-dominated ecosystems hinges on the question of "How did particular grassland dominants (as opposed to C_4 grasses in general) come to occupy such a large fraction of the land surface?" A phylogenetic approach can immediately help to organize new research questions about the evolution of grassland dominants. Below, we highlight key examples of how knowledge from other fields can be integrated within this framework.

How do environmental niche preferences differ between dominant and nondominant grasses? A fundamental question is whether the Late Miocene–to–Pliocene spread of grasslands resulted from the evolution of new environmental niche tolerances in a handful of C_4 grasses, or whether climatological changes promoted the geographical expansion of particular niches already inhabited by those grasses. The truth is likely to be some combination of both scenarios. We currently have little quantitative information on how environmental niches are distributed within the PACMAD clade. By reconstructing the evolution of environmental niche space across the grass phylogeny (*42*), we will be able to address when and how key changes occurred. Is the environmental niche relatively conserved across certain clades of PACMAD, or is it evolutionarily labile? Have dominants from different lineages converged on similar niche space when living in the same biogeographical region? Do dominant species always exhibit broader ecological ranges than their nondominant sister taxa?

How do grass species dominate grasslands? Understanding the timing and phylogenetic distribution of C_4 origins in grasses is obviously fundamental to understanding the rise of C_4 grasslands. The same is true for other traits that promote species' dominance, highlighted in the rich ecological literature on competitive interactions in grasslands (*50*). Multiple comparisons of dominant and subdominant sister taxa will help to identify traits associated with shifts to dominance, and broader surveys of how those traits are distributed across PACMAD will improve our understanding of their evolutionary history. In fire-driven grasslands of African monsoonal climate regions, for instance, dominant grasses typically have well-protected buds and storage reserves, and they resprout quickly after defoliation (*31*). Other traits act to induce fire: High wet-season growth rates and slow rates of leaf decomposition result in large fuel accumulation (*31*). When and where

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in grasses did these traits evolve, and in what order? Dating the appearance of such important ecological traits, in addition to shifts in photosynthetic pathway, may bridge the time gap between the origins of C_4 grasses and the origins of C_4 grasslands.

What role does C_4 photosynthesis play in the evolution of dominance? Clearly, the C_4 pathway must fundamentally influence the ability of PACMAD grasses to establish dominance, as evidenced by the dearth of C_3 PACMAD species with high abundances in grasslands (16 of 145 species sampled for Fig. 4). However, C_4 photosynthesis will also probably confer different advantages in different circumstances. For instance, in the fire-adapted example above, the C_4 pathway would facilitate biomass accumulation by supporting high photosynthetic rates and nitrogen-use efficiencies, especially in the high-light environment after a fire (*37*, *46*). In contrast, the North American short-grass prairies are water-limited and far less productive than tropical savannas; in this case, the higher water-use efficiency afforded by C_4 metabolism might provide the competitive edge (*37*). These two ecosystem types are dominated by different C_4 PACMAD lineages. In African Andropogoneae, C_4 works within an organismal context of a large, fire-adapted plant that quickly accumulates biomass between fires. In North American Chloridoideae, C_4 operates within a short-statured, drought-resistant plant. C_4 photosynthesis is a well-integrated component of each of these strategies, resulting in two grassland systems that are dominated by C_4 grasses, but for very different reasons.

Establishing these patterns of trait assembly for modern grasses could permit specific ecological inferences to be made about C_3/C_4 lineages that are recognized in the fossil record using phytoliths and other plant fossils. Such inferences will allow detailed reconstruction of the grass communities present in the Miocene and, by inference, the type of environment that may have promoted C_4 grass expansion in different regions (*37*).

Research Priorities

The current acceleration of computing power and molecular-sequence accumulation makes a completely sampled grass phylogeny a realistic near-term goal, which will greatly facilitate a lineage-centered focus on the C_4 grassland problem. Linking the grass phylogeny to databases detailing climatic, ecological, morphological, and physiological information for individual grass taxa is already possible, permitting the application of tools developed for ecological bioinformatics and spatial ecology. Similarly, reconstructions of turnover in paleocommunities are becoming much more refined, using phytolith data to track major grass lineages through time. This growing body of paleobotanical evidence also promises the ongoing improvement in dating of key events in grass evolutionary history. To keep pace with these developments, comparative biological investigations of grass species are urgently required to understand trait evolution in grassland dominants. A stronger synthesis between evolutionary processes, plant function, and ecosystem composition will provide new opportunities for sharpening our hypotheses about the factors that drove the Late Miocene–to–Pliocene emergence of C_4 grassland biomes.

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C_4 Grasses Consortium

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