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The Evolutionary Ecology of C_4 Plants

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Summary

C_4 photosynthesis is a physiological syndrome resulting from multiple anatomical and biochemical components, which function together to increase the CO_2 concentration around Rubisco and reduce photorespiration. It evolved independently multiple times and C_4 plants now dominate many biomes, especially in the tropics and subtropics. The C_4 syndrome comes in many flavours, with numerous phenotypic realizations of C_4 physiology and diverse ecological strategies. In this work, we analyse the events that happened in a C_3 context and enabled C_4 physiology in the descendants, those that generated the C_4 physiology, and those that happened in a C_4 background and opened novel ecological niches. Throughout the manuscript, we evaluate the biochemical and physiological evidence in a phylogenetic context, which demonstrates the importance of contingency in evolutionary trajectories and shows how these constrained the realized phenotype. We then discuss the physiological innovations that allowed C_4 plants to escape these constraints for two important dimensions of the ecological niche, growth rates and distribution along climatic gradients. This review shows that a comprehensive understanding of C_4 plant ecology can be achieved by accounting for evolutionary processes spread over million of years, including the ancestral condition, functional convergence via independent evolutionary trajectories, and physiological diversification.

Keywords: C_4 photosynthesis, physiology, evolution, ecological niche, co-option, contingency

Introduction

C_4 photosynthesis is a complex phenotype, formed from multiple anatomical and biochemical components that together increase the concentration of CO_2 around Rubisco (Hatch, 1987; Figure 1). This evolutionary innovation increases the carbon-fixation efficiency under all conditions that restrict CO_2 supply to Rubisco, and has its greatest effects at high light and temperature (Ehleringer & Bjorkman, 1977; Ehleringer, 1978; Ehleringer *et al.*, 1991, 1997). However, the distributions of C_4 plants cannot be comprehensively explained by individual environmental variables, and C_4 species thrive across a diversity of habitats, ranging from the tropics to the boreal zone, from deserts to submerged conditions, from open grasslands to forest understoreys, and from nutrient-depleted to fertile soils. This ecological diversity results from the rich evolutionary history of this physiological trait, which evolved many times in distantly related groups (Sage *et al.*, 2011).

Since its discovery in the 60s, C_4 photosynthesis has been the subject of many studies, from the fields of biochemistry, physiology, organismal biology, ecology and evolution (reviewed in Langdale, 2011). In the last fifteen years, our understanding of evolutionary aspects of C_4 photosynthesis has been boosted by the accumulation of molecular phylogenies, which have identified more than 62 monophyletic C_4 groups (e.g. Kellogg, 1999; GPWG, 2001; Giussani *et al.*, 2003; Kadereit *et al.*, 2003; McKown *et al.*, 2005; Besnard *et al.*, 2009; Sage *et al.*, 2011; GPWGII, 2012). Phylogenetic trees allow us to disentangle the events that led to the evolution of C_4 physiology (McKown *et al.*, 2007; Christin *et al.*, 2011b, 2013b; Khoshravesh *et al.*, 2012; Griffiths *et al.*, 2013; Box 1), and the accumulated evidence shows that some C_4 constituents evolved in a C_3 context and enabled the transition to C_4 physiology via the gradual addition of other C_4 constituents (Sage, 2001, 2004; Christin & Osborne, 2013). The availability of robust and densely sampled phylogenetic trees has also revolutionized our understanding of C_4 ecology, with the possibility of dating C_4 origins and placing them on the geological timeline (e.g. Christin *et al.*, 2008a; Vicentini *et al.*, 2008; Kadereit *et al.*, 2010), and the capacity to differentiate ecological properties that were inherited from C_3 ancestors from those that represent departures from ancestral conditions (e.g. Edwards *et al.*, 2007, 2008; Osborne & Freckleton, 2009; Edwards & Smith, 2010; Taylor *et al.*, 2010, 2012; Kadereit *et al.*, 2012; Box 1).

In this review, we integrate knowledge acquired during the last 50 years and recent modelling efforts into a phylogenetic context, to infer the most plausible events occurring during the evolutionary transition from C_3 to C_4 photosynthesis, and discuss their physiological and ecological consequences. Throughout, we evaluate the evidence in the context of two non-mutually exclusive hypotheses. First, that evolutionary trajectories towards novel traits cannot vary in any direction, but are highly constrained by the phenotype and genotype of the organism. Secondly, that evolutionary innovation unlocks new phenotypic opportunities for the organism and shifts the fundamental niche, by removing constraints on the trait space that can be occupied.

1. Which properties are common to all C_4 plants?

C_4 physiology

The main effect of C_4 photosynthesis is an elevated concentration of CO_2 relative to O_2 in the vicinity of Rubisco, increasing the ratio of carboxylation to oxygenation reactions catalyzed by the enzyme, and therefore lowering the rate of photorespiration (Chollet & Ogren, 1975; Hatch & Osmond, 1976). It also near-saturates Rubisco with its CO_2 substrate, which increases the rate of carbon assimilation per unit of Rubisco protein and gives the potential for very rapid photosynthetic rates under high light conditions (Schmitt & Edwards, 1981; Long, 1999). The ratio of oxygenation by Rubisco relative to carboxylation rises with temperature because the solubility of CO_2 decreases relative to O_2, and the specificity of Rubisco declines faster for CO_2 than O_2 (Long, 1991). At high temperatures and low CO_2, the C_4 cycle therefore increases the number of CO_2 molecules fixed per absorbed photon (quantum efficiency), but also per unit of Rubisco protein invested, and consequently improves the photosynthetic nitrogen-use efficiency (Ehleringer & Bjorkman, 1977; Brown, 1978; Skillman, 2008). However, the C_4 cycle consumes metabolic energy, and C_3 plants therefore retain a higher quantum efficiency when photorespiration is low, especially at low light and low temperature (Ehleringer & Bjorkman, 1977). These physiological properties are common to all C_4 plants. However, they emerge through a complex assemblage of anatomical and biochemical components. When investigating the evolution of C_4 photosynthesis, it is useful to distinguish phenotypic characters arising from individual developmental changes or biochemical reactions, from the functional properties that emerge through the coordinated action of several such characters (Table 1).

C_4 phenotypic functions

The C_4 syndrome is defined by the primary fixation of carbon by phosphoenolpyurvate carboxylase (PEPC) during the day and its refixation by Rubisco (Kellogg, 1999). These metabolic functions are achieved via the segregation of PEPC and Rubisco into two distinct compartments within the leaf, with the compartment containing Rubisco largely isolated from the external environment (Hatch & Osmond, 1976). In addition, a number of biochemical functions are required to sustain the C_4 cycle (Figure 1a): a) the action of carbonic anhydrase (CA) for converting CO_2 to HCO_3^-, and its fixation into organic acids by PEPC; b) a cascade to transform the oxaloacetate produced by PEPC into other C_4 organic acids, and transport them to the Rubisco compartment; c) a system to release CO_2 in the Rubisco compartment; and d) a cascade to regenerate the acceptor molecules for carbon in the C_4 cycle (Hatch, 1987). Besides these biochemical functions, the fixation of carbon by PEPC and its later refixation by Rubisco requires a series of functions linked to the plant structure that are present in all C_4 plants (Hattersley & Watson, 1975; Edwards & Voznesenskaya, 2011; Lundgren *et al.*, 2014). These include two compartments separated by a short distance, into which PEPC and Rubisco reactions can be segregated (Figure 1).

C_4 characters

The anatomical and metabolic functions listed above are present in all C_4 plants, independently of their taxonomic origin, but each of these functions arises from multiple characters, which result from independent modifications in the characteristics of their components (Table 1). Unlike the functions generated, these underlying characters and characteristics vary among C_4 lineages, and each time the C_4 syndrome evolved, it was assembled using one of numerous possible sets of anatomical and biochemical characters (Sinha & Kellogg, 1996; Kellogg, 1999). This leads to a number of important distinctions among C_4 lineages. First, the two compartments used to segregate PEPC and Rubisco reactions vary among C_4 plants, and may be cell types derived from the same or different meristematic tissues, or even different compartments within the same cell (Brown, 1975; Dengler *et al.*, 1985; Edwards *et al.*, 2004). Similarly, the close contact between the PEPC and Rubisco compartments can be achieved by modifying the vein architecture through different developmental pathways (reviewed by Lundgren *et al.*, 2014). The biochemical cascade that transforms and transports the product of PEPC, releases CO_2 and regenerates the intermediate compounds (Figure 1a, steps b-d), is also well known to vary among C_4 lineages, with different enzymes involved, especially in the release of CO_2 from C_4 acids in the Rubisco compartment (Figure 1a, step c; Andrews *et al.*, 1971; Gutierrez *et al.*, 1974). In conclusion, the phenotypic characters that are known to be common to all C_4 plants are a high activity of CA and PEPC in the cytosol of the first compartment and a high activity of Rubisco within chloroplasts in the second compartment (Figure 1), and most, if not all, of the others vary among C_4 lineages (Kellogg, 1999).

2. What is unique to C_4 plants?

Individual C_4 components in non-C_4 plants

The emergent physiological properties associated with the C_4 syndrome are unique to C_4 plants, but several of the underlying functions and all the components can be found in plants using other photosynthetic pathways. Close contact between the two leaf compartments usually used for PEPC and Rubisco reactions is found in several C_3 grasses (Lundgren *et al.*, 2014), and in many plants that use a C_2 pathway, a low efficiency CO_2-scavenging mechanism based on glycine decarboxylase localization (Sage *et al.*, 2012). Similarly, a concentration of Rubisco in bundle sheath chloroplasts is observed in C_2 plants as well as closely related C_3 taxa (Sage *et al.*, 2013). The biochemical functions that generate the C_4 cycle are not found as such in other plants, except for CAM plants, which use a similar pathway with a temporal segregation of reactions. However, all the enzymes of the C_4 cycle, and the catalyzed reactions, exist in C_3 plants (Aubry *et al.*, 2011). In these species, the enzymes are responsible for different functions in basal metabolism (reviewed by Aubry *et al.*, 2011). Most of these enzymes are encoded by multigene families, and the different isoforms vary in their catalytic properties and expression patterns (Tausta *et al.*, 2002; Svensson *et al.*, 2003). The ancestral functions generally still exist in C_4 plants, but some isoforms now operate in the C_4 cycle, which requires specific spatial and temporal regulation, as well as specific kinetic properties. At least some of these expression and kinetic characteristics however exist in C_3 plants. For instance, decarboxylating enzymes are active around the vascular tissue in a phylogenetically diverse range of C_3 species (Hibberd and Quick, 2002; Osborne & Beerling, 2006; Brown *et al.*, 2010), and most of the genes for the enzymes of the C_4 cycle can be found at significant levels in C_3 leaves (Christin *et al.*, 2013a; Bräutigam *et al.*, 2014).

Gradual C_4 assembly through repeated co-option of components

All of the components that together generate C_4 physiology can therefore be found in other photosynthetic types, but their characteristics vary both quantitatively and qualitatively, and C_4 lineages each present unique combinations of the resulting characters (Table 1). The presence of all components in C_3 or C_2 species implies that the evolution of C_4 photosynthesis required their co-option into a new function and, in many cases, their adaptation for the novel metabolic context. The different C_4 components were not co-opted simultaneously, but must have been added sequentially. The exact order of this process is still to be elucidated and is very likely to vary among lineages (Williams *et al.*, 2013), but recent insights have come from phylogenetic reconstructions (e.g. Christin *et al.*, 2011b; Khoshravesh *et al.*, 2012) and modelling efforts (Heckmann *et al.*, 2013; Williams *et al.*, 2013; Mallmann *et al.*, 2014). These studies differ in the characters that are considered, sometimes modelling the whole C4 cycle as a simple component (Heckmann *et al.*, 2013) or transforming quantitative traits into discrete binary variables (Christin *et al.*, 2011b; Williams *et al.*, 2013), but they all converge on similar conclusions. For instance, it is now widely accepted that several C_4 characters, especially anatomical ones, were acquired before C_4 physiology (Sage, 2001, 2004; McKown *et al.*, 2007; Christin *et al.*, 2011b; Khoshravesh *et al.*, 2012;

Heckmann *et al.*, 2013; Williams *et al.*, 2013). Similarly, several C_4 characters were probably acquired once plants were already fixing the majority of their carbon via PEPC, thereby optimizing the syndrome and adapting it to diverse environments (Christin *et al.*, 2011b; Heckmann *et al.*, 2013). The whole history of events that led to optimized C_4 descendants was likely spread over many million years (Christin & Osborne, 2013; Figure 2), and the ecological drivers and biological consequences are likely to differ among these events. In the following sections, we discuss first the events that happened in a non-C_4 context and enabled the transition to C_4 physiology (previously referred to as preconditions; Sage, 2001, 2004), then the process that generated the C_4 physiology itself, and finally the modifications that likely happened within a C_4 context. For each of these, the potential physiological and ecological consequences are discussed.

3. What happened before C_4 physiology?

Origin of enzymes of the C_4 pathway

All enzymes of the C_4 pathway originated in bacteria, hundreds of millions or billions of years before they were co-opted for C_4 photosynthesis. In angiosperms, they are usually encoded by gene families, with multiple isogenes that appeared through successive whole genome or single gene duplications (Wang *et al.*, 2009; Christin *et al.*, 2013a). The different isoforms generally diversified and came to fulfil a variety of functions, mostly anaplerotic (Drincovich *et al.*, 2001; Lepiniec *et al.*, 2003). This diversification also involved changes in expression patterns (spatial, temporal, and quantitative), as well as kinetic properties and responses to regulators (e.g. Blasing *et al.*, 2002; Tausta *et al.*, 2002; Christin *et al.*, 2013a; John *et al.*, 2014). This functional diversification was not driven by C_4 photosynthesis, but might have predisposed some plants for a later C_3-to-C_4 transition. Indeed, a function in the C_4 cycle requires specific expression patterns as well as catalytic properties (Hibberd & Covshoff, 2010), and the existence in some genomes of genes encoding enzymes with characteristics partially suitable for the C_4 cycle might have facilitated C_4 evolution. This hypothesis is supported by the observation that independent C_4 origins preferentially co-opted specific isogenes, suggesting that these were more suitable for a function in C_4 photosynthesis (Christin *et al.*, 2013a; John *et al.*, 2014). It has been shown that some C_3 plants possess isoforms with C_4-like expression patterns (Hibberd & Quick, 2002; Brown *et al.*, 2010). For instance, genes for bundle sheath-specific glycine decarboxylase were already present in the C_3 ancestors of the genus *Flaveria* (Schulze *et al.*, 2013), and mechanisms for the cell specificity of NAD-ME and NADP-ME enzymes might have evolved long before the C_4 pathway (Brown *et al.*, 2011). While the drivers of these characters remain to be elucidated, their co-option would drastically reduce the number of steps separating C_3 ancestors from C_4 descendants.

Evolution of C_4-like anatomical characters

In most C_4 lineages, PEPC and Rubisco functions are segregated within leaves into mesophyll and bundle-sheath cells, respectively (Figure 1b), the latter being specialized cells surrounding the vascular tissue. In this common variant of the C_4 syndrome, a short distance between mesophyll and bundle sheath cells is usually achieved via high vein density. Vein density first increased during the early diversification of angiosperms (Feild *et al.*, 2011), and was followed by several further increases in diverse groups of C_3 plants (Figure 2; Christin *et al.*, 2013b). In a C_3 context, a high density of major veins provides alternative paths for water transport in case of xylem embolism and might confer higher tolerance to damage and drought (Sack *et al.*, 2008, 2012). In addition, higher densities of minor veins enable high rates of photosynthesis and are advantageous in productive environments, such as high irradiance conditions (McKown *et al.*, 2010). High vein density therefore represents an adaptation to high photosynthetic rates or a high risk of xylem embolism or damage. However, vein density is only indirectly relevant to C_4 photosynthesis. Indeed, the absolute distance between veins (interveinal distance; IVD) is less important than the number of mesophyll cells separating consecutive vascular bundles (Hattersley & Watson, 1975). This latter characteristic is only partially correlated to IVD, which is also influenced by the size of mesophyll cells, the thickness of the bundle sheath, and the diameter of vascular tissue. Similar IVD values can therefore emerge through different combinations of mesophyll cell size and number (Lundgren *et al.*, 2014), and the environmental drivers of these cellular properties are yet to be identified.

Bundle-sheath cells evolved early in the history of vascular plants, with the function of regulating water and metabolite fluxes from and into the leaves, and a variety of additional metabolic tasks (Leegood, 2008; Griffiths *et al.*, 2013; Aubry *et al.*, 2014). The ecological significance of bundle-sheath cell size is still unclear, although it has been proposed that larger cells might provide protection against or rapid repair of cavitation (Sage, 2001; Griffiths *et al.*, 2013), and hence confer an advantage when transpiration exceeds water supply (Osborne & Sack, 2012). However, C_4 photosynthesis does not necessarily require large bundle-sheath cells, but only a large relative amount of bundle-sheath tissue (Hattersley, 1984; Dengler *et al.*, 1994), which may be achieved via a proliferation of small bundle-sheath cells, for instance through the development of abundant minor veins (Lundgren *et al.*, 2014). The proportion of bundle-sheath tissue varies among clades of C_3 grasses, with large fractions increasing the likelihood of evolving C_4 physiology (Christin *et al.*, 2013b; Griffiths *et al.*, 2013). Since this leaf property results from multiple characteristics of distinct components, and in particular the size of bundle-sheath cells and the number of mesophyll cells between consecutive vascular bundles (Christin *et al.*, 2013b), it could be dictated by multiple drivers, including those that influence vein density.

Concentration of Rubisco activity in bundle-sheath cells and the C_2 pathway

A high Rubisco activity in chloroplasts of the bundle-sheath is probably necessary for the evolution of C_4 photosynthesis, since any C_4 cycle in its absence would be futile. Determinants of the relative abundance of chloroplasts among mesophyll and bundle-sheath cells are poorly understood. However, it has been clearly established that enhanced Rubisco activity in the bundle-sheath can be related to the C_2 pathway (Sage *et al.*, 2012). The C_2 cycle arises through a concentration in the bundle sheath of glycine decarboxylase (GDC), the enzyme responsible for CO_2-liberation in photorespiration (Sage *et al.*, 2012). In *Flaveria* species, mesophyll and bundle-sheath GDC are encoded by different isogenes, so that a decrease of GDC expression in the mesophyll increases the relative activity of GDC in the bundle-sheath (Schulze *et al.*, 2013). This localization forces photorespiration to release CO_2 in the bundle-sheath cells, meaning that the CO_2 is less likely to diffuse back to the atmosphere before being refixed by Rubisco (Sage *et al.*, 2012). The rate of refixation is higher if Rubisco is abundant in the bundle-sheath cells, and an increased confinement of Rubisco and GDC activities to these cells might co-evolve to optimize the C_2 physiology.

The C_2 pathway has been seen as an intermediate stage between C_3 and C_4 photosynthesis for a long time (Monson *et al.*, 1984; Hylton *et al.*, 1988), a hypothesis later supported by phylogenetic analyses in different taxonomic groups (McKown *et al.*, 2005; Khoshravesh *et al.*, 2012). However, phylogenetic analyses and molecular dating have also shown that the C_2 trait can be stable, having existed in some groups for more than 10 million years without producing any known C_4 descendant (Christin *et al.*, 2011a). Although most plants using the C_2 pathway are limited in range (Sudderth *et al.*, 2009), others, like *Mollugo verticillata*, are widespread and colonize numerous ecological conditions. While some C_2 plants possess C_4-like biochemical characters (e.g. *Mollugo verticillata*; Kennedy & Laetsch, 1974), others, such as *Mollugo nudicaulis*, have no C_4 activity (Kennedy *et al.*, 1980), which shows that C_2 physiology can evolve and be maintained independently of any C_4 cycle. The main physiological effect of the C_2 pathway is to slightly decrease photorespiration, and consequently increase the net carbon gain in conditions where photorespiration is important (Vogan & Sage, 2011; Way *et al.*, 2014).

Selective pressures

The assembly of C_4 physiology via natural selection requires environmental conditions where C_4 photosynthesis is advantageous compared to the ancestral conditions. This is believed to have happened after atmospheric CO_2 reached very low levels some 30 million years ago during the

Oligocene (Pagani *et al.*, 2005; Beerling & Royer, 2011), which exacerbated photorespiration (Ehleringer *et al.*, 1991). Molecular dating places C_4 origins in the last 30 million years (Box 1; Figure 2), and phylogeny-based models have shown that the probability of C_3-to-C_4 transition increased during this time (Christin *et al.*, 2008a, 2011a; Vicentini *et al.*, 2008; Besnard *et al.*, 2009). However, depending on the taxonomic/phylogenetic placement of some microfossils, the earliest C_4 origin, in the grass subfamily Chloridoideae, might have happened in a high-CO_2 world (Prasad *et al.*, 2011; Christin *et al.*, 2014), and fossilized pollen grains from a couple of million years before the Oligocene CO_2 decline have been assigned to C_4 species (Urban *et al.*, 2010). Despite this possibility of some C_4 origins before the Oligocene CO_2 decline, the vast majority of C_4 origins happened in a low-CO_2 world. (Christin *et al.*, 2014). However, a low atmospheric CO_2 level is not sufficient to select for C_4 photosynthesis (Ehleringer and Bjorkman, 1977; Osborne and Beerling, 2006), and other environmental factors that increase photorespiration likely promoted each of the numerous origins of C_4 physiology (Sage, 2001; Roalson, 2008). Comparative analyses have shown that transitions to C_4 physiology occurred in grass lineages from open habitats of warm regions (Osborne & Freckleton, 2009; Edwards & Smith, 2010), while in Chenopodiaceae *sensu stricto*, the evolution of C_4 photosynthesis was more likely in lineages inhabiting saline and coastal environments (Kadereit *et al.*, 2012).

4. What happened during the transition to C_4 photosynthesis?

Increase of PEPC activity and new selective pressures

If the appropriate leaf functions are in place and a significant fraction of Rubisco activity is concentrated in the BS cells, the C_4 cycle can theoretically evolve through the gradual increase of C_4 reactions (Heckmann *et al.*, 2013). The order in which the C_4 enzymes are incorporated is not known with precision, and the order might differ among lineages (Williams *et al.*, 2013). An increase in the rate of transformation and transport of the C_4 intermediates, release of CO_2, or regeneration of the intermediates would not generate any kind of C_4 cycle in the absence of a sufficiently high concentration of oxaloacetate, the product of the PEPC reaction (Figure 1b). An increased activity of the other enzymes could however evolve before enhanced PEPC activity for reasons unrelated to C_4 photosynthesis (Williams *et al.*, 2013; Mallmann *et al.*, 2014). The very first step in the establishment of a proper C_4 cycle must be an increase in the rate of fixation of atmospheric CO_2 by the coupled action of PEPC and CA. CA is already present at high levels in many C_3 plants, where it plays a role in carbon assimilation (Majeau & Coleman, 1994). An increase of PEPC activity in the mesophyll might thus be sufficient to generate high concentrations of oxaloacetate. This oxaloacetate would however need to be transformed and transported by several enzymes before feeding Rubisco with released CO_2. It has been established that at least some enzymes of the C_4 cycle are already present in some C_3 plants in the areas of the leaf required for a C_4 cycle (Hibberd & Quick, 2002). Their expression levels in C_3 plants can moreover be significant, although below those observed in C_4 plants (Christin *et al.*, 2013a; Bräutigam *et al.*, 2014). Furthermore, the activities of PPDK and decarboxylating enzymes increase in some C_2 plants before PEPC (Williams *et al.*, 2013), potentially to rebalance nitrogen metabolism in C_2 plants (Mallmann *et al.*, 2014). The enzymes already present in the cells of some C_3 or C_2 species may be sufficient to process the oxaloacetate produced by an increased PEPC activity, especially if their activity is induced by an increase in substrate concentrations. Transfer of intermediates between cells could initially be made via simple diffusion, so that increased PEPC activity might, in plants already possessing C_4-like characters, be sufficient to generate a C_4 physiology.

The establishment of a weak C_4 cycle through an increased activity of PEPC and the co-option of other enzymes is a key event, because it can significantly decrease photorespiration and consequently lead to a gradual improvement of the efficiency of the C_4 pathway through natural selection (Heckmann *et al.*, 2013), fixing mutations that enhance activities of C_4 enzymes and adapt their catalytic properties for the new metabolic context (Nakamoto *et al.*, 1983; Bauwe, 1984; Svensson *et al.*, 2003). In the case of PEPC, the past action of selection left traces as an excess of non-synonymous mutations that are mostly concentrated on branches leading to each C_4 group (Christin *et al.*, 2007; Besnard *et al.*, 2009). This distribution of C_4-driven amino acid changes suggests that the adaptation of PEPC for the C_4 function occurred over a short time that overlaps with changes in the enzyme's activity (Figure 2). In most phylogenies, the first C_4 descendant is separated from its last C_3 ancestor by several million years (Christin *et al.*, 2008a, 2011a; Besnard *et al.*, 2009), so that the different characters that together generate C_4 physiology cannot be disentangled. However, some exceptional groups maintained a diversity of photosynthetic phenotypes that might represent the footprint of gradual modifications during the evolution of C_4 physiology.

Insights from Flaveria

In the genus *Flaveria*, the transition from the last C_3 ancestor to the first C_4 descendant spanned about 2-3 million years (Christin *et al.*, 2011a), and extant taxa represent a range of anatomical, biochemical and physiological states (Bauwe, 1984; Ku *et al.*, 1991; McKown & Dengler, 2007; Sudderth *et al.*, 2007; Vogan & Sage 2011). We compiled data from the literature for different C_4-related traits and reconstructed their evolution on the time-calibrated phylogeny for the genus (from Christin *et al.*, 2011a). Ancestral reconstructions for nodes separating the C_3 ancestor of all *Flaveria* from the extant C_4 species *Flaveria trinervia* suggest that C_4 anatomy, biochemistry and physiology were acquired in parallel in this group (Figure 3), although ancestral reconstructions come with large confidence intervals. A higher PEPC activity can be observed in some *Flaveria* species that do not have a typical C_4 metabolism (Bauwe, 1984), as also shown for other groups (Murphy *et al.*, 2007), and this results in an increase in the proportion of carbon fixed as C_4 acids (Monson *et al.*, 1986; Moore *et al.*, 1987; Vogan & Sage, 2011). The increased C_4 activity in these plants might result from a need to rebalance the nitrogen metabolism between bundle sheath and mesophyll cells, putting some C_2 plants on a highway towards C_4 (Mallmann *et al.*, 2014). An effect of this enhancement of C_4 activity on water-use efficiency has not been detected (Vogan & Sage, 2011). There are, however, indications of a rise in photosynthetic nitrogen-use efficiency (PNUE) in parallel with the enhancement of C_4 activity in *Flaveria*, associated with the clear decrease in CO_2 compensation point that accompanies the accumulation of C_4 functions (Vogan & Sage, 2011; Figure 3).

The C_4 characters that accumulated before the transition to a C_4 physiology are likely to vary among taxonomic groups (Williams *et al.*, 2013). The increase of PEPC activity might happen in plants that already have C_4 functional properties, but the establishment of a weak C_4 cycle might also be possible in plants with components that are more distant from the C_4 requirements. In the former case, few changes might be needed besides the increase in C_4 cycle activity, while in the latter case C_4 functions would be reinforced by selection for a more efficient C_4 cycle, as seen for leaf anatomical characteristics in *Flaveria* (Figure 3). The changes required in both expression patterns and catalytic properties will also depend on the properties of the enzyme inherited from the C_3 ancestor and co-opted for the C_4 cycle. The timing of origin for C_4 characters will consequently vary among C_4 lineages (Williams *et al.*, 2013), with the same changes happening in some cases within a C_3 context, while in other lineages they might happen during the evolution of a C_4 physiology, or even slightly later.

5. What happened after C_4 evolution?

Optimization of Rubisco and PNUE

The relative specificity of Rubisco for CO_2 compared to O_2 is negatively correlated with its catalytic efficiency, and the two parameters are thought to be finely tuned to allow the highest catalytic rate while minimizing O_2 fixation (Tcherkez *et al.*, 2006). In C_3 plants and a low-CO_2 atmosphere, this trade-off results in more specific but slower enzymes that have to be highly expressed to fix sufficient CO_2, and Rubisco represents up to one third of all leaf soluble proteins and 20% of the total nitrogen budget (Evans & Poorter, 2001). The higher concentration of CO_2 around Rubisco generated by C_4 physiology relaxed selection for enzymes with a higher specificity for CO_2, and enabled the evolution of faster Rubiscos (Seeman *et al.*, 1984; Tcherkez *et al.* 2006; Kubien *et al.* 2008; Kapralov *et al.* 2011). A more efficient enzyme, together with increased CO_2 concentrations at its active site, means that fewer protein molecules are needed, and the abundance of Rubisco is reduced by 60-80% in some C_4 species (Ku *et al.*, 1979). Although the C_4 cycle itself requires additional enzymes, large quantities of proteins are not necessary if their catalytic rates are high, and the C_4 cycle thus allows for lower total protein and nitrogen amounts if the proteins are optimized, which increases photosynthetic nitrogen-use efficiency (PNUE; Schmitt & Edwards 1981; Sage & Pearcy 1987; Ghannoum *et al.*, 2005).

Models suggest that the adaptation of Rubisco kinetics started in parallel with increased C_4 enzyme activity, but continued once the plants were in a C_4 physiological state (Heckmann *et al.*, 2013; Williams *et al.*, 2013). In *Flaveria*, the Rubisco kinetics of C_4 species differ from those of related C_3 taxa, but those of C_3 and intermediate taxa were not consistently different (Kubien *et al.*, 2008). The continuous adaptation of Rubisco after C_4 evolution is supported by the footprint of adaptive evolution on genes encoding Rubisco, with an excess of non-synonymous mutations spread across branches within C_4 lineages in various groups of angiosperms (Christin *et al.*, 2008b; Kapralov *et al.*, 2012). The decreased nitrogen costs of Rubisco thus evolved very gradually, and continued long after the initial diversification of C_4 groups. The ranges of Rubisco kinetics almost overlap between C_3 and C_4 species (Seeman *et al.*, 1984), and variation in the catalytic rate of Rubisco affects PNUE among C_4 grasses, with higher catalytic rates increasing PNUE (Ghannoum *et al.*, 2005). For instance, the PNUE increase in C_4 lineages compared to C_3 sister-groups varies from 25% in the C_4 grass lineage *Aristida* to 42% in Chloridoideae and 60% in Andropogoneae (Taylor *et al.*, 2010).

The capacity to grow with limited access to nitrogen is key to ecological success on infertile soils, and a more efficient use of nitrogen acquired during the diversification of C_4 lineages might have contributed to the rise to ecological dominance of some C_4 species (Edwards *et al.*, 2010). For example, recovery after fire in mesic savannas requires rapid resprouting in a nitrogen-depleted soil, and these environments are dominated by grasses from the Andropogoneae clade (Forrestel *et al.*, 2014), which have the highest PNUE values among C_4 grasses (Taylor *et al.*, 2010). The number of species for which PNUE has been measured is limited, and it is thus not known whether the evolution of high PNUE coincided with the rise to ecological dominance better than the origin of C_4 photosynthesis. It is however likely that C_4 physiology enabled the evolution of very high PNUE in some cases, and hence the colonization of competitive habitats, like savannas.

Adaptation of stomatal conductance and plant hydraulics

CO_2 partial pressures within the leaf intercellular air spaces are sufficient to saturate the coupled CA-PEPC enzyme system at 25-33% of the atmospheric value, and maximum rates of C_4 photosynthesis can thus be maintained despite large decreases in stomatal conductance (Wong *et al.*, 1979; Long, 1999). C_4 plants consequently evolved lower stomatal conductance for a given rate of photosynthesis, a property that is amongst the most consistently associated with C_4 photosynthesis in grasses (Taylor *et al.*, 2010). Decreased stomatal conductance could theoretically arise directly from the emergence of a C_4 cycle if stomatal aperture is regulated in response to the intercellular CO_2 partial pressure and photosynthetic rate (e.g. Messinger *et al.*, 2006). Changes in the stomatal response to internal CO_2 concentrations are already visible in some C_3-C_4 species of *Flaveria* (Huxman & Monson, 2003), but in the longer term, the maximum capacity for stomatal conductance is adjusted downwards via developmental changes in the density and/or size of the stomata (Taylor *et al.*, 2012). The diversity of strategies used to decrease stomatal conductance within some C_4 grass lineages (i.e. smaller versus less numerous stomata; Taylor *et al.*, 2012) suggests continuing adjustments after the emergence of a C_4 cycle, although an initial decrease of stomatal number might result directly from the elevated vein density in C_4 species (Way, 2012; Figure 3).

A lower stomatal conductance decreases leaf transpiration relative to hydraulic supply, thereby improving leaf water status if the hydraulic system remains unchanged (Osborne & Sack, 2012). This effect remains if any subsequent reduction in hydraulic conductance is of a smaller magnitude than the change in stomatal conductance. In keeping with this expectation, comparisons within common garden, glasshouse and controlled environments show that soil-leaf water potential gradients are smaller in C_4 grass lineages compared to their close C_3 relatives under well-watered conditions (Taylor *et al.*, 2010, 2011, 2014). This can be advantageous in environments where evaporative demand exceeds hydraulic supply, including conditions of high evaporative potential where solar radiation is high or the atmosphere is dry (Osborne & Sack, 2012). The advantage of reducing stomatal conductance is greater in low CO_2 atmospheres, where the stomatal aperture of both C_3 and C_4 species tend to increase, thereby augmenting the risk of hydraulic failure (Osborne & Sack, 2012).

The effects of stomatal conductance on plant tolerance of water deficits are complex (Ghannoum, 2009). During the initial stages of soil drying, stomatal conductance decreases more sensitively in C_3 than C_4 grasses (Ripley *et al.*, 2010; Taylor *et al.*, 2011, 2014). This observation is consistent with a hypothesis of hydropassive stomatal control, mediated via a higher ratio of evaporative demand to hydraulic supply in C_3 than C_4 species (Osborne & Sack, 2012), but may also follow from differences in the optimization of stomatal aperture relative to photosynthesis in C_3 and C_4 species (Taylor *et al.*, 2014). In a common garden experiment of closely related grasses adapted to similar habitats in the same regional flora, this difference in stomatal behaviour unexpectedly led to higher stomatal conductance in C_4 than C_3 species during the early stages of drought (Taylor *et al.*, 2014). However, during chronic drought, non-stomatal limitation of carbon assimilation becomes more important in C_4 than closely related C_3 grasses, and may reduce or eliminate the differences in photosynthesis between them (Ghannoum *et al.*, 2003; Ripley *et al.*, 2007, 2010; Ibrahim *et al.*, 2008; Ghannoum, 2009; Taylor *et al.*, 2011). The mechanisms underlying this behaviour are unknown, but seem to correlate with low water potential in C_4 leaves (Ibrahim *et al.*, 2008; Ripley *et al.*, 2010; Taylor *et al.*, 2014), and could correspond to a failure of the C_4 cycle.

In some C_4 eudicots, modifications in the xylem architecture, including narrower and shorter vessels, decrease the leaf conductivity, which provides protection against cavitation and thus enhanced drought tolerance (Kocacinar & Sage, 2003, 2004). It might be assumed that the higher water-use efficiency conferred by the C_4 physiology enabled decreases in leaf conductivity. However, xylem modifications are already visible in the C_3-C_4 intermediates of *Flaveria* that have water-use efficiencies similar to the C_3 species, suggesting that xylem modifications predated C_4-related higher water-use efficiency, at least in this genus (Kocacinar *et al.*, 2008). It has been hypothesized that the decreased conductivity actually drove the evolution of a C_2 pathway in these species (Kocacinar *et al.*, 2008), and might therefore be seen as a C_2 precondition. This emphasizes difficulties in generalizing the order of events during the transition from C_3 to C_4 photosynthesis, such that some modifications might have evolved before C_4 physiology and favored its evolution in some lineages, while they were enabled by C_4 physiology in others.

Addition of alternative carbon shuttles

The action of a decarboxylase is necessary directly after PEPC becomes responsible for a significant part of atmospheric CO_2 fixation. The evidence accumulated so far however indicates that the shuttling of carbon between PEPC and Rubisco (Figure 1a, steps b-d) diversified after plants were already in a C_4 physiological state. The variation in the carbon shuttles among C_4 plants belonging to the same C_4 groups (Gutierrez *et al.*, 1974; Wang et al., 2014) indeed indicates either that some shuttles present in the common C_4 ancestor were lost in some of the descendants, or that shuttles were added in some descendants only. The second hypothesis receives strong support from comparative analyses of genes encoding decarboxylating enzymes (Christin *et al.*, 2009a, 2009b).

In particular, strong signatures of positive selection are associated with the evolution of C_4-specific PCK in grasses, and this selection is detected on branches nested within several of the C_4 groups (Christin et al., 2009a; Figure 2).

The C_4 biochemical pathway can be plastic and respond to the environment (Furbank, 2011). For example, leaves of maize change the balance between NADP-ME and PCK shuttles when subject to shade (Bellasio & Griffiths, 2014; Sharwood *et al.*, 2014), and models suggest that the addition of alternative carbon shuttles increases the range of light conditions tolerated by the plant (Wang *et al.*, 2014). These attributes often evolved long after the initial origins of C_4 photosynthesis, and might thus have allowed the colonization of habitats differing in their vegetation cover. These adaptations consequently allowed C_4 plants to expand their niches compared to the ancestors that first acquired a C_4 pathway, and contributed to the ecological diversity found within C_4 groups.

6. Contingency and the ecological diversity of C_4 plants

The evolution of C_4 photosynthesis is a long process, beginning with the acquisition of C_4 anatomical and biochemical functions in a C_3 context, and continuing long afterward with the development of novel attributes enabled by the C_4 pathway (Figure 2). Following the establishment of C_4 physiology, each C_4 lineage has subsequently diversified, in some cases producing more than a thousand extant species (GPWGII, 2012). The diversity of environments occupied by C_4 plants means that the C_4 syndrome cannot be associated with a simply defined ecological strategy, but only partially affects the ecological preference of each plant, which is also influenced by other attributes inherited from the C_3 ancestors or that evolved after C_4 photosynthesis (Stowe & Teeri 1978; Stock *et al.*, 2004; Edwards *et al.*, 2010). The ecological diversity of C_4 species is therefore contingent upon (i) the ecology of ancestral C_3 lineages, which has subsequently been modified by (ii) physiological changes imparted by C_4 photosynthesis and then (iii) radiation into new niche space. In recent years, a phylogenetic perspective has enabled these three interacting factors to be teased apart, to bring a deeper understanding of the ecological diversity of C_4 species. In the next two sections, we illustrate how these processes have operated, using the examples of growth rate and sorting along environmental gradients.

Phenotypic integration – the example of growth

Growth rate varies significantly among plant species, with fast growth especially important for the persistence of species in resource-rich or disturbed habitats, and slow growth associated with persistence in resource-limited environments (Grime & Hunt, 1975; Grime *et al.*, 1997). C_4

photosynthesis increases the efficiency of canopy photosynthesis across a range of temperatures (Long, 1999), especially in open environments, and allows a higher maximum conversion efficiency of intercepted light energy into biomass compared with C_3 photosynthesis (Monteith, 1978). If all else were equal, the acquisition of C_4 photosynthesis would therefore increase the rate of plant growth under hot, sunny conditions. However, experimental comparisons have surprisingly failed to discern a clear general difference in growth between C_3 and C_4 species.

Snaydon (1991) compiled published aboveground productivity data for 34 herbaceous species across 88 sites, and found no significant difference between C_3 and C_4 species when latitude (and, by proxy, temperature and growing season length) were taken into account. The most productive species in this analysis were however all C_4, consistent with previous results (Monteith, 1978), and supporting the hypothesis that C_4 photosynthesis confers the potential for higher maximum productivity than in C_3 species (Hatch, 1999; Long, 1999). Indeed, work by Piedade *et al.* (1991) showed that productivity in the C_4 hydrophyte *Echinochloa polystachya* growing in nutrient-rich Amazon floodwaters approaches the theoretical limit predicted from the efficiencies of physiological processes. However, in general, direct comparisons between C_3 and C_4 plants have failed to show consistently faster growth in C_4 species under controlled environments (e.g. Öztürk *et al.*, 1981; Pearcy *et al.*, 1981; Hunt *et al.*, 1996; Reich *et al.*, 2003), natural climate conditions (e.g. Öztürk *et al.*, 1981; Gebauer *et al.*, 1987; Reich *et al.*, 2001), or in comparisons between closely related C_3 and C_4 species (Slatyer, 1970; Rajendrudu & Das, 1982; Taylor *et al.*, 2010). For example, Taylor et al (2010) compared 34 closely related species of C_3 and C_4 grass, sampling multiple independent C_4 lineages. Although leaf photosynthesis was higher in the C_4 species, as expected, there were no differences in relative growth and net assimilation rates between these C_3 and C_4 species. The evidence from multiple experiments is clear: the large differences in leaf photosynthesis typically observed between C_3 and C_4 species do not generally translate into faster rates of growth.

This apparent paradox might result from the way that C_4 photosynthesis is integrated into the phenotype of the whole organism. In particular, interactions among processes operating at the organismal scale mean that growth often does not depend strongly on area-normalized leaf photosynthesis (Poorter *et al.*, 1990). First, a limited number of pairwise comparisons between ecologically similar or closely related species have shown that the leaves of C_4 plants may be shorter-lived than those in C_3 species (reviewed by Long, 1999), suggesting that higher photosynthesis may be associated with more rapid leaf turnover, with a negative effect on growth. In addition, the allocation of growth to leaves versus heterotrophic tissues (e.g. roots and stems) and the area to mass ratio of leaves (specific leaf area), each have major effects on growth that may partially offset or fully obscure the effects of higher rates of leaf photosynthesis (Körner, 1991). These effects are illustrated by work on the recently diverged C_3 and C_4 subspecies of *Alloteropsis semialata*. Leaf photosynthetic rates differ between these taxa as expected from theory (Osborne *et al.*, 2008). However, the associated differences in growth rates are partially offset by a lower allocation of growth to leaves, and a smaller specific leaf area in the C_4 than C_3 subspecies (Ripley *et al.*, 2008), which both tend to oppose the effects of C_4 photosynthesis. More generally, comparative work indicates that each of these growth traits may show phylogenetic patterns (e.g. Burns & Strauss, 2012), which means that closely related species share similar attributes, and the growth rates of C_4 species may be contingent upon characters inherited from their C_3 ancestors.

An altered partitioning of growth from leaves to roots in C_4 plants has been noted in a number of pairwise comparisons between ecologically similar or closely related species (Slatyer, 1970; Long & Mason, 1983; Ripley *et al.*, 2008; Taylor *et al.*, 2010). In each documented case, the shift in partitioning is achieved alongside similar or faster rates of growth in the C_4 species. It has been hypothesized that this shift in allocation could arise from the higher PNUE of C_4 plants and may depend on the ecological context (Long, 1999). C_4 species of fertile and/or disturbed habitats may use the same investment of nitrogen to produce a larger leaf area than their C_3 counterparts, thereby promoting more rapid growth. In contrast, C_4 plants of infertile habitats may adopt a more conservative strategy by producing the same leaf area as their C_3 counterparts with less nitrogen, but investing the resultant surplus of nitrogen into root development to better acquire this limiting resource. The hypothesis is supported by studies of growth allocation in plants adapted to fertile and infertile habitats (reviewed by Long, 1999). In summary, although C_4 photosynthesis offers the potential for faster growth, there is little published evidence for a consistent general translation of higher rates of leaf photosynthesis into greater productivity. Instead, the effects of C_4 photosynthesis on growth are mediated by changes in allocation and turnover, and may depend on the ecological context in which they evolve.

Ecological sorting at the global scale – temperature and water availability

Temperature is the primary determinant of species distributions at the global scale (Woodward, 1987), and hot conditions have long been considered important for C_4 plant ecology (Black, 1971). Global distribution patterns in relation to temperature are especially strong for grasses, where the classic pattern is turnover from C_4 to C_3 species with declining temperature, along both latitudinal (Teeri & Stowe, 1976) and altitudinal (Rundel, 1980) gradients. However, phylogenetic analyses show that C_3 grasses closely related to C_4 lineages also inhabit warm environments, which is the ancestral condition for this taxonomic group (Edwards & Still, 2008; Edwards & Smith, 2010;

Figure 4). Differences in land surface temperature can be detected between the habitats of closely related C_3 and C_4 grasses (Still *et al.*, 2013), but the classical global patterns arise largely because one lineage of C_3 grasses, the Pooideae, acquired cold adaptations in the Oligocene and subsequently diversified at high latitudes and altitudes (Edwards & Still, 2008; Sandve *et al.*, 2008; Edwards & Smith, 2010; Pau *et al.*, 2013; Visser *et al.*, 2014; Figure 4). These observations have prompted a re-evaluation of how C_4 taxa are distributed in relation to climate.

Because of the extra metabolic cost of C_4 photosynthesis, net leaf photosynthesis under light-limited conditions is lower for C_4 than C_3 plants at low temperatures, where the energetic benefit of suppressing photorespiration is limited (Ehleringer & Bjorkman, 1977; Collatz *et al.*, 1998). Model simulations of leaf or canopy photosynthesis that account for this effect therefore predict a “crossover temperature” below which C_3 plants outperform their C_4 counterparts (Ehleringer, 1978; Collatz *et al.*, 1998). However, under light-saturated conditions, energy is absorbed in excess of that required to drive the C_4 cycle and, for a given investment in Rubisco, leaf photosynthesis is higher at all temperatures in a C_4 than C_3 leaf (Long, 1999). As a consequence, a more complex photosynthesis model accounting for the penetration of direct light as sunflecks into the canopy shows that photosynthesis may be higher in a C_4 than C_3 canopy at temperatures down to 10 °C (Long, 1999). However, a lower concentration of Rubisco in C_4 than C_3 leaves leads to a temperature trade-off in light-saturated photosynthesis, with a crossover temperature similar to that observed under light-limitation (Still *et al.*, 2003). Thus, according to theory, if C_4 plants maintain a high investment in Rubisco, there is no intrinsic energetic cost that would prevent them from colonizing open habitats in cool environments, particularly if they also have an open canopy.

C_4 physiology evolved in warm climatic regions of the subtropics (Ehleringer *et al.*, 1991; Sage, 2004; Edwards & Smith, 2010), and the leaves of many C_4 species suffer chilling and freezing damage in common with other tropical and subtropical plants (Pearce, 2001). However, after evolving the C_4 syndrome, a number of plant lineages migrated into cool climate regions (Edwards & Smith, 2010; Figure 5), and now inhabit high temperate latitudes (Bjorkman *et al.*, 1975; Long *et al.*, 1975) and montane habitats (Sage & Sage, 2002). Absolute minimum winter temperatures impose a stringent climatic filter on the species that can persist in these environments, and adaptation requires the prevention or tolerance of ice formation within tissues during extreme low temperature episodes (Woodward, 1987). Many C_4 species of cold environments survive winter freezing events by either adopting an annual life history (e.g. weeds) or being deciduous (e.g. prairie grasses), in both cases overwintering in a dormant state, which is a common strategy adopted by plants to avoid episodic freezing (Zanne *et al.*, 2013). However, there seems to be no intrinsic barrier to freezing tolerance in a C_4 leaf, with species developing protection via constitutive or facultative cold acclimation mechanisms (Sage & Sage, 2002; Liu & Osborne, 2008, 2013). The leaves of other C_4 species are intolerant of freezing, but have physiological mechanisms for protection against light-mediated damage during chilling events in the range 0-10 °C (Long, 1983; Naidu *et al.*, 2003). In conclusion, C_4 photosynthesis evolved in hot environments because there was a strong selective pressure for decreased photorespiration in these conditions. However, it can offer smaller benefits at low temperatures under high light conditions, so that C_4 plants can colonize cooler regions following the acquisition of cold adaptations, increasing the ecological diversity within C_4 groups (Figure 5).

The water-saving and hydraulic benefits of the C_4 syndrome outlined in Section 5 lead to the prediction that C_4 species should occupy drier habitats and environments with higher potential evaporation than C_3 species. It has long been known that C_4 eudicots sort into drier climate regions than their C_3 counterparts (Ehleringer *et al.*, 1997). There seems however to be a phylogenetic effect, with C_4 eudicots that are extremely well adapted to arid conditions having evolved from C_3 ancestors that already inhabited dry conditions (Stowe & Teeri, 1978), and, in several groups of eudicots, the distributions of related C_3 and C_4 lineages along environmental gradients largely overlap (Sudderth *et al.* 2009; Edwards & Ogburn, 2012; Figure 4). Similarly, in the Chenopodiaceae group, C_3 plants that were more tolerant of salinity gave rise to C_4 halophytes (Kadereit *et al.*, 2012). Early studies failed to detect an overall relationship between the distribution of C_4 grasses and rainfall (Hattersley, 1983; Ehleringer *et al.*, 1997), despite the clear differences in water relations between C_4 and C_3 grass species. A phylogenetic perspective has resolved this paradox by revealing a complex picture in which contingency, physiological innovation, and subsequent ecological radiation have each played important parts.

Phylogenetic patterns in the precipitation (Edwards & Smith, 2010) and habitat water requirements of grasses (Osborne & Freckleton, 2009) mean that closely related species tend to occupy similar environments, and both the global and regional distributions of major grass lineages thus differ in relation to precipitation (Taub, 2000; Edwards & Smith, 2010; Visser *et al.*, 2012, 2014; Figure 4). This latter pattern has long been recognized in the differing geographical and climate space occupied by different taxonomic groups (Hartley, 1950). When C_4 photosynthesis evolved against this background, it modified physiological relationships with the environment, but plants nonetheless tended to retain attributes of their ancestors (Figure 4). The variation among groups of C_4 grasses might therefore result from the ecological diversification of grasses before C_4 evolution. For instance, the groups of C_4 grasses that prosper in more arid conditions, such as Aristidoideae and Chloridoideae (Edwards & Smith, 2010; Visser *et al.*, 2012, 2014), have C_3 relatives that inhabit similarly arid habitats (Gibbs Russell & Le Roux, 1990; Cerros-Tlatilpa *et al.*, 2011). Despite this phylogenetic effect, the transition to C_4 physiology was still accompanied by changes in the ecological niche. Ancestral state reconstructions show that C_4 evolution in grasses led to consistent shifts into drier and more seasonal niche space (Edwards & Smith, 2010), and C_4 grasses are more likely to migrate into arid or saline habitats than their C_3 counterparts (Osborne & Freckleton, 2009; Brohman & Bennett, 2014), suggesting that C_4 photosynthesis facilitates adaptation to conditions of low soil water potential, probably through the continuous adaptation of stomatal conductance and plant hydraulics, and thereby allows plants to more readily access dry niche space (Edwards & Donoghue, 2013; Figure 5). In sedges however, many clades of C_3 species that prosper in more humid habitats produced C_4 descendants that share this preference (Stock *et al.*, 2004). Water-use efficiency is likely irrelevant for sedges of infertile wetlands, where the C_4 advantage might result from the associated nitrogen-use efficiency (Li *et al.*, 1999; Stock *et al.*, 2004). On the other hand, a high maximum rate of growth may be critical for sedges of fertile wetlands (Muthuri *et al.*, 1989), highlighting the diversity of ecological strategies enabled by the C_4 syndrome.

In summary, phylogenetic analyses show that contingency has played an important role in shaping the ecological niche of C_4 plants. This is classically illustrated by island colonists, like the C_3 *Scaevola* and C_4 *Euphorbia* lineages of Hawaii. Each is likely derived from a single island colonist, but has radiated into a similar diversity of habitats ranging from wet, closed forest to dry, open scrub, irrespective of the difference in their photosynthetic pathway (Robichaux & Pearcy, 1984). However, ecological diversification into the vacant niches offered by volcanic islands represents a special case. Generally, the ecological preferences inherited from C_3 ancestors have been affected by C_4 physiological novelty in subsequent diversification. This process of diversification is exemplified by the large C_4 group of Paniceae, which evolved from a C_3 ancestor inhabiting tropical seasonal forests but came to colonize diverse conditions after the evolution of C_4 physiology (Figure 5). Despite similar evolutionary times (Figure 5, left panel), the C_3 species in this group remained in a relatively small portion of the environmental space, with the exception of members of the *Dichanthelium* genus, which adapted to cold habitats (Figure 5). The transitions between C3 and C4 photosynthesis (blue branches leading to red branches in Figure 5) are associated with a slight shift to drier habitats in the same temperature range. This shift has already been reported and interpreted as a migration from forests in the aseasonal moist tropics to more open habitats in the seasonal subtropics, such as woodlands and savannas (Edwards & Smith, 2010; Figure 5). Following this shift, the C_4 species from this group rapidly dispersed into habitats ranging from dry and hot deserts to temperate grasslands and deciduous forests, and tropical rainforests (Figure 5). This pattern highlights the niche-opening effect of C_4 photosynthesis, which enables adaptation to new environments, probably through the adaptive integration of other attributes of the plants with the C_4 syndrome.

7. Conclusions

The evolutionary history of each C_4 taxon is rich and unique. It starts with the acquisition by its ancestors of characters that are required to build a C_4 system, but which evolve for completely unrelated reasons. Once all the characters exist in a given plant, these can be co-opted to create a weak C_4 cycle following an increase of PEPC activity. This key event creates new selective pressures toward the optimization of the C_4 pump, but it is not the end of the evolutionary process. The ecological preference of each C_4 group initially depends on the attributes inherited from its C_3 ancestors, but changes that happened during and after the transition to C_4 physiology allow plants to escape this heritage. The ecological strategies of specific C_4 plants are best understood by considering their whole evolutionary history, including the characters that were present in the C_3 ancestors, the way the C_4 apparatus was assembled, and the modifications to this apparatus that happened during the diversification of the C_4 group.

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Box 1. Phylogenetic analyses and the evolution of complex phenotypes

Comparisons among groups of species that differ in specific traits is complicated by two factors: i) other attributes of each species alter the effects of the traits; and ii) species are not statistically independent, because of their shared evolutionary history. These problems can be partially solved by taking the evolutionary history into account in comparative analyses. Phylogenetic trees are primarily used to reconstruct the relationships among species, but have also become important in comparative analyses. Their integration into statistical tests of differences among species can remove the variance due to shared evolutionary history, and thus identify properties that are associated with given traits independently of this history (Freckleton *et al.*, 2002). In the case of C_4 photosynthesis, this approach can differentiate attributes that are directly conferred by the C_4 physiology from those that are usually associated with it, but might be inherited from their C_3 ancestors (Edwards & Smith, 2010). The origin of a trait on a phylogenetic tree can be mapped through different ancestral reconstruction methods, which estimate the character state for each speciation event, represented by each node in a phylogenetic tree (Figure 6). For instance, parsimony methods identify scenarios that minimize the number of transitions between character states, and methods based on likelihood estimate the most likely scenario given a set of assumptions (Figure 6). While these are powerful for testing specific hypotheses, such as the statistical association between sets of traits (e.g. Pagel, 1994; Osborne and Freckleton, 2009; Kadereit *et al.*, 2012), the inferred ancestral states are dependent on the underlying model (Maddison, 2006; Christin *et al.*, 2010). This problem can be partially solved by decomposing a complex trait into its constituents, so that the modelled entities are relatively simple properties and not complex phenotypes that result from multiple underlying characters (Christin *et al.*, 2010; Roalson, 2011). Changes in discrete or quantitative characteristics can be estimated with different methods (Christin *et al.*, 2013b; Figure 6). The timing of these changes can then be estimated either relative to each other, by comparing the order of nodes (Figure 6), or in absolute terms, based on the age associated with the branch on which they happened (Figure 6). In addition, phylogenetic analyses of DNA sequences encoding genes of interest can identify past episodes of adaptive evolution (Zhang *et al.*, 2005), and their positioning on phylogenetic trees can highlight periods of protein adaptation linked to an adaptive shift (Figure 2). Each of these methods comes with caveats, and considering multiple sources of information is crucial when inferring the evolutionary history of complex traits.

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Table 1: Hierarchical deconstruction of the C_4 syndrome into different phenotypic levels, from the cell or enzyme to the whole organism.

Term	Definition	Examples
Niche	Environmental conditions in which the organism grows naturally	Warm and open environments
Physiology	Attribute of the whole organism that is generated by a combination of functions	C_4 photosynthesis, growth rate, water-use efficiency
Function	Action at the cellular or tissue level that is enabled by a combination of underlying characters	Rapid transport of C_4 intermediates, fixation of atmospheric carbon by CA + PEPC
Character	Emergent property of one component that is determined by multiple characteristics	Distance between consecutive bundles, activity of PEPC in the mesophyll
Characteristic	Property of one component that is theoretically independent from the others	Length of bundle-sheath cells, expression level of PEPC
Component	One cellular or enzymatic element	Bundle-sheath cell, PEPC

Figure captions

Figure 1. Schematic of the C_4 cycle.

(**a**) Simplified diagram representing the functional properties of the C_4 cycle (Table 1), which is consequently applicable to all C_4 plants. The main biochemical steps are indicated by circled letters. Atmospheric CO_2 enters the first compartment (dashed grey line) by diffusion. It is fixed into the C_4 cycle (a), which results in C_4 acids (in red) that are transformed and transported (b) to the second compartment (grey line), where CO_2 is released (c). The C_4 cycle is completed by the regeneration of the resulting C_3 acid (d). (**b**) One of the realizations of the C_4 cycle, with the example of the grass *Zea mays*, based on Tausta *et al.* (2014). As in most C_4 species, reactions are segregated between the mesophyll and bundle-sheath tissues of the leaf. The C_4 acids are in red, and the circled numbers represents enzymes. The black circles indicate enzymes that are involved in all C_4 types. Ala = alanine, Asp = aspartate, mal = malate, OAA = oxaloacetate, PEP = phosphoenolpyruvate, pyr = pyruvate, 1 = carbonic anhydrase (CA), 2 = PEP carboxylase (PEPC), 3 = NADP malate dehydrogenase, 4 = NADP-malic enzyme (NADP-ME), 5 = alanine aminotransferase (ALA-AT), 6 = pyruvate, phosphate dikinase (PPDK), 7 = aspartate aminotransferase (ASP-AT), 8 = phosphoenolpyruvate carboxykinase (PCK), 9 = Rubisco and the C_3 cycle (Calvin-Benson cycle).

Figure 2. Gradual accumulation of C_4 characters inferred for grasses.

The dated phylogenetic tree for grasses was obtained from Christin *et al.* (2013b), with the time scale in million years ago (Ma). All groups containing only C_3 or C_2 species are compressed and in black. Monophyletic C_4 groups are compressed in red, with their numbering on the right following GPWGII (2012). The two main grass clades are delimited on the right (BEP and PACMAD). Important changes in anatomical characters are reported based on Christin *et al.* (2013b). Episodes of adaptive evolution of C_4 enzymes are based on Christin *et al.* (2007, 2009a, 2009b). The changes shown here represent only a fraction of all changes linked to C_4 evolution and their positioning is approximate because the species sampling was not identical in the different studies. The grey box represent the last 30 million years, when atmospheric CO_2 stayed below 500 ppm. OS = outer bundle sheath, BSD = distance between consecutive bundle sheaths.

Figure 3. Changes inferred during the transition from a C_3 ancestor to the C_4 species *Flaveria trinervia*.

Six different variables were reconstructed on the time-calibrated phylogeny for *Flaveria* from Christin *et al.* (2011a). The values inferred for each node between the root of the tree and *Flaveria trinervia* are plotted against the estimated age of the node. Dashed lines indicate the 95%

confidence interval for the reconstructed ancestral values. The coloured background indicates the estimated photosynthetic state through time, with C_3 in white, C_3-C_4 intermediate in yellow, C_4-like in orange and C_4 in red. The vein density values (in mm/mm^2) come from McKown *et al.* (2007), the PEPC activities (in µmol/mg Chl*h) come from Bauwe (1984) and Sudderth *et al.* (2007) for *F. kochiana*, the percentages of carbon fixed to C_4 acids were summarized from various sources by Vogan & Sage (2011), the CO_2 compensation points come from Ku *et al.* (1991) and Sudderth *et al.* (2007) for *F. kochiana*, and the photosynthetic water-use efficiency (PWUE; in mmol CO_2/mol H_2O) and photosynthetic nitrogen-use efficiency (PNUE; in µmol CO_2/mmol N*s) come from Vogan and Sage (2011).

Figure 4. Ecological distribution of some C_4 taxa compared to their C_3 relatives.

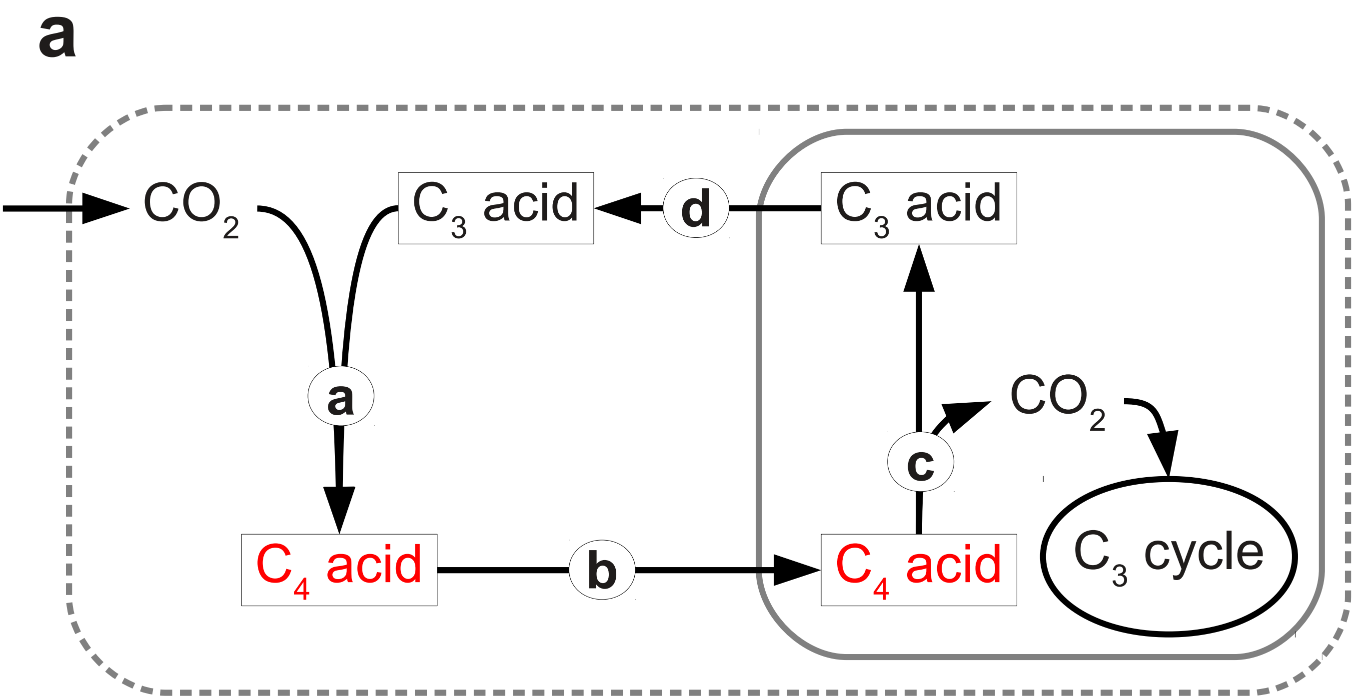
For two distantly related groups that contain C_4 taxa (grasses and Molluginaceae), the mean annual temperature (MAT; in ºC) is plotted against the mean annual precipitation (MAP; in mm year^{-1}). For grasses, environmental variables were extracted from Edwards & Smith (2010), with one point per species. For Molluginaceae, environmental variables were taken from Edwards & Ogburn (2012), with multiple localities per species. Grey points represent localities for C_3 species that belong to the sister-group of the clade with C_4 species (the BEP clade of grasses and the Portulacineae clade, respectively). Localities for C3 species that are closely related to C4 taxa are in black (C_3 grasses from the PACMAD clade and C_3 Molluginaceae, respectively), and those C_4 taxa in each group are in red.

Figure 5. Ecological diversity in C_3 and C_4 Paniceae.

The mean annual temperature (MAT; in ºC) and mean annual precipitation (MAP; in mm year^{-1}) were extracted from the ecological dataset of Edwards & Smith (2010) for those members of the grass tribe Paniceae that were also present in the time-calibrated phylogeny of Christin *et al.* (2013b). In the phylogenetic tree on the left, dots at the tips are coloured according to the species means for MAT on the left and MAP on the right. Branches are coloured based on photosynthetic types, with C_4 clades in red and C_3 branches in blue. The phylogenetic relationships are projected into climatic space on the right. For clarity, the lower part of the tree that includes the C_4 clades *Echinochloa* and *Alloteropsis* is presented independently from the upper part of the tree that includes the C_4 clade 'MCP' (Melinidinae, Cenchrinae and Panicinae; GPWGII, 2012). In the righthand panels, each segment connects the values estimated for two consecutive nodes in the phylogenetic tree (see Box 1). The blue point indicates the root (also indicated on the phylogeny), while tips are indicated by blue arrows when C_3 and red arrows when C_4. The major biomes are approximately delimited with dashed grey lines. They follow Ricklefs (2008), and are numbered in the lower panel; 1 = temperate rain forest, 2 = temperate deciduous forest, 3 = temperate grassland and desert, 4 = tropical rainforest, 5 = tropical seasonal forest, 6 = savanna, 7 = subtropical desert.

Figure 6: Examples of phylogenetic inference.

A. Hypothetical time-calibrated phylogenetic tree for a group of C_4 species nested within a C_3 clade. **B.** Hypothetical quantitative character mapped onto the tree using a maximum likelihood method. The estimated value for each node comes with confidence intervals, but only the optimum is presented as the dot size. **C.** Hypothetical binary character mapped on the tree using a maximum likelihood method. The likelihood of each state at each node is represented by pie charts. In the most parsimonious scenario, the origin of C_4 photosynthesis in this group could be estimated between time units 4 and 3 (bold branch, Figure 6A). The increase in the quantitative trait happened between time units 6 and 4 (bold branch, Figure 6B), before the change in the binary trait, which would be estimated between time units 4 and 3 based on a maximum likelihood model (bold dashed branch, Figure 6C) or between time units 3 and 1 based on a maximum parsimony approach (bold solid branch, Figure 6C).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C3 acid
d
C3 acid
a
CO2
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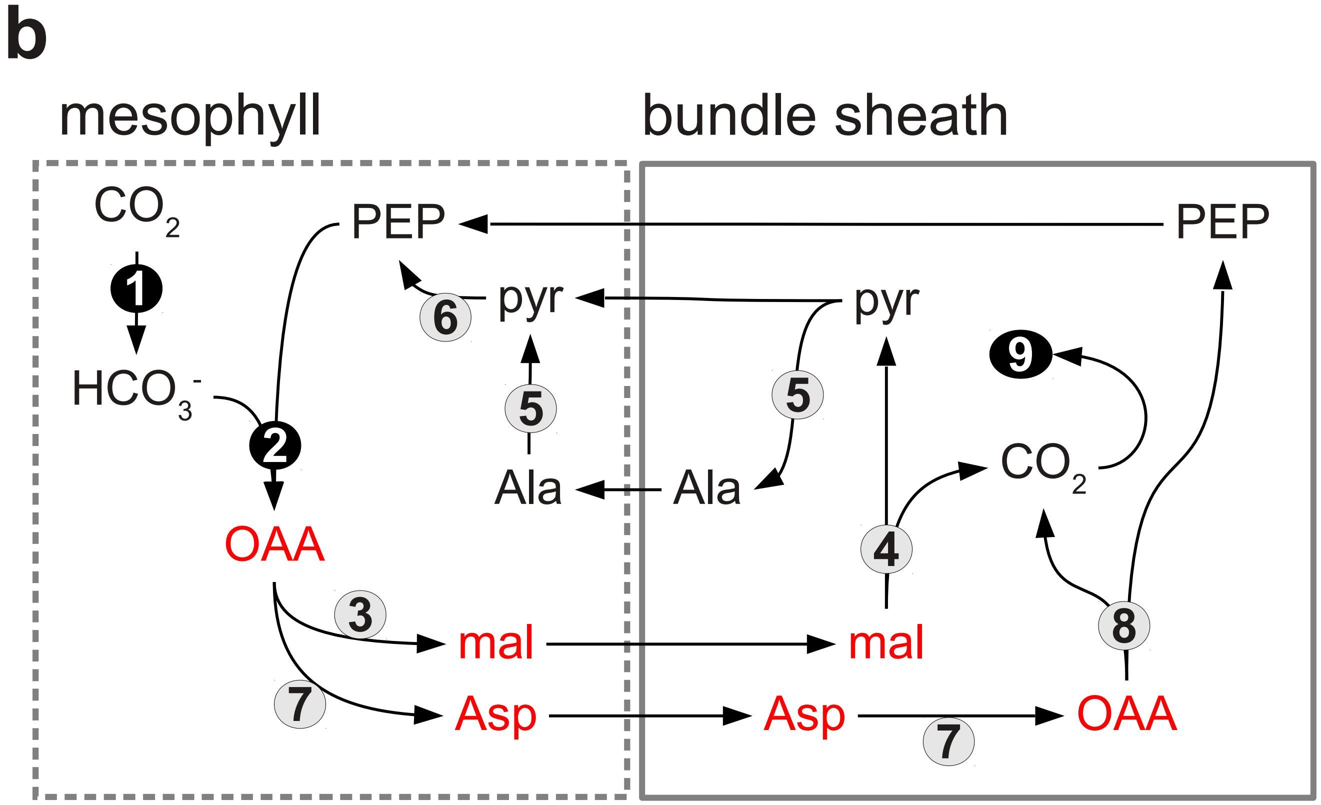
C4 acid
b
C4 acid


b
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bundle sheath
CO2
1
HCO3-
2
OAA
PEP
6
pyr
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mal
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Ala
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Asp
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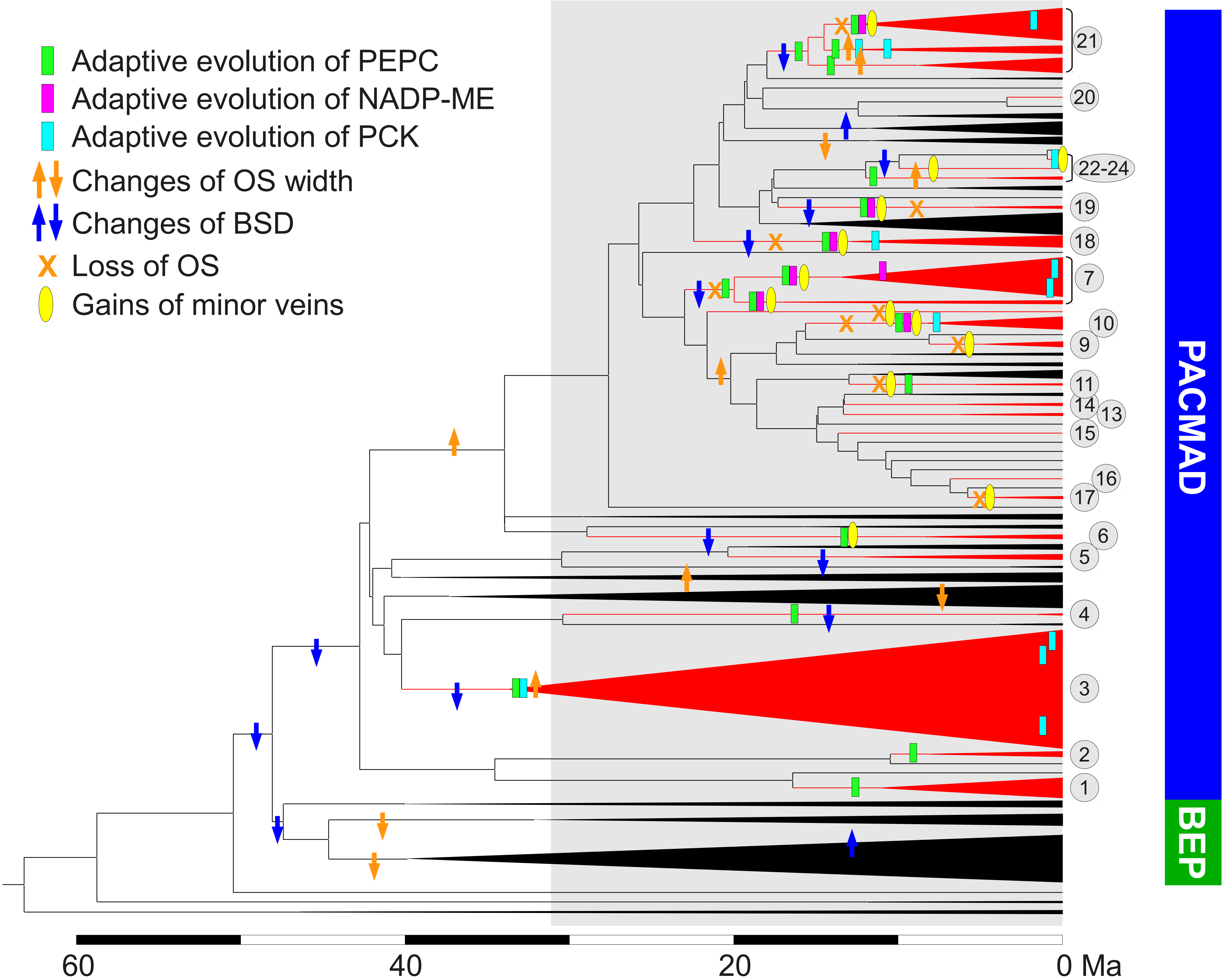
OAA


Adaptive evolution of PEPC
Adaptive evolution of NADP-ME
Adaptive evolution of PCK
Changes of OS width
Changes of BSD
Loss of OS
Gains of minor veins
21
20
22-24
19
18
7
10
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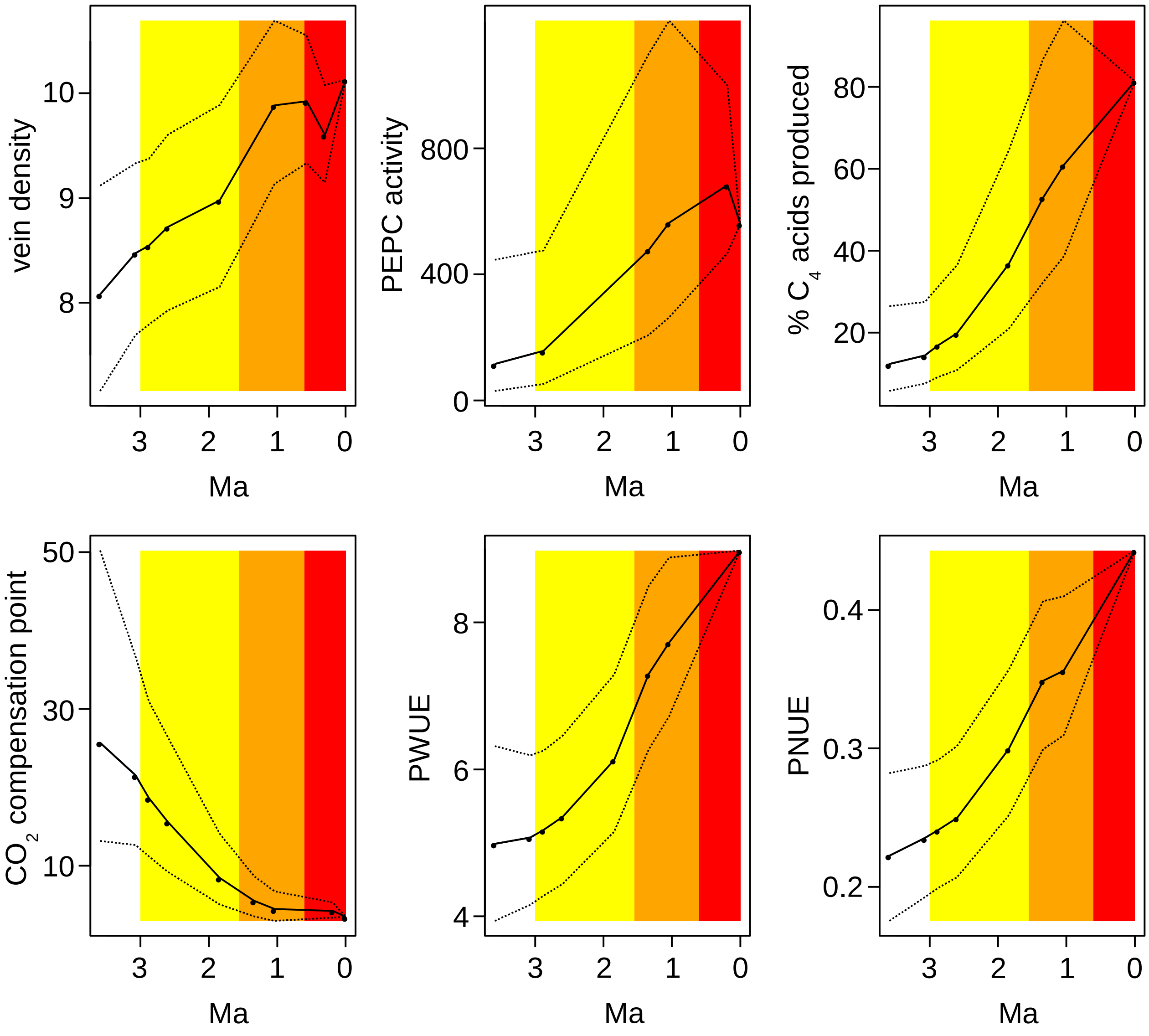
vein density
10
9
8
3
2
1
0
Ma
PEPC activity
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400
0
3
2
1
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Ma
% C4 acids produced
80
60
40
20
3
2
1
0
Ma
CO2 compensation point
50
30
10
3
2
1
0
Ma
PWUE
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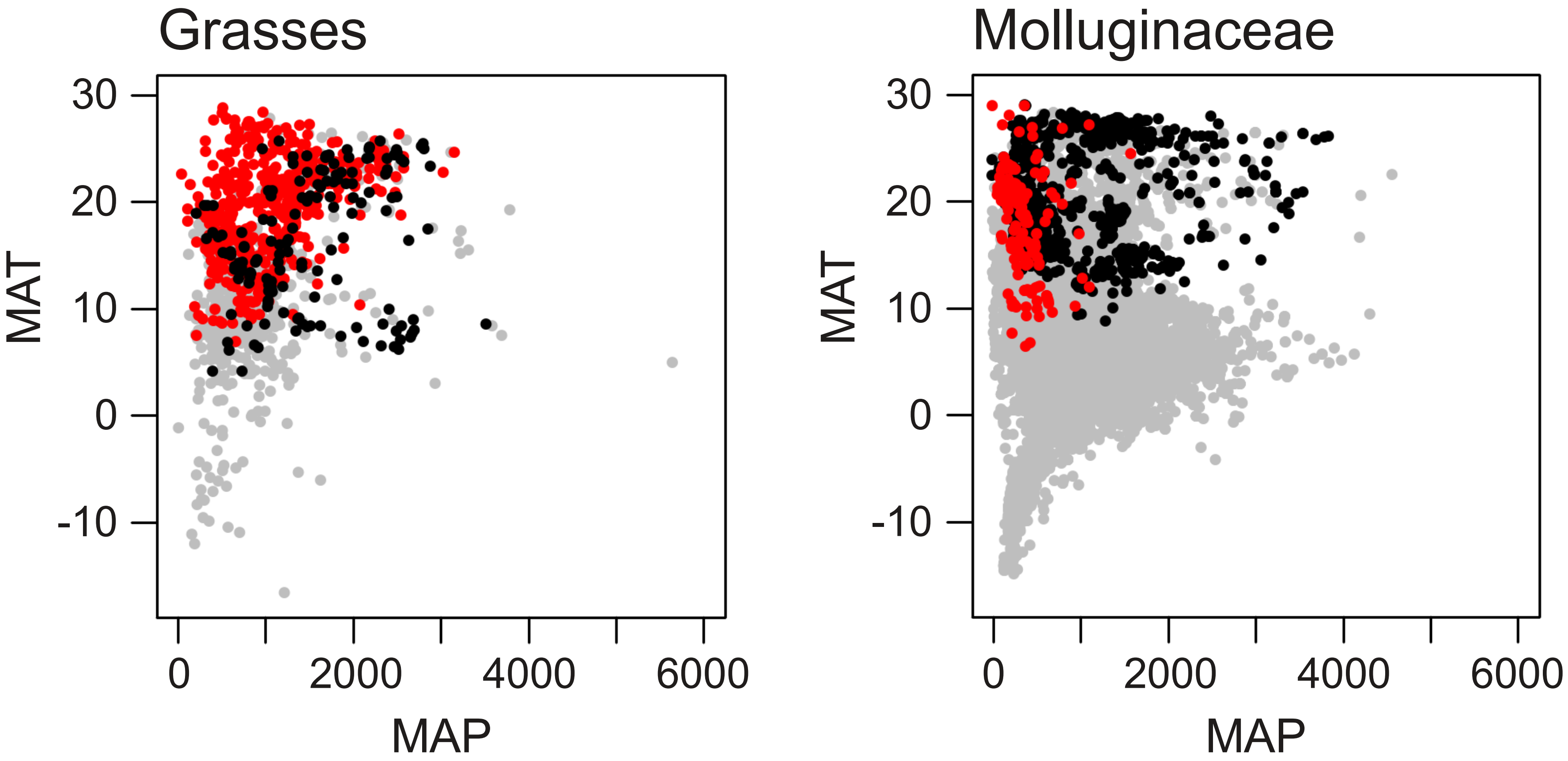
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-10
MAT
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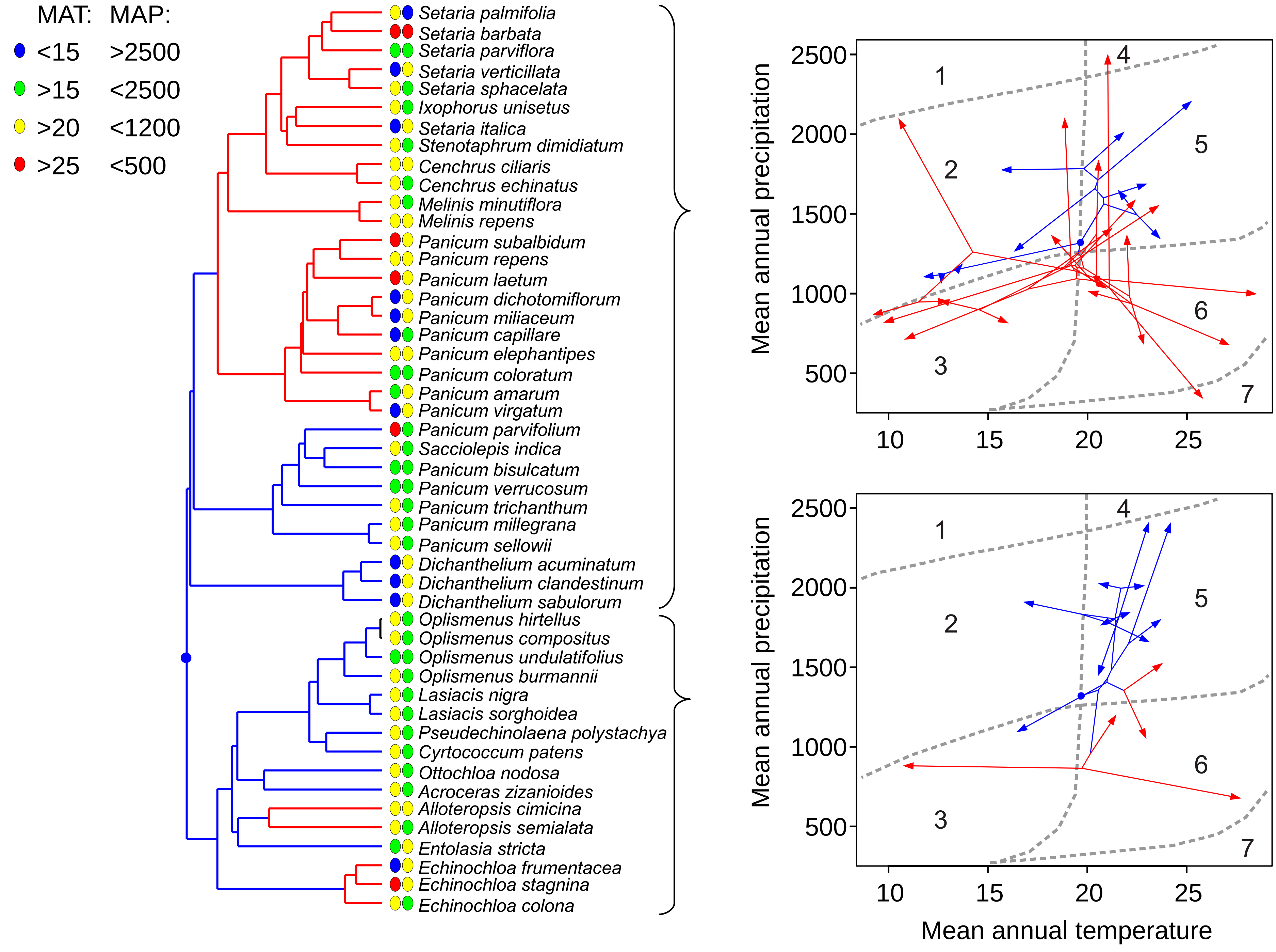
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>15 <2500
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>25 <500
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Setaria sphacelata
Ixophorus unisetus
Setaria italica
Stenotaphrum dimidiatum
Cenchrus ciliaris
Cenchrus echinatus
Melinis minutiflora
Melinis repens
Panicum subalbidum
Panicum repens
Panicum laetum
Panicum dichotomiflorum
Panicum miliaceum
Panicum capillare
Panicum elephantipes
Panicum coloratum
Panicum amarum
Panicum virgatum
Panicum parvifolium
Sacciolepis indica
Panicum bisulcatum
Panicum verrucosum
Panicum trichanthum
Panicum millegrana
Panicum sellowii
Dichanthelium acuminatum
Dichanthelium clandestinum
Dichanthelium sabulorum
Oplismenus hirtellus
Oplismenus compositus
Oplismenus undulatifolius
Oplismenus burmannii
Lasiacis nigra
Lasiacis sorghoidea
Pseudechinolaena polystachya
Cyrtococcum patens
Ottochloa nodosa
Acroceras zizanioides
Alloteropsis cimicina
Alloteropsis semialata
Entolasia stricta
Echinochloa frumentacea
Echinochloa stagnina
Echinochloa colona
Mean annual precipitation
2500
2000
1500
1000
500
1
2
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7
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15
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Mean annual precipitation
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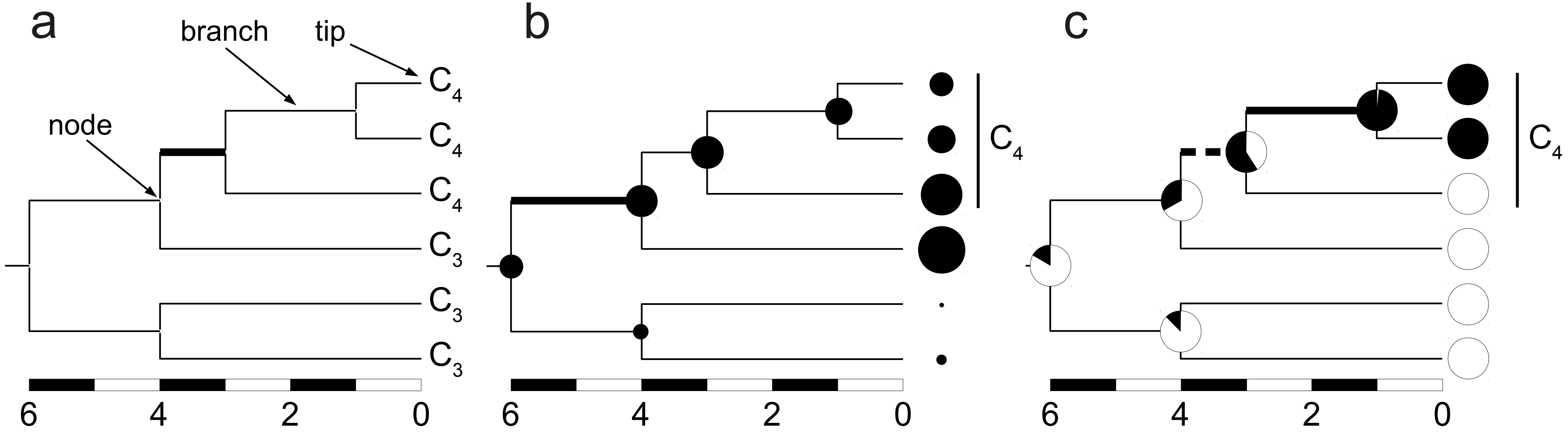
Mean annual temperature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