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RESEARCH PAPER

C_4 eudicots are not younger than C_4 monocots

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Abstract

C_4 photosynthesis is a plant adaptation to high levels of photorespiration. Physiological models predict that atmospheric CO_2 concentration selected for C_4 grasses only after it dropped below a critical threshold during the Oligocene (~30 Ma), a hypothesis supported by phylogenetic and molecular dating analyses. However the same models predict that CO_2 should have reached much lower levels before selecting for C_4 eudicots, making C_4 eudicots younger than C_4 grasses. In this study, different phylogenetic datasets were combined in order to conduct the first comparative analysis of the age of C_4 origins in eudicots. Our results suggested that all lineages of C_4 eudicots arose during the last 30 million years, with the earliest before 22 Ma in Chenopodiaceae and Aizoaceae, and the latest probably after 2 Ma in *Flaveria*. C_4 eudicots are thus not globally younger than C_4 monocots. All lineages of C_4 plants evolved in a similar low CO_2 atmosphere that predominated during the last 30 million years. Independent C_4 origins were probably driven by different combinations of specific factors, including local ecological characteristics such as habitat openness, aridity, and salinity, as well as the speciation and dispersal history of each clade. Neither the lower number of C_4 species nor the frequency of C_3–C_4 intermediates in eudicots can be attributed to a more recent origin, but probably result from variation in diversification and evolutionary rates among the different groups that evolved the C_4 pathway.

Key words: C_4 photosynthesis, eudicots, evolution, molecular dating, multiple origins, phylogeny.

Introduction

C_4 photosynthesis is a highly convergent trait that has evolved more than 60 times in at least 18 families of flowering plants (Sage *et al.*, 2011a). It consists of anatomical and biochemical innovations that increase the internal CO_2 concentration around the carboxylating enzyme Rubisco, thereby suppressing oxygenation of RuBP and photorespiration. C_4 photosynthesis thus provides an advantage in all conditions where photorespiration levels are deleteriously high, especially in warm, dry, and/or saline habitats (Sage, 2004). However, the energetic requirements of the C_4 pathway can represent a net cost in conditions where photorespiratory rates are low, most notably at low temperature or in atmospheres of elevated CO_2. One index that effectively describes the relationship between the efficiency of C_3 and C_4 photosynthesis and the environment is the maximum quantum yield of photosynthesis (QY) measured at low light intensities (Ehleringer *et al.*, 1997). Maximum QY (hereafter referred to as simply QY) is a measure of the maximum light use efficiency of photosynthesis, calculated as the number of CO_2 molecules fixed per absorbed photon. As an index of photorespiratory inhibition, QY is useful in C_3 and C_4 comparisons both at low light where variation in light use efficiency is directly proportional to carbon gain, and at high light, where QY is inversely proportional to the ratio of photorespiration to photosynthesis (Sage and Kubien, 2003). Due to the increase in photorespiration, QY in C_3 plants declines with decreasing CO_2 levels and increasing temperature. By contrast, C_4 QY is little affected by variation in CO_2 and temperature, but is below that of C_3 plants at high CO_2 and/or low temperature due to the two ATP equivalents

needed for each turn of the C_4 metabolic cycle (Ehleringer *et al.*, 1997).

Because of its simplicity and close relationship with the ratio of photorespiration to photosynthesis, QY has been widely used to compare C_3 and C_4 species across a range of environments, and to model conditions, both present and past, when the C_4 pathway would be advantageous over the C_3 pathway. Using a QY model, Ehleringer and co-workers (Ehleringer *et al.*, 1991, 1997; Cerling *et al.*, 1997) predicted that C_4 plants would have greater evolutionary fitness under the low CO_2 levels of recent geological time than C_3 species, leading to the hypothesis that low CO_2 was a selection factor for the rise of the C_4 pathway. With improved estimates of paleo atmospheric CO_2 and evidence of C_4 plants occurring in the Mid-Miocene, the date proposed for the earliest C_4 plants has been placed in the late Oligocene period, when global climate underwent important changes and atmospheric CO_2 levels fell to near or below current levels (Sage, 2001, 2004; Pagani *et al.*, 2005). This hypothesis has been empirically supported in C_4 monocots (grasses and sedges), where phylogenetic modelling indicates a marked increase in the probability of C_4 evolution after the Oligocene CO_2 decline (Christin *et al.*, 2008; Besnard *et al.*, 2009). It has not been tested in the numerous eudicot lineages with C_4 species, however. QY comparisons generally demonstrated lower values of light use efficiency in C_4 eudicots than C_4 monocots (Ehleringer and Pearcy, 1983). This observation led to the hypothesis that C_4 evolution in eudicots would have only occurred at lower atmospheric CO_2 levels than in monocots, constraining C_4 evolution in eudicots to the Pleistocene (Ehleringer *et al.*, 1997). This prediction can now be evaluated using phylogenetic information accumulated in the past 15 years for eudicot lineages where C_4 photosynthesis appears.

Phylogenetic data have increased exponentially in recent years, leading to a better understanding of the relationships between C_4 taxa and affiliated C_3 taxa. The grasses (Poaceae) have received the lion's share of phylogenetic attention and the number and timing of C_4 origins in this group are now relatively well understood, with a minimum of 17 C_4 origins, starting around 30 million years ago (Ma) and continuing to more recent geological time (Giussani *et al.*, 2001; Christin *et al.*, 2008; Vicentini *et al.*, 2008; Edwards *et al.*, 2010; Roalson, 2011). This pattern, along with considerations of the ecological setting for C_4 evolution, has led to the current view that Oligocene CO_2 decline met an essential environmental precondition for the evolution of C_4 photosynthesis, while individual C_4 origins were probably driven by additional local factors, such as warmth, seasonality, and habitat openness (Sage, 2001, 2004; Roalson, 2008; Osborne and Freckleton, 2009; Edwards and Smith, 2010). Besides grasses, five C_4 origins in Cyperaceae (sedges) were estimated to have occurred between 20 and 4 Ma (Besnard *et al.*, 2009; Roalson, 2011). The C_4 origins in sedges thus also occurred in a low CO_2 world, perhaps as a response to increasing disturbance and fire frequencies in the wetter parts of the warm biomes (Linder and Rudall, 2005). Together, sedges and grasses encompass about 80% of all C_4 species, but represent only a minority of C_4 origins, the majority of which (about 60%) occurred in eudicots (Sage *et al.*, 2011a). Some of these eudicot groups serve as model systems for the study of C_4 genetics and evolution, including members of the Cleomaceae, Amaranthaceae, and, especially, *Flaveria* in the Asteraceae (Kadereit *et al.*, 2003; Svensson *et al.*, 2003; Brown *et al.*, 2005; McKown *et al.*, 2005; McKown and Dengler, 2007). It has long been postulated that C_4 eudicots were much younger than C_4 monocots, based mainly on the physiological models described above, but also on the presence of C_3–C_4 intermediates and the lower number of C_4 species in eudicot groups (Ehleringer *et al.*, 1997; Sage, 2004). Some well-resolved and relatively well-sampled phylogenies are now available for several groups of C_4 eudicots (McKown *et al.*, 2005; Kapralov *et al.*, 2006; Sage *et al.*, 2007; Feodorova *et al.*, 2010; Kadereit *et al.*, 2010; Ocampo and Columbus, 2010; Christin *et al.*, 2011). However, accurate time calibration of these phylogenies has been hampered by the lack of fossil records for the studied groups.

The first attempt to date C_4 eudicots used a phylogeny based on *rbcL* for Amaranthaceae/Chenopodiaceae, which suggested that the first C_4 origins in this group could have occurred more than 14.5 Ma (Kadereit *et al.*, 2003), challenging the hypothesis of a Pleistocene origin of C_4 eudicots. This potential early origin of C_4 Amaranthaceae/Chenopodiaceae has been confirmed by more densely sampled phylogenetic analyses (Kadereit *et al.*, 2010; Kadereit and Freitag, 2011). Very recently, two more studies have used time estimates obtained for the major angiosperm lineages to calibrate phylogenies for Molluginaceae and Cleomaceae (Feodorova *et al.*, 2010; Christin *et al.*, 2011). Surprisingly, these C_4 groups with very few species (two, and less than five, respectively) were found to be up to 10 million years (My) old. These new lines of evidence demonstrate the need for a re-evaluation of the timing of C_4 evolution in eudicots and their relationship with past fluctuation of atmospheric CO_2 levels.

The goal of the present study was to estimate the potential ages of the different eudicot C_4 lineages described in the literature. First, a phylogenetic tree containing the major eudicot clades was inferred and dated and then these time estimates were used to calibrate a series of smaller and more detailed phylogenies inferred from fast-evolving markers. The resultant time-calibrated phylogenetic framework contains many of the postulated C_4 origins in eudicots and is used to address variation in the probability of C_4 evolution through geological time. This study also identified groups for which phylogenetic information is limited or completely absent, but are critical for understanding the timing, evolvability, and reversibility of transitions between C_3 and C_4 photosynthesis in angiosperms.

Materials and methods

General methodology and eudicot phylogeny

The GenBank database was screened for genes available for different C_4 lineages (according to Sage *et al.*, 2011a). Non-coding

genes, such as nuclear internal transcribed spacers (ITS) and intergenic spacers from the plastid genome, are available for numerous C_4 groups. Unfortunately, these fast-evolving markers cannot be unambiguously aligned among distantly related plant groups. Therefore, slower-evolving coding markers were selected that allowed the largest number of C_4 taxa to be incorporated. The two markers settled upon were genes from the plastid genome encoding *matK* and *rbcL*. They have been used in various attempts to reconstruct the angiosperm phylogeny (Soltis *et al.*, 2000; Hilu *et al.*, 2003) and are relatively well sampled for several groups that contain C_3/C_4 transitions (Cuénoud *et al.*, 2002; Kadereit *et al.*, 2003; Sage *et al.*, 2007; Christin *et al.*, 2011). These two markers were retrieved for species spanning the main eudicot lineages, the purported sister group of eudicots (*Ceratophyllum*) and one monocot, which was used to root the tree (*Acorus*). Clades previously reported to contain C_4 taxa were densely sampled and, where possible, the closest C_3 relatives of each C_4 group was included in order to improve the estimates of divergence times. This sampling was completed by *de novo* sequencing of *matK* and *rbcL* for several Caryophyllales species in order to improve the resolution of the phylogeny and, in particular, the relationships between C_3 and C_4 taxa (see Supplementary Document S1 at *JXB* online). These markers were amplified and sequenced with the primers developed in Christin *et al.* (2011), following the procedure described therein.

Sequences were aligned with ClustalW (Thompson *et al.*, 1994) and the alignment was then manually edited. A phylogenetic tree was inferred simultaneously from *rbcL* and *matK* through a Bayesian procedure implemented in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). The substitution model used for the analyses was a general time-reversible model with a gamma shape parameter and a proportion of invariant sites (GTR+G+I). Two analyses, each consisting of four parallel chains, were run for 7 000 000 generations after a burn-in period of 3 000 000, and a tree was sampled each 1000 generations. The consensus phylogeny resulting from the 14 000 sampled trees was used for molecular dating with a Bayesian method that accounts for changes in rates of evolution among branches, following the recommendations of Rutschmann (2006). Model parameters were estimated with baseml (Yang, 2007) for the two genes separately. Branch lengths and the variance–covariance matrix were then optimized using estbranches (Thorne *et al.*, 1998). A Bayesian MCMC procedure implemented in multidivtime (Kishino *et al.*, 2001; Thorne and Kishino, 2002) approximated the posterior distributions of substitution rates and divergence times, given a set of time constraints. The MCMC procedure was run for 1 000 000 generations after a burn-in of 100 000 generations, with a sampling frequency of 100 generations. The outgroup (*Acorus americanus*) was removed during the analysis. Calibration points, based on reported fossils, were set as described in Christin *et al.* (2011), with lower bounds on the stems of Buxales at 102.2 Ma, of Malpighiales at 91.2 Ma, of Fabales at 59.9 Ma, of Malvales at 69.7 Ma, of Myrtales at 88.2 Ma, of Ericales at 91.2 Ma, a lower bound on the divergence of Polycarpon from the higher Caryophyllaceae at 34 Ma, a lower bound on the stem of eudicots at 120 Ma and an upper bound on the crown of eudicots at 130 Ma.

This eudicot phylogeny gave relatively good estimates for several C_4 origins, but many groups were poorly represented. For this reason, additional phylogenies were reconstructed with fast-evolving markers for several groups containing C_4 taxa. Since good fossils are not available for most groups at this taxonomic scale, time estimates for nodes of the eudicot phylogeny, together with their associated confidence interval, were used to calibrate these lower scale phylogenies. With this approach, the ages estimated for the different groups are not independent, increasing the probability of accurate estimates of the relative ages, even in the presence of misleading calibration points. For constrained nodes, the lower bound was set to the estimated divergence time minus the standard deviation and the upper bound to the estimated divergence time plus the standard deviation. All trees were inferred and calibrated with the method described above. Specific details for each group are detailed below.

To account for recent suggestions that angiosperms could be older than previously thought (Smith *et al.*, 2010), dating of the eudicot tree was repeated by removing the upper constraint on the crown of eudicots and setting the maximal age of the root (eudicots+*Ceratophyllum*) to 200 Ma. Divergence times estimated in this analysis were then used to calibrate lower taxonomic scale phylogenies under the hypothesis of an earlier origin of angiosperms.

Detailed analyses of selected clades

The phylogeny for *Flaveria* was reconstructed using the nuclear ITS and the plastid intergenic spacer *trnL-trnF*. Sequences for *Flaveria* were extracted from the dataset of McKown *et al.* (2005) and other Asteraceae were added to allow for more calibration points. Only one accession per *Flaveria* species was considered. *Scaevola aemula* (Goodeniaceae) was used as the outgroup (removed during the dating analysis). The inferred phylogeny was calibrated with the estimated divergence times between Cichorioideae (represented by *Lactuca* in the eudicot phylogeny) and Asteroideae (i.e. *Helianthus* and *Flaveria*) and between *Helianthus* and *Oyedaea*. The photosynthetic types were determined by McKown *et al.* (2005) and McKown and Dengler (2007).

A phylogeny for the Chenopodiaceae was inferred from nuclear ITS and the non-coding plastid marker *psbB-psbH*, from data originating from previous studies (Kapralov *et al.*, 2006; Akhani *et al.*, 2007; Wen *et al.*, 2010). The phylogeny was rooted with Chenopodioideae (*Atriplex* and *Chenopodium*, removed during the dating analysis), according to previous phylogenies (Kadereit *et al.*, 2003; Kapralov *et al.*, 2006). It was calibrated with the estimated divergence time between Salsoloideae (*Salsola*, *Camphorosma*, and *Bassia*) and Suaedoideae/Salicornioideae (*Bienertia*, *Allenrolfea*, and *Halocnemum*). Photosynthetic types were reported from the literature (Jacobs, 2001; Kadereit *et al.*, 2003, and references therein, 2010; Kapralov *et al.*, 2006; Akhani *et al.*, 2007).

A phylogenetic tree for C_4 and related C_3 Nyctaginaceae was inferred from four markers, the nuclear ITS, the plastid coding gene *ndhF*, and the plastid non-coding genes *rpl16* and *rps16*. These data come from a densely sampled phylogenetic dataset by Douglas and Manos (2007). The phylogenetic tree was rooted with *Colignonia scandens*, according to Douglas and Manos (2007). It was calibrated with the estimated divergence times between *Bougainvillea glabra* and the other species and between the *Mirabilis* genus and the group composed of *Allionia*/*Okenia*. Photosynthetic types were deduced from carbon isotope ratio measurements (RF Sage, unpublished data).

A phylogeny was inferred using ITS for Sesuvioideae (data generated by Hassan *et al.*, 2005), other Aizoaceae, and Montiaceae (used as the outgroup, removed in the dating analysis). The resulting phylogeny was calibrated using the estimated divergence time between *Galenia*+*Delosperma*+*Trichodiadema* and Sesuvioideae and the estimated divergence time between *Galenia* and *Delosperma*+*Trichodiadema*. Photosynthetic types were reported from previous studies (Sankhla *et al.*, 1975; Kocacinar and Sage, 2003; RF Sage, unpublished data).

GenBank was screened for markers representing *Euphorbia* species previously identified as C_4. ITS was selected as it was available for a large number of C_4 as well as C_3 *Euphorbia*. *Euphorbia* species spread across the phylogeny were selected together with *Manihot esculenta* and *Jatropha curcas* (used as the outgroup, removed during the dating analysis). The resulting phylogeny was calibrated with the estimated divergence time between *Manihot esculenta* and the *Euphorbia* genus. Photosynthetic types were reported from the literature (Pearcy and Troughton, 1975; Webster *et al.*, 1975; Batanouny *et al.*, 1991; Sage *et al.*, 2011b).

Modelling of photosynthetic transition probabilities

Two models of transition between C_3 and C_4 photosynthesis were optimized on the eudicot phylogeny inferred from *rbcL* and *matK*, with branch lengths estimated from molecular markers and incorporating the estimated divergence times. The null model allows different transition rates from C_3 to C_4 and from C_4 to C_3, but these rates are constant through time. The alternative model allows for one change in the transition rates, independently optimizing transition probabilities between C_3 and C_4 before and after a given time threshold (Christin *et al.*, 2008). The two models are nested and can be compared through likelihood ratio tests. Species were coded as C_4 or C_3 (including C_3–C_4 intermediates) and all parameters were estimated from the data, using MLtree software (Christin *et al.*, 2008).

Results

Eudicot phylogeny

The phylogeny inferred from *matK* and *rbcL* (see Supplementary Fig. S1A, B at *JXB* online) is congruent with the known relationships among angiosperms (APG III, 2009; Brockington *et al.*, 2009), and our age estimates for major clades are consistent with recent estimates (see Supplementary Fig. 1A at *JXB* online; Bell *et al.*, 2010; Moore *et al.*, 2010). However, to account for recent suggestions that angiosperms could be older than previously thought (Smith *et al.*, 2010), the dating analysis was repeated with relaxed constraints on the maximal age of the eudicots and the root. Removing these constraints affected the estimated ages of the major clades, but did not significantly change those for nodes closer to the tips, which included all potential C_4 origins (see Supplementary Table S1 at *JXB* online).

Our sampling of *rbcL* and *matK* incorporated C_4 taxa forming a total of 31 C_4-like or C_4 groups (Table 1). Of the 36 C_4 lineages of eudicots hypothesized by Sage *et al.* (2011a), eight are missing because data in GenBank were insufficient (*Blepharis*, *Anticharis*, *Heliotropium*, *Pectis*, *Chrysanthellum*/*Isostigma*, *Polycarpaea*, and two C_4 groups of *Cleome*). The two C_4 *Atriplex*, for which only *rbcL* was available, were not monophyletic, congruent with previous analysis of this marker (Kadereit *et al.*, 2003). However, more recent analyses with other markers and a denser sampling have shown the monophyly of the C_4 *Atriplex* (Kadereit *et al.*, 2010). All other relationships are perfectly congruent with previous phylogenies for C_4 eudicots (Kadereit *et al.*, 2003; Kapralov *et al.*, 2006; Akhani *et al.*, 2007; Sage *et al.*, 2007; Christin *et al.*, 2011).

For each C_4 group, C_4 photosynthesis could have originated at any time between the divergence of the C_4 group and its C_3 sister group (stem group node), and the earliest evidence of diversification within the C_4 group (crown group node; Table 1). Thus, the limited species sampling in our *rbcL*/*matK* tree produces large uncertainties about the timing of some C_4 origins. In addition, many groups are only represented by a single species, which makes it impossible to define a minimum age bracket with a crown group. The upper estimate for the C_4 origin in *Portulaca* is at 28.8 Ma (with a minimum age for this origin at 9.7 Ma; Table 1). Dates for the Portulacineae suborder (which contains *Portulaca*) are older than in a previous study that calibrated the phylogeny with the appearance of the Hawaiian islands (Ocampo and Columbus, 2010). C_4 photosynthesis could be 63.5 My old in the *Tribulus* group (with a minimum age of 13.8 Ma), but the only markers available for these species were *rbcL* or a small fragment of *matK*, decreasing the accuracy of the age estimation and leading to very wide confidence intervals (Table 1). All other C_4 origins are estimated to have occurred during the last 30 My (Table 1).

Detailed analyses of selected clades

According to our time estimates, the appearance of C_3–C_4 photosynthesis in *Flaveria* occurred either once between 3.6 and 3.1 Ma (with a reversal in *F. robusta*) or twice, between 2.8 Ma and the present in *F. sonorensis,* and between 3.1 and 2.9 in the common ancestor of clades A and B (see Supplementary Fig. S1C at *JXB* online). This C_3-C_4 type was co-opted twice to evolve a C_4-like trait, between 0.4 Ma and the present in *F. brownii* and between 1.8 and 1.3 Ma in clade A (Fig. 1). The number of C_4 origins from the C_4-like type are difficult to infer, since different scenarios are equally probable; in all cases, they occurred in the last 2 My (Table 1).

The reconstruction of C_3/C_4 transitions is highly ambiguous in the inferred Chenopodiaceae phylogeny and the most parsimonious scenarios would imply C_4 to C_3 reversals (see Supplementary Fig. S1D at *JXB* online). Such reversions have been hypothesized (Pyankov *et al.*, 2001), but other studies concluded that the family contains mainly C_4 origins based on anatomical variation (Kadereit *et al.*, 2003, 2010; Kadereit and Freitag, 2011). According to our estimate, the evolution of single-celled C_4 photosynthesis (Edwards *et al.*, 2004) occurred between 20.8 and 2.6 Ma in *Bienertia* and between 7.7 Ma and the present in *Suaeda aralocaspica* (Table 1). If two transitions from C_3 to C_4 are assumed in *Suaeda*, they would have occurred between 9.9 and 7.0 Ma and between 5.6 and 4.5 Ma, respectively. In Salsoloideae, a single origin followed by reversals would be estimated between 28.3 and 26.2 Ma. In the case of multiple C_4 origins in Salsoloideae, these would be spread across the last 25 My (Table 1). The oldest lower estimate for a C_4 origin in the eudicots is in the Caroxyloneae at 22.1 Ma. Dates for C_4 Chenopodiaceae are slightly younger than previous estimates based on phylogenetic trees encompassing only Amaranthaceae/Chenopodiaceae (Kadereit *et al.*, 2003, 2010; Kadereit and Freitag, 2011).

In Nyctaginaceae, C_4 taxa form two groups (see Supplementary Fig. S1E at *JXB* online). If they correspond to two C_4 origins, the *Allionia* lineage evolved the C_4 pathway between 6.1 Ma and the present, while C_4 appeared between 4.7 and 2.2 Ma in the *Boerhavia*/*Okenia* group (Table 1).

The evolutionary history of photosynthetic types in Sesuvioideae is difficult to reconstruct (see Supplementary Fig. S1F at *JXB* online). The observed distribution of C_4 taxa is compatible with a single C_4 origin, which would

Table 1. Estimated ages (in millions of years, with standard deviation in brackets) of stem and crown nodes for the different photosynthetic transitions, assuming no reversal

Clade[a]	Transition	Stem age	Crown age	Remarks
Portulacaceae				
Portulaca	C_3 to C_4	28.8 (5.0)	9.7 (3.3)	For details, see Ocampo *et al.* (2010)
Molluginaceae				
Mollugo cerviana/ *Hypetertelis spergulacea*	C_3 to C_3–C_4	17.1 (3.5)	7.0 (2.0)	For details, see Christin *et al.* (2011)
Mollugo cerviana group	C_3–C_4 to C_4	7.0 (2.0)	0.5 (0.4)	For details, see Christin *et al.* (2011)
Mollugo fragilis group	C_3–C_4 to C_4	5.3 (1.6)	1.4 (0.6)	For details, see Christin *et al.* (2011)
Gisekiaceae				
Gisekia	C_3 to C_4	4.8 (3.6)	0	
Amaranthaceae				
Gomphreneae	C_3 to C_4	8.6 (2.5)	7.0 (2.2)	
Tidestromia	C_3 to C_4	13.0 (3.3)	3.2 (1.7)	
Alternanthera	C_3 to C_3–C_4	10.3 (2.9)	7.5 (2.4)	
Alternanthera	C_3–C_4 to C_4	7.5 (2.4)	5.9 (2.1)	
Aerva	C_3 to C_4	14.5 (3.7)	2.5 (1.5)	
Amaranthus	C_3 to C_4	15.4 (4.1)	1.8 (1.2)	
Chenopodiaceae				
Atriplex	C_3 to C_4	12.9 (2.4)	4.7 (2.3)	Recent evidence suggests one C_4 origin in *Atriplex* 14.1-10.9 Mya (Kadereit *et al.*, 2010)
Suaeda sect. *Salsina*	C_3 to C_4	9.9 (3.1)	7.0 (2.4)	
Suaeda sect. *Schoberia*	C_3 to C_4	5.6 (2.1)	4.5 (1.8)	
Bienertia	C_3 to single-celled C_4	20.8 (3.9)	2.6 (2.0)	
Suaeda aralocaspica	C_3 to single-celled C_4	7.7 (3.2)	0	
Halosarcia indica	C_3 to C_4	6.5 (3.9)	0	
Camphorosmeae	C_3 to C_4	16.7 (4.6)	13.1 (4.1)	Recent evidence suggests two C_4 origins, 21.3-8.3 and 13.8-9.8 Mya (Kadereit and Freitag, 2011)
Caroxyloneae	C_3 to C_4	24.6 (3.4)	22.1 (3.6)	
Salsola kali group	C_3 to C_4	22.9 (3.5)	17.9 (4.0)	
Halothamnus	C_3 to C_4	21.2 (3.5)	7.2 (3.3)	
Haloxylon/*Anabis*	C_3 to C_4	13.7 (2.9)	12.7 (2.8)	
Nyctaginaceae				
Boerhavia	C_3 to C_4	4.7 (0.9)	2.2 (0.5)	
Allionia	C_3 to C_4	6.1 (1.0)	0	
Aizoaceae				
Trianthema	C_3 to C_4	22.1 (4.9)	20.2 (4.7)	Postulating all *Trianthema* are C_4
Zaleya	C_3 to C_4	20.1 (4.8)	4.2 (3.4)	Postulating all *Zaleya* are C_4
Sesuvium	C_3 to C_4	4.8 (3.3)	0	
Cypselea	C_3 to C_4	11.3 (4.1)	0	
Polygonaceae				
Calligonum	C_3 to C_4	19.9 (5.2)	1.2 (1.1)	
Asteraceae				
Flaveria	C_3 to C_3–C_4	3.1 (0.9)	2.9 (0.8)	
Flaveria sonorensis	C_3 to C_3–C_4	2.8 (0.8)	0	
Flaveria brownii	C_3–C_4 to C_4-like	0.4 (0.2)	0	
Flaveria clade A1	C_3–C_4 to C_4-like	1.8 (0.6)	1.3 (0.5)	
F. campestris	C_4-like to C_4	0.5 (0.3)	0	
F. trinervia group	C_4-like to C_4	1.0 (0.4)	0.2 (0.2)	
F. bidentis	C_4-like to C_4	0.6 (0.3)	0	
F. kochiana	C_4-like to C_4	0.3 (0.2)	0	
Cleomaceae				
Cleome gynandra	C_3 to C_4	17.7 (4.1)	0	Recent evidence suggests five C_4 origins in *Cleome* in the last 10 My (Feodorova *et al.*, 2010)

Table 1. *Continued*

Clade[a]	Transition	Stem age	Crown age	Remarks
Zygophyllaceae				
Zygophyllum	C_3 to C_4	8.6 (5.3)	0	
Tribulus	C_3 to C_4	63.5 (7.6)	13.8 (7.2)	
Euphorbiaceae				
Chamaesyce	C_3 to C_4	10.4 (2.7)	7.4 (2.2)	

[a] Clade names follow Sage *et al.* (2011a).

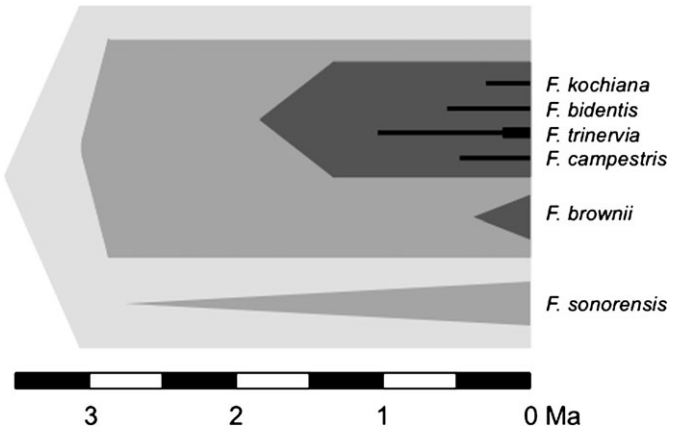

Fig. 1. Temporal representation of the gradual C_3 to C_4 transition in *Flaveria*. According to McKown and Dengler (2007), the ancestor of the *Flaveria* group evolved increased vein density that represent a C_4 precondition (light grey). This was co-opted twice to evolve C_3-C_4 photosynthesis (grey), which in turn was co-opted twice to evolve a C_4-like trait (dark grey). The C_4-like ancestors, in turn, gave rise to multiple C_4 descendants (black). Note that this scenario assumes strictly directional C_3 to C_4 transitions but reversals between the different states cannot be excluded. For each group, triangles or thin bars represent the interval between the stem and crown nodes (interval in which the trait could have evolved).

have occurred between 29.5 and 22.1 Ma, followed by two reversals to a C_3 state. Alternatively, in the case of multiple C_4 origins, the *Trianthema* genus would have acquired its C_4 trait between 22.1 and 20.2 Ma and the other genera during the last 20 My (Table 1). In either scenario, C_4 photosynthesis in Sesuvioideae is at least 20 My old.

All the *Euphorbia* species reported in the literature as C_4 and for which genetic data were available in GenBank form a single clade in the phylogeny (see Supplementary Fig. S1G at *JXB* online), sister to a group that contains C_3 and C_3-C_4 taxa (Sage *et al.*, 2011*b*). According to our estimates, C_4 photosynthesis originated between 10.4 and 7.4 Ma in this group (Table 1).

Modelling of photosynthetic transition rates

The model allowing variation of transition rates through time was marginally better than the null model (log-likelihood=-86.65; χ^2=7.49; df=3; P value=0.058). The significance of this alternative model should be re-evaluated when more data are available, but the present result, with limited taxon sampling, suggests that transition probabilities were not constant through time. The optimal threshold for the change of transition probabilities was at 28.8 Ma (Fig. 2), essentially identical to what was estimated for grasses (27.6 Ma; Christin *et al.*, 2008). According to this model, the probability of C_4 evolution was almost zero before 28.8 Ma, but strongly increased after this threshold. After 28.8 Ma, the probability of C_4 to C_3 reversal was positive in eudicots while it was null in grasses (Christin *et al.*, 2008).

Discussion

Timing of C_4 origins in the eudicots

C_4 eudicots have been proposed to be of recent origin compared to C_4 monocots (grasses and sedges), based on arguments such as a low number of C_4 species and the existence of numerous C_3–C_4 intermediates (Ehleringer *et al.*, 1997; Kellogg, 1999; Sage, 2004). A combination of physiological models and estimations of past climatic conditions even led to suggestions of a Pleistocene origin of C_4 eudicots (Ehleringer *et al.*, 1997). While our phylogenetic sampling was limited, which hampered an accurate estimate of the timing of C_4 origins in several eudicot lineages, narrow confidence intervals were still obtained for others. Our analysis provided little evidence for Pleistocene origins of C_4 photosynthesis in the eudicots. The only C_4 origin for which the maximal bound was inferred in the Pleistocene is *Flaveria* (Table 1). Several origins, within *Portulaca*, *Euphorbia*, and most C_4 groups of Aizoaceae, Amaranthaceae, and Chenopodiaceae, are estimated to have occurred in the Miocene or even the late Oligocene, supporting previous estimates (Kadereit *et al.*, 2003, 2010; Ocampo and Columbus, 2010). Excluding the Chloridoideae subfamily of grasses (first estimated C_4 origin 32–25 Ma; Christin *et al.*, 2008), C_4 origins in monocots are also spread throughout the last 25 My and largely overlap with C_4 origins in eudicots (Fig. 3). C_4 eudicots should thus no longer be considered young compared to monocots.

The contemporaneous nature of C_4 monocots and eudicots emphasizes that neither the number of species nor the presence of C_3–C_4 intermediates accurately predicts the age of the different C_4 groups. A recent study suggests that C_3–C_4 intermediacy has been evolutionarily stable for more

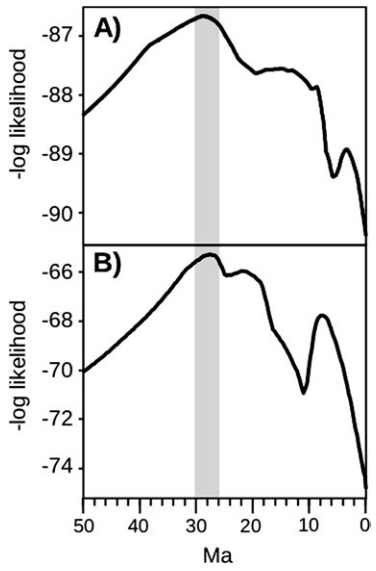

Fig. 2. Comparison of transition models for eudicots and grasses. The likelihood of the model allowing different rates of C_3/C_4 transitions after a time threshold is presented for all possible thresholds between 50 and 0 million years ago (Ma), for (A) eudicots and (B) grasses. The graph for grasses was redrawn from Christin *et al.* 2008, and reprinted by kind permission of Elsevier Ltd © 2008. The vertical grey bar shows the overlap between the optima for grasses and eudicots.

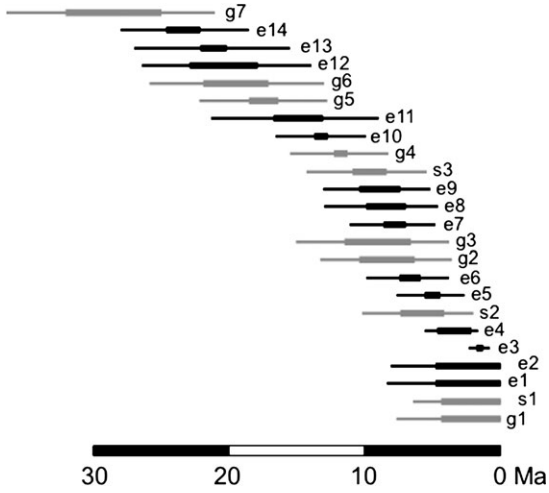

Fig. 3. Comparison of the ages of C_4 monocots and C_4 eudicots. The estimated age is indicated for each C_4 origin where the interval between the stem and crown nodes is smaller than 5 My, and for the putative oldest C_4 origin in the subfamily Chloridoideae of grasses. For each group, thick bars represent the interval between stem and crown nodes, and thin bars the confidence interval. Monocots are in grey and eudicots in black. For *Flaveria*, only one of the transitions to the C_4-like state is represented. Ages of monocots are based on Christin *et al.* (2008) and Besnard *et al.* (2009). Names on the right are numbered for eudicots, sedges, and grasses; e1, *Gisekia*; e2, *Sesuvium sesuvioides*; e3, C_4 and C_4-like *Flaveria*; e4, *Boerhavia/Okenia*; e5, *Suaeda* sect. *Schoberia;* e6, C_4 *Alternanthera*; e7, Gomphreneae; e8, *Suaeda* sect. *Salsina;* e9, *Chamaesyce*; e10, *Haloxylon/Anabasis;* e11, Camphorosmeae; e12, Kali; e13, *Trianthema*; e14 Caroxyloneae; s1, *Eleocharis vivipara*; s2, C_4 *Rhynchospora*; s3, C_4 Cypereae; g1, *Neurachne munroi*; g2, *Panicum prionitis* group; g3, *Eriachne*; g4, *Mesosetum* clade; g5, main C_4 Paniceae; g6, Andropogoneae; g7, Chloridoideae.

than 20 My in some Molluginaceae (Christin *et al.*, 2011) and, according to our present results, the C_3–C_4 type evolved at least 7.7 Ma in *Alternanthera* (Table 1). With regard to the species richness of various C_4 clades, the *Trianthema* genus contains fewer than 20 species, but could have appeared more than 20 Ma. Conversely, *Cyperus* and affiliated taxa in sedges acquired the C_4 pathway at less than 11 Ma but encompass more than 550 C_4 species (Bruhl and Wilson, 2007; Besnard *et al.*, 2009). Thus, there appears to be no relationship between the age of a C_4 clade and the number of species it contains. The larger number of C_4 species in monocots may instead be the consequence of grass and sedge families being prone to high diversification rates, since both lineages also contain highly diversified and widespread groups of C_3 species (Bruhl and Wilson, 2007; Edwards and Smith, 2010).

Ecological drivers of C_4 photosynthesis in eudicots

Past variations of atmospheric CO_2 levels have long been viewed as instrumental in driving the evolution of C_4 photosynthesis (Ehleringer *et al.*, 1991, 1997; Sage, 2004). Our results provide further evidence in support of this hypothesis; however, the proposal that C_4 eudicots evolved in the Pleistocene, only after CO_2 fell to lower levels than promoted C_4 evolution in the grasses, is not supported by our data. The atmospheric CO_2 concentration is estimated to have drastically decreased around 30 Ma to below current levels (Pagani *et al.*, 2005). Further CO_2 declines during the last 10 My are difficult to reconstruct with certainty (Edwards *et al.*, 2010), although recent evidence suggests that atmospheric CO_2 fell below 350 ppm between 15 and 8 Ma and reached its lowest levels during glacial episodes in the Pleistocene (Tripati *et al.*, 2009). Several C_4 eudicot lineages evolved more than 20 Ma (Table 1), and our modelling of C_3/C_4 transitions suggested that the probability of C_4 evolution increased at the same time in monocots and eudicots, around 28 Ma (Fig. 3). These time estimates are consistent with a global effect of the decline in atmospheric CO_2 during the Oligocene, and could explain the widespread C_4 origins in many different geographic regions beginning around 30 Ma (Sage *et al.*, 2011a).

The similarity in the timing of C_4 origins in eudicots and monocots is inconsistent with the physiologically-based predictions on the estimated lower QY of eudicots. A number of possibilities could explain this discrepancy. First, most C_4 species occur in high light environments where QY is not directly limiting. At moderate to high light levels, photoprotective processes such as zeaxanthin quenching of excess light energy become engaged, reducing QY below the maximum values used in interspecies comparisons (Sage and Kubien, 2003). Hence, QY differences between C_4 eudicots and monocots would not be directly related to

carbon gain and fitness. Second, past CO_2 levels cannot be reconstructed with certainty when lower than 500 ppm (Edwards *et al.*, 2010), and post-Oligocene levels could have been below the threshold proposed for both the evolution of C_4 monocot and eudicots, based on QY models (Ehleringer *et al.*, 1997). Third, QY differences between C_4 monocots and eudicots are small, and are not consistent between all groups (Skillman, 2008). While QY of C_4 Amaranthaceae/Chenopodiaceae and *Flaveria* are clearly lower than those of C_4 grasses analysed by the same authors, some C_4 *Euphorbia* had higher QY than many C_4 grasses (Ehleringer and Bjorkman, 1977; Ehleringer and Pearcy 1983; Monson *et al.*, 1986). In this light, QY differences among taxonomic groups could have been of limited importance for C_4 evolution. Rather, ecological factors such as heat, aridity, and salinity might have been key determinants of C_4 evolvability once the low CO_2 precondition was met.

Length of the transition from C_3 to C_4

It has been proposed that the multiple transformations in anatomy and biochemistry required for C_4 photosynthesis have been acquired gradually (Griffiths, 1989; Sage, 2004). The time required for the whole transition from a C_3 ancestor to completely C_4 descendants is difficult to estimate, since the precise moment at which the different C_4 characteristics appeared cannot be easily mapped onto a phylogenetic tree (Christin *et al.*, 2010). One approach is to consider the time that separates the first appearance of C_4 characters from the first fully C_4 node of the same lineage. In *Flaveria*, the first C_3–C_4 ancestor emerged between 3.1 and 2.9 Ma, but anatomical preconditions appeared earlier, between 3.6 and 3.1 Ma. The first C_4 plants of *Flaveria* (if the possibility of reversals is excluded) evolved between 1 and 0.2 Ma (Table 1). This indicates that the transition from a C_3 ancestor to C_4 plants took at least 2 My in this clade (Fig. 1). In Molluginaceae, enlarged bundle-sheath cells evolved more than 20 Ma, probably in a C_3 context, and were co-opted to evolve a C_4 trait that was optimized in the last 1 My (Christin *et al.*, 2011). This suggests that the complete transition from the appearance of C_4 preconditions in a C_3 context to completely developed C_4 plants took at least 15 My (Fig. 4). The presence of anatomical C_4 preconditions in taxa related to C_4 lineages has also been suggested for *Heliotropium* and *Cleome* (Marshall *et al.*, 2007; Vogan *et al.*, 2007). Unfortunately, phylogenies are not available for *Heliotropium* and the taxonomic distribution of these preconditions is uncertain for *Cleome* (Feodorova *et al.*, 2010).

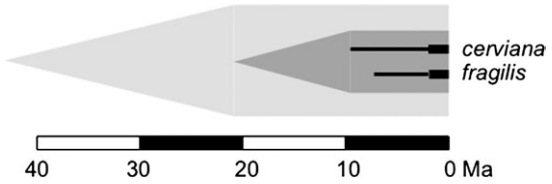

Fig. 4. Temporal representation of the gradual C_3 to C_4 transition in *Mollugo*. This diagram is drawn based on the conclusions of Christin *et al.* (2011). The ancestor of the group evolved enlarged bundle sheath cells that represent a C_4 precondition (light grey) more than 20 Ma. This was co-opted to evolve C_3–C_4 photosynthesis (grey), which served twice to evolve a C_4 trait (black). For each group, triangles or thin bars represent the interval between the stem and crown nodes (interval in which the trait could have evolved).

Conclusions

By adopting a multi-faceted phylogenetic approach, it has been shown that the numerous C_4 origins in eudicots have been spread across the last 30 My, and were contemporaneous with those in monocots. So far, no conclusive evidence of pre-Oligocene C_4 plants have been found by molecular dating or analysis of fossilized organic remains (Edwards *et al.*, 2010, Urban *et al.*, 2010). The period extending from the Oligocene to the present has thus seen an exceptional burst of C_4 origins in numerous distantly related groups of flowering plants (Sage *et al.*, 2011a), suggesting the action of global environmental triggers. The decline of atmospheric CO_2 probably increased the probability of C_4 evolution by establishing photorespiration that inhibited C_3 photosynthesis on the one hand, while on the other creating an internal pool of photorespired CO_2 that could serve as resource for driving evolutionary innovation (Hylton *et al.*, 1988; Sage, 2004). Low CO_2 alone was probably insufficient, as shown by the relative lack of C_4 species in habitats such as moist, shaded forests. Other environmental changes, notably increased seasonality, fire, and aridification possibly enhanced the probability of C_4 evolution by opening up landscapes, reducing competition, and restricting stomatal aperture. Together with anatomical and genetic factors that enhanced C_4 evolvability in some clades, these environmental factors might have been instrumental in driving the repeated origins of C_4 photosynthesis in a variety of angiosperms, and profoundly changing the plant communities of many biomes across the planet.

Supplementary data

Supplementary data can be found at JXB online

Supplementary Document S1. List of sequences used for phylogenetic reconstructions.

Supplementary Fig. S1. Calibrated phylogenetic trees.

Supplementary Table S1. Comparison of estimates of stem and crown ages (in millions of years, with standard deviation in brackets) with those obtained with less calibration constraints.

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