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Genetic enablers underlying the clustered evolutionary origins of C_4 photosynthesis in angiosperms

Running title: Co-option of genes for C_4 photosynthesis

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Abstract

The evolutionary accessibility of novel adaptations varies among lineages, depending in part on the genetic elements present in each group. However, the factors determining the evolutionary potential of closely related genes remain largely unknown. In plants, CO_2-concentrating mechanisms such as C_4 and CAM photosynthesis have evolved numerous times in distantly related groups of species, and constitute excellent systems to study constraints and enablers of evolution. It has been previously shown for multiple proteins that grasses preferentially co-opted the same gene lineage for C_4 photosynthesis, when multiple copies were present. In this work, we use comparative transcriptomics to show that this bias also exists within Caryophyllales, a distantly related group with multiple C_4 origins. However, the bias is not the same as in grasses and, when all angiosperms are considered jointly, the number of distinct gene lineages co-opted is not smaller than that expected by chance. These results show that most gene lineages present in the common ancestor of monocots and eudicots produced gene descendants that were recruited into C_4 photosynthesis, but that C_4-suitability changed during the diversification of angiosperms. When selective pressures drove C_4 evolution, some copies were preferentially co-opted, probably because they already possessed C_4-like expression patterns. However, the identity of these C_4-suitable genes varies among clades of angiosperms, and C_4 phenotypes in distant angiosperm groups thus represent genuinely independent realizations, based on different genetic precursors.

Keywords**:** C_4 photosynthesis, crassulacean acid metabolism, transcriptomics, phylogenetics, co-option, evolvability

Introduction

During the evolutionary diversification of organisms, novel adaptations emerge through the re-assignment of genes inherited from ancestors to novel developmental or biochemical pathways, a process named co-option. The evolutionary accessibility of novel traits can therefore depend on the genomic content of the ancestor (Blount *et al.* 2008, 2012; Harms and Thornton 2014), together with mutations that produce the new phenotype. But the role of historical contingency in determining the evolutionary potential of specific taxonomic groups remains largely unexplored.

Adaptive traits that independently evolved multiple times represent excellent systems to study the constraints that dictate evolutionary trajectories toward novel adaptations (Fong *et al.* 2005; Weinreich *et al.*, 2006; Blount *et al.* 2008, 2012; Marazzi *et al.* 2012). Among plants, CO_2-concentrating mechanisms (CCMs) rank amongst the best examples of convergent evolution (Sage *et al.* 2011). These complex traits consist of numerous anatomical and biochemical components that function together to increase the internal concentration of CO_2 before its fixation by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Osmond 1978; Hatch 1987), and provide an advantage in warm and arid environments, in the low-CO_2 atmosphere that prevailed for the last 30 million years (Sage 2004; Beerling and Royer 2011; Edwards and Ogburn, 2012; Sage *et al.* 2012). They rely on the segregation of the initial fixation of atmospheric CO_2 into organic compounds, which is mediated by the coupled action of carbonic anhydrase (CA) and phosphoenolpyruvate carboxylase (PEPC), and its secondary refixation by Rubisco, which starts the Calvin-Benson cycle (Figure 1). This segregation occurs spatially among distinct compartments within the leaf during the day in C_4 plants, and temporally between night and day in CAM plants. Besides anatomical requirements, an efficient CCM therefore relies on specific spatial and diurnal expression of CCM-specific genes, as well as suitable catalytic properties of the encoded enzymes (Hibberd and Covshoff 2010; Mallona *et al.* 2011). Despite their apparent complexity, the C_4 CCM evolved in more than 62 lineages of flowering plants (Sage *et al.* 2011), and the number of the CAM lineages might be even higher (Edwards and Ogburn 2012). These origins are, however, not randomly distributed in the angiosperm phylogeny, but are clustered within certain clades, while other large clades contain no C_4 or CAM species (Sage, 2001; Sage *et al.* 2011; Edwards and Ogburn 2012). This pattern has been attributed to differences in the evolvability of CCMs among angiosperm subclades, because of factors hypothesized to include ecology, life history and genomic content (Sage 2001; Monson 2003). The association of C_4 origins with particular ecological conditions has since received statistical support (Osborne and Freckleton 2009; Edwards and Smith 2010; Kadereit *et al.* 2012), but the effect of genetic factors is still unknown. The importance of

gene duplication for C_4 evolvability has not received empirical support when evaluated via the number of gene copies in complete genomes (Williams *et al.* 2012). However, the expression profiles and catalytic properties of properties encoded by a given gene family diversified following speciation events, as well as after gene-specific or whole genome duplications, and the individual genes present in a given taxonomic group might thus influence the accessibility of the CCMs.

The numerous C_4 origins in the PACMAD subclade of grasses have been statistically associated with the presence of C_4-like anatomical characters in their common ancestor, which were then recurrently co-opted for C_4 evolution, decreasing the number of changes required to generate the C_4 phenotype (Christin *et al.* 2013b; Griffiths *et al.* 2013). The genetic mechanisms responsible for these anatomical properties are still unknown, but the enzymes responsible for the main reactions of the C_4 and CAM biochemical pathways are well documented (Figure 1; Osmond 1978; Hatch 1987). Their phenotypic variation among taxonomic groups is however not easily quantified, and it is not known whether certain gene lineages encoding these enzymes possess characteristics that facilitate the evolution of the C_4 phenotype. Insights into this question have recently been gained by the comparative analyses of the transcriptomes of three independently-evolved C_4 grasses in a phylogenetic context, which showed that only a subset of the genes encoding seven core enzymes were repeatedly co-opted during the evolution of C_4 photosynthesis (Christin *et al.* 2013a), suggesting that C_4-suitable genes were present in the common ancestor of at least some grasses and have been transmitted to most descendants. However, the properties that predisposed certain gene lineages for a CCM function might be more ancient, which could explain the great phylogenetic breadth of origins within angiosperms (Figure 2): perhaps all origins incorporated the same potentiated genes inherited from their common ancestor? This hypothesis can be evaluated by comparing the genetic determinants of CCMs that evolved in distantly related clades of angiosperms.

In this study, we use transcriptome analyses to identify putative CCM-specific gene lineages in different families of Caryophyllales, the clade of angiosperms with the highest recorded number of CCM origins (Figure 2; Sage *et al.* 2011; Edwards and Ogburn 2012; Kadereit *et al.* 2012). We then use phylogenetic analyses to identify co-ortholog gene clusters: that is, monophyletic groups of genes that are descended from each of the genes present in the common ancestor of a given clade, through speciation and possibly subsequent gene or genome duplication. These groups of co-orthologs are identified specificically for Caryophyllales, and also for monocots+eudicots. The data available in the literature for other C_4 species, and especially C_4 grasses, is also incorporated to test whether (i) there is a bias in gene co-option for CCMs in multiple groups of angiosperms, and (ii) the bias is the same for all angiosperms. These analyses shed new light on the importance of

historical contingency during evolution and the genetic evolvability of adaptive novelties through time.

Results

Phylogenetic analyses

Phylogenetic trees were reconstructed for ten gene families encoding enzymes of the C_4/CAM biochemical cycles (Figures 3 and S1). Between one (for genes encoding ALA-AT, NADP-ME, PCK and PPDK) and three (for genes encoding ASP-AT, and NAD-MDH) co-ortholog groups across monocots+eudicots were identified. Several of these contain multiple co-ortholog groups specific to eudicots, grasses, or Caryophyllales (Figures 3 and S1). Relationships among co-orthologs are compatible with the expected species relationships (Figure S2), despite limited support in some cases and potential problems near the tips due to the presence of tandem repeats that can occasionally recombine (e.g. Wang *et al.*, 2009).

Phylogenetic trees were similarly inferred for ten gene families encoding proteins related to the C_4/CAM traits, but that are not responsible for the core biochemical reactions (Figure S3). The number of monocots+eudicots co-ortholog groups identified for these families ranged from one (*pepck*, *ppdkrp*, *nhd*, and *tdt*) to five (s*bas*; Figure S2).

Expression patterns of core C_4/CAM enzymes

For six of ten gene families encoding core C_4/CAM enzymes (ALA-AT, β-CA, NAD-MDH, NADP-ME, PEPC and PPDK), genes with expression patterns expected for C_4-specific forms were identified for all four C_4 Caryophyllales (*Amaranthus*, *Boerhavia*, *Trianthema*, and *Portulaca*; Tables 1 and S1). The presence of NADP-ME in this list is surprising, given that *Amaranthus* and *Portulaca* use the NAD-ME decarboxylating enzyme (Muhaidat *et al.* 2007). A high diurnal abundance of *nadpme-1E1* genes could be independent of an involvement in the C_4 cycle, although it is not observed in *Nopalea* and *Mesembryanthemum* (Table S1). Two of the six enzymes with C_4-like expression in the four species are encoded by a single group of co-orthologs in Caryophyllales (ALA-AT and PPDK; Figure S1). For the four other enzymes, all four species used the same group of co-orthologs in each case (*βca-2E3*, *nadmdh-3C1*, *nadpme-1E1*, and *ppc-1E1*). An additional *βca* gene (*βca-1E2*), not detected at a significant level in the other species, was however present at high levels in *Portulaca*, although it was still more than twenty times less abundant than *βca-2E3*. An additional *nadmdh* gene (*nadmdh-2*) was similarly present at significant abundance in *Portulaca*, although it was well below the other *nadmdh* gene (Table S1). In addition to these gene families with a significant abundance in all four C_4 species, different lineages encoding ASP-AT

have apparently been co-opted in the three C_4 groups with a significant activity (*aspat-1E1*, *aspat-2* and *aspat-3C1*). Variation in the co-option of genes for ASP-AT was also reported for grasses (Christin *et al*. 2013a). C_4-like expression was also identified for *nadpmdh-1* in *Boerhavia*, *Trianthema* and *Portulaca* (Tables 1 and S1). The genes encoding NAD-ME above the rpkm threshold were different in *Amaranthus* and *Portulaca*, and in each case, the second gene lineage was just below the threshold (Tables 1 and S1). Proteins encoded by the different *nadme* lineages are known to form heterodimers in *Arabidopsis* (Tronconi *et al.* 2008), which is also likely the case in these C_4 species. The species *Trianthema portulacastrum* has been described as having a high PCK activity (Muhaidat and McKown 2013), but no *pck* gene was present at high transcript abundance in the samples analyzed here (Table S1). This might indicate that PCK activity varies among *T. portulacastrum* individuals or with environmental conditions.

The levels of *ppc-1E1c* genes encoding PEPC increased in the *Portulaca* samples expressing a CAM cycle, as reported in Christin *et al.* (2014; Table S1). The gene *nadpmdh-3* increased at night, and is likely involved in the CAM pathway of *Portulaca*, together with *nadpmdh-1*, which was present at high levels during both day and night in one of the well-watered samples and reached high levels in all samples expressing a CAM cycle (Table S1). One of the genes encoding NADP-ME (*nadpme-1E1a*) was present at high transcript abundance in *Nopalea* although it did not reach 300 rpkm in both individuals (Table S1). However, putative CAM-specific PPDK and NADP-MDH encoding genes were easily identified during the day period (*ppdk-1C1a* and *nadpmdh-3*; Tables 1 and S1) and another gene encoding NADP-MDH was present at high levels at night (*nadpmdh-1*). Finally, two closely related PEPC-encoding genes were present at high transcript abundance at night (*ppc-1E1c* and *ppc-1E1d*). While the abundance of *ppc-1E1d* dramatically decreased during the day, the abundance of *ppc-1E1c* remained at similar levels (Table S1).

Expression patterns of other enzymes

One of the genes for AK (*ak-1*) was above 300 rpkm during the day in *Boerhavia*, *Trianthema* and *Portulaca*, and one of the genes for PPa (*ppa-1*) had a high transcript abundance during the day in *Amaranthus* and *Boerhavia*, while *ppa-2* was above 300 rpkm during the day in *Nopalea* (Table S1). The single lineage encoding PEPC-K reached high transcript abundance during the day in *Amaranthus* but stayed below 300 rpkm in the other species. However, there was a clear diurnal increase of *pepck-1* in *Trianthema* and *Portulaca*, and a nocturnal increase in *Nopalea* as well as *Portulaca* and *Mesembryanthemum* samples watered less frequently (Table S1). Genes for PPDK-RP stayed at moderate transcript abundance in all samples, with little day/night fluctuations. Regarding the transporters, s*bas-1* was present at high levels in the C_4 *Amaranthus*, *Trianthema* and

Portulaca, while it was at very low abundance in the other species (Table S1), suggesting it is involved in the C_4 pathway of these three taxa (Table 1). Similarly, some genes for DIC, NHD and TPT/PPT were present at high diurnal abundance in some or all C_4 species (Table S1), supporting their involvement in the C_4 cycle of some species (Table 1; Bräutigam *et al*. 2011, 2014; Külahoglu *et al.* 2014). The gene *dic-2* for DIC was also present at very high diurnal transcript abundance in *Nopalea* (Table S1), suggesting an involvement in the CAM cycle of this species.

Test for a bias in gene co-option

Given the size of each gene family, the number of co-ortholog groups in each species, and the number of times each gene family has been co-opted for C_4 photosynthesis (Table 2), 18 different gene lineages are expected to be co-opted at least once by chance across seven C_4 origins in monocots and eudicots (Figure 4). The sixteen gene lineages identified as C_4-specific across grasses and Caryophyllales (Table 2) is not significantly lower than expected by chance (p-value = 0.10). The theoretical minimum is 10 genes co-opted at least once (Table 2). The observed number is far from this theoretical minimum, and values up to 15 would have been significant (Figure 2), which indicates that the non-significance of the test is not simply due to a lack of statistical power.

Considering only Caryophyllales allows a greater number of co-ortholog groups to be delimited (Table S2). In this clade, an average of 20 different gene lineages that are co-opted at least once during four C_4 origins is expected by chance (Figure 4). The fourteen different gene lineages co-opted for C_4 photosynthesis observed in Caryophyllales (Table S2) is significantly lower than expected by chance (p-value < 0.0005), indicating that gene co-option for C_4 photosynthesis was non-random, a pattern previously reported within grasses (Figure 4; Christin *et al.* 2013a).

The CAM cycles of *Nopalea* and *Portulaca* use *nadpmdh-3* for part of their cycle, a gene that is not used by any of the sampled C_4 species (Table S1). The co-option of a second *nadpmdh* gene might have been dictated by the need for distinct, diurnally regulated isoforms. Besides this dissimilarity, the other putative CAM-specific genes (*βca-2E3*, *nadpmdh-1*, *ppc-1E1* and *ppdk-1*) are also used for C_4 photosynthesis in Caryophyllales (Table 1), and the co-option of genes for C_4 and CAM photosynthesis can consequently be considered convergent, which might suggest that C_4 and CAM evolution share some genetic predispositions (Christin *et al.* 2014). Given the size of the four gene families and the co-options observed in C_4 species, the minimum possible total of gene lineages co-opted at least once across the CAM and C_4 origins is five (Table S2), which is only one below the total observed from the real data, and the difference is due to *nadpmdh-3*. If the presence of two distinct *nadpmdh* genes is indeed required, then the six observed co-options is the absolute

minimum possible number of different co-options given the co-options in C_4. This small number is however not significantly smaller than expected by chance (Figure S4; p-value = 0.22). There is a good chance that this negative result is due to a lack of statistical power. First, only one origin of CAM was considered. The second CAM sample in our study (*Portulaca*) uses the same gene lineages or very recent duplicates encoding the core CAM enzymes (Table S1), but the CAM pathways of the two groups might not be independent (Christin *et al.*, 2014). In addition, CAM-specific forms were identified for only four enzymes, one of which is a single-gene family (Table S2), and so the theoretical maximum number of gene lineages that might have been co-opted (10) is also very close to the theoretical minimum (Figure S4). Finally, the number of subgroups of co-orthologs for some of these is high in *Nopalea* (five for *ppc-1E1* vs. one for *ppc-1E2* and *ppc-2*), making the number of co-orthologs co-opted at least once low even in the absence of a co-option bias (Figure S4). Testing the hypothesis that CAM origins preferentially co-opted the same genes as C_4 origins in Caryophyllales will require the inclusion of additional independent CAM origins, which exist in Aizoaceae.

Discussion

All major angiosperm gene lineages could be recruited into C_4 photosynthesis

An assessment of gene orthology depends strongly on taxonomic scale, as it is affected by clade-specific gene duplications. The divergence between grasses and Caryophyllales (monocots and eudicots) is an early event in the history of angiosperms, which marks the last common ancestor of the majority of species (Soltis *et al.* 2011). As a consequence, the co-orthologs defined for monocots and eudicots together represent the number of gene copies that existed in their common ancestor, some 130 million years ago (Bell *et al.* 2010; Smith *et al.* 2010). Multiple groups of deep co-orthologs exist for six out of ten enzymes of the C_4/CAM cycles (Table 2) and, when considering these, the recruitment of genes for C_4 photosynthesis is not significantly different from random (Figure 4). The gene lineages co-opted by at least one C_4 origin in either grasses or Caryophyllales cover most of the gene lineages present in the common ancestor of monocots and eudicots (Tables 2 and S3). The only exceptions are *ppc, nadmdh* and *nadpmdh*, where one distantly related group of co-orthologs (*ppc-2*, *nadmdh-1* and *nadpmdh-2*, respectively) was never co-opted, but this is not statistically unexpected given the higher number of gene duplications in the other gene lineages in grasses and Caryophyllales (Figures S1 and 4). All the other co-ortholog groups for all CCM-related gene families have been co-opted at least once for C_4 photosynthesis, despite sometimes being highly divergent. Most of the gene lineages present in the common ancestor of monocots and

eudicots therefore could evolve to encode an enzyme responsible for a C_4 function, at least after tens of million years of further diversification.

...but C_4-potentiation evolved later, during the diversification of monocots and eudicots

The different gene families expanded to various degrees after the split of monocots and eudicots via a combination of gene-specific and whole-genome duplications (e.g. Blanc *et al.* 2003; Flagel and Wendel 2009; Fischer *et al.* 2014; Rensing 2014), producing up to six distinct co-ortholog groups in certain clades (for example, *ppc-1* in grasses). For the ten enzymes of the C_4/CAM cycles, the number of gene lineages expanded from 19 to 27 in Caryophyllales, of which only fourteen have been co-opted at least once for C_4 photosynthesis (Table S2), which is far smaller than expected by chance (Figure 4). Gene co-option was thus not random, with some genes more likely to become C_4-specific, a pattern also detected within grasses (Figure 4; Christin *et al.* 2013a; John *et al.* 2014). It has been hypothesized that the presence of closely related copies of one gene following gene duplication favors functional diversification (Zhang 2003), and might have facilitated C_4 evolution (Monson 2003). While it is certainly true that repeated gene duplications contributed to the functional diversity within gene families, the pattern reported here cannot be simply due to the number of closely related duplicates within each group of co-orthologs, as this was accounted for in our simulations. Other genetic properties must therefore explain the higher C_4-suitability of some of the genes encoding similar enzymes.

As with any complex trait, C_4 photosynthesis must evolve through successive evolutionary steps, each of which has to confer greater fitness than the ancestral one (Heckmann *et al.* 2013; Mallmann *et al.* 2014; Williams *et al.* 2014). Since different isoforms of CCM-related enzymes are known to differ in their catalytic properties (Tausta et al. 2002, Svensson et al. 2003, Alvarez et al. 2013), the preferential co-option of genes encoding proteins with C_4-like kinetics might have facilitated C_4 evolution by decreasing the number of mutations required. However, the ability to be directly incorporated into the C_4 cycle might be more important than the kinetic properties of the encoded enzymes. A primitive C_4 cycle might emerge through an increase of PEPC activity sustained by the other enzymes that are already active in the C_3 ancestors (Christin and Osborne 2014). Indeed, some enzymes of the C_4 cycle already exhibit a C_4-like spatial expression in C_3 plants (Hibberd and Quick 2002; Brown *et al.* 2010; Kajala *et al.* 2012; Mallmann *et al.* 2014), which then enables the establishment of a weak C_4 cycle via a few key genetic changes. Once a C_4-pump is acting, natural selection can act to increase its efficiency (Heckmann *et al.* 2013), through multiple changes to the expression and catalytic properties of each of its constituent enzymes, and the optimization of cellular anatomy. The adaptation of each enzyme through natural selection can

however occur only if the enzyme is already involved in the C_4 pathway, making isoforms with C_4-like expression profiles, including cellular and subcellular localization as well as expression levels, more likely to be co-opted. We hypothesize that spatial and temporal expression patterns in the C_3 ancestors affected the likelihood of a given group of co-orthologs being co-opted for CCMs.

Although the exact cause of the co-option bias is not known with confidence, we have clearly established that such a bias exists and that it varies between the two major clusters of C_4 origins in angiosperms. This conclusion is moreover supported by the analysis of other groups of eudicots containing C_4 species (Table S3). Within eudicots, *Cleome* belongs to the Rosidae and *Flaveria* to the Asteridae, while Caryophyllales represents an additional lineage (Soltis *et al.* 2011). These three clades with C_4 species consequently represent ancient splits within eudicots (Figure 3; Bell *et al.* 2010), and some C_4 enzymes for which the same gene lineage was consistently co-opted within Caryophyllales are encoded by other co-ortholog groups in *Flaveria* (e.g. *ppc-1E2* and *βca-2E1*; Table S3), indicating that the bias evidenced for Caryophyllales does not extend to other eudicots. Homologous transcription factors are apparently involved in C_4 development in monocots and eudicots (Aubry *et al.* 2014), and comparative studies have established that the mechanism allowing the cell-specificity of some C_4 enzymes was shared between monocots and eudicots, suggesting that they evolved before their split (Brown *et al.* 2011). However, the functional diversification that happened during the expansion of each gene family has either decreased the C_4-suitability of some gene lineages or produced gene lineages that are more suitable for a C_4 function, through modifications that predate C_4 photosynthesis and consequently evolved for unrelated reasons. The modifications that favored co-option for C_4 photosynthesis are probably different for each gene family, and likely happened at different times. Interestingly, the same gene lineages are used for the C_4 and CAM pathways in the closely related species included in this study (Table 1). Although more CAM origins are needed to confirm this hypothesis, it might indicate that genes more likely to be co-opted for C_4 are equally suitable for CAM photosynthesis, which might result from shared requirements for high expression levels in photosynthetic organs (for some enzymes in the same cells and same time of day; e.g. PPDK and MDH) and kinetic properties adapted to high concentrations of substrates and products.

Conclusions

The repeated evolution of CO_2-concentration mechanisms in flowering plants represents one of the best examples of convergent adaptation to changing environments, and constitutes an extraordinary system to test hypotheses about the effect of ancestral states on the evolutionary trajectories of

descendants. It has been shown that C_4 origins in grasses were constrained to subclades with anatomical enablers (Christin *et al.* 2013b), and the observation of a gene co-option bias within the same group suggested that genetic enablers might also have contributed to C_4 evolvability (Christin *et al.* 2013a). By expanding the sampling to distantly related lineages that also contain multiple C_4 origins, we have shown here that the gene co-option bias is not restricted to grasses, but is also present in the distantly related Caryophyllales, where it might even extend to CAM origins. However, while both clades show such a bias, it is not present when they are analyzed jointly, which indicates that the bias is clade-specific. The changes that increased the suitability of some genes for the C_4 function happened during the diversification of angiosperms and not before the divergence of eudicots and monocots, nor before the divergence of the major groups of eudicots (e.g. Rosidae, Asteridae and Caryophyllales). Therefore, the C_4 origins found in tight phylogenetic clusters might be best considered as parallel realizations of a C_4 syndrome, which have repeatedly evolved from a potentiated state that was inherited from their C_3 ancestor. The C_4 origins observed in Caryophyllales are clearly independent from those observed in grasses. This is likely also true of the other, smaller clusters of C_4 origins present in angiosperms (Figure 3; Sage *et al.* 2011), revealing the complex contributions of parallelism and convergence throughout the evolutionary history of C_4 photosynthesis.

Material and Methods

Plant material

A total of six species of the Caryophyllales were selected for quantitative transcriptome analyses. The species *Portulaca oleracea* is constitutively C_4 but a complementary CAM pathway can be triggered by drought stress (Kraybill and Martin 1996; Lara *et al.* 2003; Winter and Holtum 2014). The transcriptome of *P. oleracea* individuals expressing the C_4 and CAM cycles respectively was sequenced in a previous paper, including analyses of selected genes (Christin *et al.* 2014). The additional species in the present study include three C_4 taxa (*Amaranthus hypochondriacus*, *Boerhavia coccinea*, and *Trianthema portulacastrum*), one constitutive CAM species (*Nopalea cochenillifera*), and one CAM-inducible plant (*Mesembryanthemum crystallinum*) from distinct Caryophyllales families. These different species are separated in the phylogeny by multiple C_3 taxa and are thought to represent different origins of the C_4 and CAM pathways (Figure 2; Arakaki *et al.* 2011; Brockington *et al.* 2011; Sage *et al.* 2011), although the CAM cycles of *N. cochenillifera* and *P. oleracea* might share a partially common origin (Christin *et al.* 2014).

The plants were grown from seeds, except for *N. cochenillifera* samples, which were

acquired as small plants. All seedlings were placed simultaneously in a Conviron E7/2 plant growth chamber (Conviron Ltd., Winnipeg, Manitoba, Canada). The conditions were as described in Christin *et al.* (2014), with 14 hours of light, and a night temperature of 22 °C, which increased to 28 °C after three hours of light and until three hours before dark. The chamber was illuminated with twelve 32W fluorescent lamps and four 60W incandescent lamps. Each plant was grown individually in a 7.5 cm pot, except for *N. cochenillifera* individuals, which were bigger and had to be placed in 445 ml pots. The pots were filled with cleaned mix for succulent plants (2 parts soil, 1 part perlite, 1 part gravel, 1 part calcined clay). Their position within the growth chamber was randomized daily for the duration of the experiment. Most plants were bottom-watered as needed to keep the soil constantly moist. However, one group of *P. oleracea* and *M. crystallinum* seedlings were selected at the beginning of the experiment and were watered less frequently to induce a CAM cycle. Nutrients were added to the water periodically at a concentration of 1:100 (w/v) of K, P, and N in equal proportions.

After one month in these conditions, leaf samples (or stem fragments for *N. cochenillifera*) were collected and flash-frozen in liquid nitrogen and stored at -80 C. For each species, two individuals were sampled after 4h of light (day sample) and after 2h of dark (night sample). *For P. oleracea* and *M. crystallinum*, two individuals of each watering regime were sampled. For each species, one individual was sampled first during the day and then on the consecutive night, and the other one was sampled first at night and then during the consecutive day. This sampling was meant to control for effects triggered by the removal of leaves. An equal proportion of young and mature leaves were sampled. Multiple leaves from each sample were randomly mixed for RNA extraction.

In addition to these 32 samples used for quantitative transcriptome analyses, eight individuals were sampled for qualitative transcriptome analyses, without controlling for the growth conditions or tissue type. These additional individuals represent different families of the suborder Portulacineae (*Talinum portulacifolium*, *Anacampseros filamentosa*, *Pereskia grandifolia*, *Pereskia bleo*, *Pereskia lychnidiflora*, *Echinocereus pectinatus* and another individual of both *Portulaca oleracea* and *Nopalea cochenillifera*). The sampled individuals came from the collection of living material available in the greenhouse of Brown University, except for *P. oleracea*, which was collected in Providence, RI, USA. For each individual, various proportions of leaf, stem, root and/or floral tissue were mixed before RNA extraction, to increase the diversity of transcripts in the sequencing results.

RNA extraction, sequencing and assembly

RNA isolation was performed using the RNeasy Plant Mini Kit (Qiagen Inc., Texas, USA), or for

the succulent tissues the FastRNA™ Pro Green Kit (MP Biomedicals US, Ohio, USA), and included a DNAse treatment. For each sample (one individual in one condition), several extractions were performed and pooled. The samples were prepared for sequencing using the Illumina TruSeq mRNA Sample Prep Kit (Illumina Inc., California, USA) and following the provider's instructions. Each sample was tagged with a specific barcode. Fragments of the cDNA libraries ranging from 400 to 450 bp were selected and sequenced as paired-end 100 bp reads using the Illumina HiSeq 2000 instrument at Brown University Genomics Core Facility. For the quantitative transcriptome analyses, sixteen samples were pooled per lane, while the samples for the qualitative transcriptome analyses were sequenced on 1/9 th of a lane. Raw reads were deposited in NCBI SRA database, under the project accession SRP050968. Accession numbers for individual samples are indicated in Table S4.

The reads from each sample were assembled individually, to decrease assembly difficulties caused by different alleles among individuals. The assemblies were performed using the software Trinity (Grabherr *et al.* 2011) as implemented in the Agalma pipeline (Dunn *et al.*, 2013; Table S4). For the quantitative transcriptome samples, reads for each individual were mapped to the assembled contigs using the software Bowtie 2 (Langmead and Salzberg 2012; Table S4), which is a reliable method to estimate transcript abundance (Marioni *et al.* 2008; Siebert *et al.* 2011). The mixed model was used, which allows unpaired alignments when paired alignments fail. Only one of the best alignments was reported per read, and the number of reads mapping each contig was used to compute reads per million or reads (rpm), which were later transformed into reads per kilobase per million (rpkm; see below). Multiple best alignments were frequent because multiple contigs were generally assembled per locus. These contigs were however merged during the phylogenetic annotation (see below), so that the rpm value per gene was not affected.

Reference datasets and phylogenetic trees

The assembled contigs corresponding to genes encoding proteins that are involved in the C_4 or CAM cycles were identified and annotated phylogenetically using an improved version of the approach developed by Christin *et al*. (2013a, 2014). A list of C_4- and CAM-related proteins was compiled from the literature (Osmond 1978; Hatch 1987; Bräutigam *et al.* 2011, 2014; Christin *et al*. 2013a). This list is not clade-specific, nor relevant for a specific biochemical subtype only. It includes ten core enzymes, which were used to test a bias in gene co-option (see below). In addition, ten gene families potentially related to CCMs were analyzed as they might be important to engineer CCMs in C_3 crops (Bräutigam *et al.*, 2014). This includes two enzymes involved in the processing of PPDK products, two regulatory proteins, as well as six metabolite transporters (one of

the gene families includes genes encoding PPT and others encoding TPT; Figure S3, Table S1). The annotation was performed in two consecutive steps. First, a reference dataset was compiled for each enzyme from sequences extracted from complete genomes, public databases, and the longest of the contigs assembled here. This was used to infer high quality phylogenetic trees and identify co-ortholog groups. These groups are defined as all the genes descending from a given speciation event, and are consequently specific for a given taxonomic group. Then, all the homologous contigs assembled here were successively compared to the corresponding reference dataset, and assigned to one of the co-ortholog groups.

For the reference datasets, coding sequences of all genes encoding each of the selected enzymes were retrieved from *Arabidopsis thaliana* based on their annotation. *Arabidopsis* was used because it has the best annotation, but any species could have been used as the starting genome. In some cases, well-annotated sequences for other species were retrieved from GenBank and added to this reference dataset. These sequences were used as the query of a BLAST search against *Arabidopsis* predicted cDNAs based on its genome with a maximal e-value of 0.0001. The identified homologous sequences were added to the reference dataset, which was recursively used as the query of a BLAST search against additional predicted cDNAs based on 17 complete genomes (Figure S2)), each time adding the homologous sequences to the reference dataset. Each dataset was manually curated and sequences that were similar on a small fragment only or that were obviously incomplete or corresponded to chimeras were removed. The dataset for each gene family was completed with sequences extracted from the transcriptomes generated in this study, using the same approach except that only contigs that matched the reference sequences on at least 2/3 of the average length of the other coding sequences were retained, and an e-value of 0.01 was used. These new reference datasets were aligned and manually inspected. The congruence between the sequences assembled here from Illumina data and those generated by other sequencing methods and extracted from public databases confirmed the quality of our assemblies. Putative introns and UTRs were identified based on homology, the GT-AG rule, and start and stop codons, and these non-coding regions were removed. All contigs that had indels affecting the reading frame were deleted. The remaining sequences were translated into amino acid sequences and aligned using ClustalW (Thompson *et al.* 1994), and a phylogenetic tree was inferred on the nucleotide sequences using Phyml (Guindon and Gascuel 2003). Groups of very similar contigs from the same species were identified and only the longest sequence of each was retained. The selected sequences were again visually inspected, and possible chimeras between closely related paralogs were identified using the software Geneconv (Sawyer 1999) and were removed. The remaining sequences extracted from either the complete genomes or transcriptomes generated here constituted the reference datasets

used for further phylogenetic annotation.

For each gene family, the alignment of the reference dataset was manually refined, and extremities were truncated to remove regions that were too variable to be unambiguously aligned. A phylogenetic tree was then computed on nucleotide sequences using Phyml and the best-fit substitution model identified through hierarchical ratio tests (GTR+G or GTR+G+I in all cases), with 100 bootstrap pseudoreplicates. After this phylogenetic tree revealed two very distant groups in the nadpmdh family, trees were computed separately for each of these groups. Each phylogenetic tree was manually inspected and co-ortholog groups common to grasses and eudicots were identified, as monophyletic groups of genes congruent with the species relationships (Figure 3). In some cases, one gene lineage identified for grasses and eudicots contained multiple co-ortholog groups in either eudicots or grasses, and these were annotated as such (Figure 3). Similarly, some groups of eudicot co-orthologs contained several co-ortholog groups in Caryophyllales and some groups of Caryophyllales co-orthologs contained several co-ortholog groups in Portulacineae. These different levels of orthology were all considered, so that gene lineages were defined for Caryophyllales and Portulacineae and could be matched to a more inclusive set of eudicot co-orthologs for comparison with distantly related clades, and to a most inclusive set of monocots+eudicots co-orthologs for comparison with grasses. Numbers attached to the gene names were used to describe these groups of monocots+eudicots co-orthologs. Grass-specific co-ortholog groups were named by adding to the angiosperm name a "P" (for Poaceae) and the number that was given previously (Christin *et al.* 2013a). In cases where co-ortholog groups specific to eudicots were detected, an "E" (for eudicots) was added to the monocots+eudicots name, and these were numbered consecutively. In some cases, lack of phylogenetic support prevented the identification of eudicot-specific co-ortholog groups, but these could be easily identified for Caryophyllales (e.g. *aspat-3*; see Results), and were consequently named using the same rules with a "C" instead of "E". Finally, in some cases, distinct co-ortholog groups specific to the Portulacineae subclade of Caryophyllales were observed and named adding lower-case letters (e.g. *ppdk-1C1a* and *ppdk-1C1b*) to the Caryophyllales-specific name. The only exception is *ppc-1E1* where the numerous Portulacineae-specific co-ortholog groups have been identified with a dense species sampling and genomic DNA and named by Christin *et al.* (2014). The identity of the C_4-specific genes for two additional lineages of C_4 eudicots (*Cleome*, Brassicales) and (*Flaveria*, Asterales) was retrieved from the literature (Table S3; Bräutigam *et al*. 2011; Gowik *et al.* 2011). These genes were assigned to co-ortholog groups identified within the phylogenetic trees inferred in this study, based on the presence of either *Flaveria* genes or *Arabidopsis* genes orthologous to *Cleome* in the trees.

*Phylogenetic annotation of all contigs and transcript abundance*Once the reference datasets and corresponding phylogenetic trees were available, all contigs from the 32 quantitative transcriptomes belonging to each gene family were identified through BLAST searches, with the reference dataset used as the query against each transcriptome, and an e-value of 0.01. Each of the identified contigs was then individually placed in the phylogenetic tree. The matching region of the contig, identified through the BLAST search, was added to the reference dataset, which was aligned with Muscle (Edgar 2004), a program that can be easily automated, and a phylogenetic tree per contig was inferred with Phyml and a GTR+G model. The phylogenetic tree was automatically inspected, and the contig was assigned to a group of Caryophyllales or Portulacineae co-orthologs identified based on the reference dataset if they formed a monophyletic group. In order to differentiate the Portulacineae-specific *ppc-1E1* copies, identified with a large sample of sequences isolated from genomic DNAs (Christin *et al*. 2014), the *ppc-1E1* transcripts from *Nopalea* and *Portulaca* were reannotated using the same method but with a reference dataset comprised of a sample of Portulacineae sequences previously isolated, and representing the different gene lineages.

The rpm values for all the contigs assigned to a given group of co-orthologs were summed to obtain the rpm value for each gene lineage. These rpm values were then transformed rpkm values, based on the length of orthologous mRNAs for model organisms. The length of the assembled contigs was not used because they do not generally cover the whole length of the transcript, and rpkm values for contigs covering parts of the same transcript can not be added. The rpkm values were used to identify the groups containing C_4- and CAM-specific genes, which are routinely identified based on quantitative gene expression with consistent results among studies (Bräutigam *et al.*, 2011, 2014; Gowik *et al.*, 2011; Mallmann *et al.*, 2014). For each species, genes were considered as putative C_4 forms if they were present at more than 300 rpkm during the day in both replicates. The same criterion was used to identify putative CAM forms, except that a predominantly nocturnal expression was expected for several of them (Figure 1). To examine whether our 300 rpkm cutoff influenced our results, we reran the test for biased co-option in Caryophyllales (see below) with an extremely conservative cutoff of 1000 rpkm and, although fewer gene lineages were identified as putatively C_4-specific, the test was still significant, as only one group of co-orthologs per mutligene family contains gene lineages with rpkm values above 1000 (see Table S1).

Tests for C_4 recruitment bias

The ten gene families encoding enzymes responsible for the main C_4 biochemical reactions were considered to test the hypothesis that the co-option of particular groups of co-orthologs within each

gene family was not random. It is well established that the activity and transcript abundance of the C_4-specific forms of these gene families differ from those observed in C_3 species (Bräutigam, *et al*. 2011, 2014; Christin *et al*. 2013a; Külahoglu *et al.* 2014; Mallmann *et al.* 2014), so that a high transcript abundance in a C_4 group can be considered as an evolutionary novelty and is consequently independent from a high transcript abundance in another C_4 group. Gene families encoding enzymes linked to the processing of PPDK products, regulation of C_4 enzymes and transport of metabolites were not included in the test because their expression level in C_3 species can also be high (Christin *et al*. 2013a) and is generally not known with confidence, so that a high transcript abundance in different C_4 lineages could be inherited from their common ancestor and would consequently be non-independent.

A bias in gene co-option for C_4 photosynthesis was first tested across monocots+eudicots. For each of the ten gene families, the number of co-ortholog groups across both monocots+eudicots was determined using phylogenetic trees (Figure S1; Table 2). The transcriptome data generated in this study was used to estimate the number of co-option events in Caryophyllales, which can exceed the number of C_4 groups if more than one gene lineage is used by the same species (e.g. genes encoding βCA and NAD-MDH in *Portulaca*). The number of different gene lineages co-opted at least once was also recorded. The same information was retrieved for three independent origins of C_4 in grasses, using the analyses of Christin *et al.* (2013a). We then generated the null distribution that would be expected if co-option of gene lineages was unbiased, by simulating a random recruitment from the total pool of available lineages across the ten gene families, using 100,000 replicates. These simulations accounted for the number of co-ortholog groups in each family as well as the number of gene duplications in each lineage, estimated by the number of clade-specific subgroups of co-orthologs. For instance, the gene *aspat-1* encoding ASP-AT is present as two co-ortholog groups in grasses (Figure S1), and is therefore twice as likely to be co-opted by chance in this group than *aspat-2* and *aspat-3*. The p-value associated with the hypothesis of co-option bias was computed as the number of replicates producing a total of gene lineages co-opted that were fewer or equal to the number observed in the real dataset.

The hypothesis of a bias in gene co-option for C_4 photosynthesis was then tested specifically for Caryophyllales. The number of gene lineages differed from the previous analysis because some co-ortholog groups defined for monocots+eudicots contain multiple co-ortholog groups in Caryophyllales (Figure S1). Again, the number of gene lineages, as well as multiple subgroups of co-orthologs in some taxa (e.g. *nadmdh-3* in Portulacineae), was accounted for. The exact same test was also applied to the dataset from grasses (Christin *et al*. 2013a).

Test for CAM recruitment bias

A similar approach was used to test the hypothesis that CAM origins preferentially co-opt genes used by C_4 groups. This test was conducted using Caryophyllales only, and C_4 gene co-option was fixed to that observed in the real data. The hypothesis tested was that the total number of gene lineages co-opted at least once across the four C_4 and the CAM origins does not differ from that expected if the co-option for CAM was independent of the identity of genes co-opted for C_4. The total number of lineages co-opted at least once across the C_4 or CAM origins expected by chance was obtained by randomly selecting genes for CAM photosynthesis, and adding them to the tally of gene lineages co-opted in the four C_4 origins. This test was based on the four enzymes for which CAM-specific isoforms were identified (βCA, NADP-MDH, PEPC and PPDK; Table S2), and the presence of multiple subgroups of co-orthologs in some species was again accounted for. Only *Nopalea* was considered, as the CAM pathway of *Portulaca* might not represent a completely independent origin (Christin *et al*. 2014), and our *Mesembryanthemum* transcriptome data did not strongly support a successful induction of a CAM cycle (Table S1). Indeed, the samples of *Mesembryanthemum* grown with different watering regimes did not differ markedly in their transcript abundances (Table S1). The duration of the drought period was probably too short to trigger a high-level CAM cycle (Winter and Holtum 2014) and these samples were consequently not used to detect CAM-specific genes. However, two *nadpmdh* genes (*nadpmdh-1* and *nadpmdh-3*), *ppc-1E1*, and *βca-2E3* increased in nocturnal abundance in the low water treatment, and *nadpme-1* increased during the day, which indicates that this independent CAM origin uses the same isoforms as *Portulaca* and *Nopalea* (see Results; Table S1).

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Figure legends

Figure 1. Simplified schematics of the C_4 and CAM cycles. The main sets of biochemical reactions of the C_4 and CAM cycles are indicated by grey circles, and are separated among the distinct compartments (space or time) that allow separation of initial atmospheric CO_2 fixation (dashed line) and the Calvin cycle (solid line). **a)** Fixation of atmospheric CO_2 into organic acids via the action of β-carbonic anhydrase (βCA) and phosphoenolpyruvate carboxylase (PEPC), **b)** transformation and transport of C_4 acids that can involve aspartate aminotransferase (ASP-AT) and malate dehydrogenase (NAD-MDH and/or NADP-MDH), **c)** decarboxylation of C_4 acids to release CO_2 that can involve malic enzymes (NAD-ME and NADP-ME) or phosphoenolpyruvate carboxylase (PCK), **d)** regeneration and transport of C_3 acids by pyruvate phosphate dikinase (PPDK) and in some cases alanine aminotransferase (ALA-AT), **e)** transformation of C_4 acids by NADP-MDH and storage in vacuoles, **f)** regeneration of C_3 acids by PPDK and storage as starch or sugars.

Figure 2. Phylogenetic position of C_4 lineages. The position of C_4 lineages is shown in red in species phylogenetic trees. **A.** Angiosperm phylogeny at the family level inferred by Soltis *et al.* (2011). The bars on the side are proportional to the estimated number of C_4 origins in each family. The groups discussed in this work are indicated. The split between eudicots and monocots is highlighted by a black circle. Branches within Caryophyllales are in bold. This figure was adapted from Christin and Osborne (2013). **B.** Time-calibrated phylogeny for Caryophyllales. The tree was inferred from plastid markers (Christin *et al.* 2011). C_4 taxa are indicated in red, and CAM taxa in blue. The species sampled in this study for transcriptome data are indicated on the right, and the taxonomic groups containing them are delimited with vertical bars.

Figure 3. Example of co-orthologs defined on a gene tree. This tree was inferred for the gene family encoding βCA, and bootstrap values are indicated near branches when above 50. Groups of co-orthologs for either eudicots or grasses (=monocots) are compressed, and their name is indicated on the right, as are those of taxonomic groups and co-ortholog groups across monocots and eudicots. Note that *Amborella* is expected to be sister to monocots+eudicots (Figure S2), and Buxales represents an early branching within eudicots (Soltis *et al.*, 2011). The corresponding gene duplications are indicated by dots, in white if they occurred before the divergence of monocots and eudicots, in grey if they happened in monocots after this divergence and in black if they happened in eudicots after this divergence. The phylogenetic tree is detailed in Figure S1.

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Figure 4. Test for recruitment bias. The histogram represents the total number of different gene lineages encoding ten enzymes with isoforms involved in the C_4 biochemical pathway, and co-opted for C_4 photosynthesis in 100,000 simulations. The test was conducted across (i) four C_4 origins in Caryophyllales and three in grasses ('angiosperms'), (ii) only the four in Caryophyllales, and (iii) only the three in grasses. For each histogram, the vertical bars indicate the observed numbers of different gene lineages co-opted, and limits of the x axis correspond to the minimum and maximum numbers theoretically possible (see Tables 2, S2 and 3; Christin *et al.* 2013a).

Table 1: Groups of co-orthologs containing putative C_4- or CAM-specific genes in Caryophyllales[a]

	C_4				CAM	
Enzyme	***Amaranthus***	***Boerhavia***	***Trianthema***	***Portulaca***	***Portulaca***	***Nopalea***
ALA-AT	1	1	1	1	-	-
ASP-AT	3C1	2	-	1E1+3C1	-	-
βCA	2E3	2E3	2E3	2E3+1E1	2E3	2E3
NAD-MDH	3C1	3C1	3C1	3C1a+2	2	-
NADP-MDH	-	1	1	1	1+3	1+3
NAD-ME	1	-	-	2	-	-
NADP-ME	1E1	1E1	1E1	1E1a	-	-
PCK	-	-	-	-	-	-
PEPC	1E1	1E1	1E1	1E1a'	1E1c	1E1c+1E1d
PPDK	1	1	1	1C1b	1C1b	1C1a
AK	-	1	1	1	-	-
PPa	1	1	-	-	-	2
PEPC-K	1	-	-	-	-	-
PPDK-RP	-	-	-	-	-	-
BASS	1	-	1	1	1	-
DIC	2	-	-	1C2	-	2
DIT	-	-	-	-	-	-
NHD	-	-	1	1	1	-
TDT	-	-	-	-	-	-
PPT	1E2	1E2	-	1E2	1E2	-
TPT	1E2	1E2	1E2	1E2	1E2	-

[a] These were identified based on their transcript abundance. See Table S1 for details of transcript abundance and Figures S1 and S3 for phylogenetic trees and identification of gene lineages.

Table 2: Gene co-option in angiosperms[a]

Enzyme	Lineages	Caryophyllales co-options[b]	Grass co-options[b]	Total co-options[b]	Total co-opted[c]
ALA-AT	1	4	3	7	1
ASP-AT	3	4	3	7	3
βCA	2	5	3	8	2
NAD-MDH	3	5	0	5	2
NADP-MDH	3	3	3	6	2
NAD-ME	2	2	0	2	2
NADP-ME	1	4	3	7	1
PCK	1	0	2	2	1
PEPC	2	4	3	7	1
PPDK	1	4	3	7	1

[a] Gene co-option in Caryophyllales is based on Table 1, while gene co-option in grasses is based on Christin *et al*. (2013a; See Table S3). Gene lineages were identified based on phylogenies (Figure S1), and are listed in Table S1; [b] Number of times gene lineages were co-opted for C_4 photosynthesis; [c] Number of different gene lineages that were co-opted at least once for C_4 photosynthesis.

Figure 1

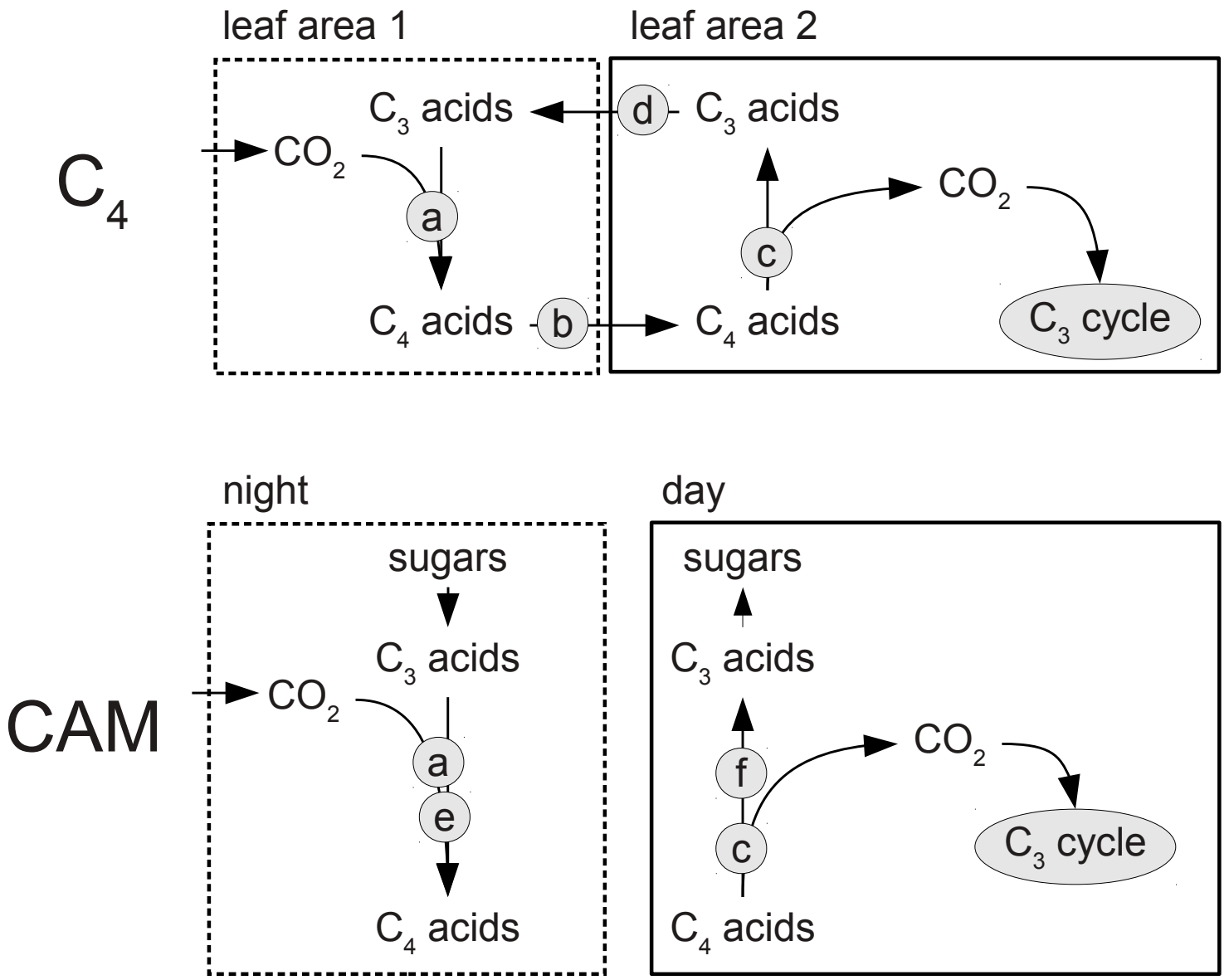

C4
leaf area 1
leaf area 2
C3 acids
CO2
a
C4 acids
b
d
C3 acids
c
CO2
C4 acids
C3 cycle
CAM
night
day
sugars
C3 acids
CO2
a
e
C4 acids
sugars
C3 acids
f
c
CO2
C3 cycle
C4 acids

Figure 2

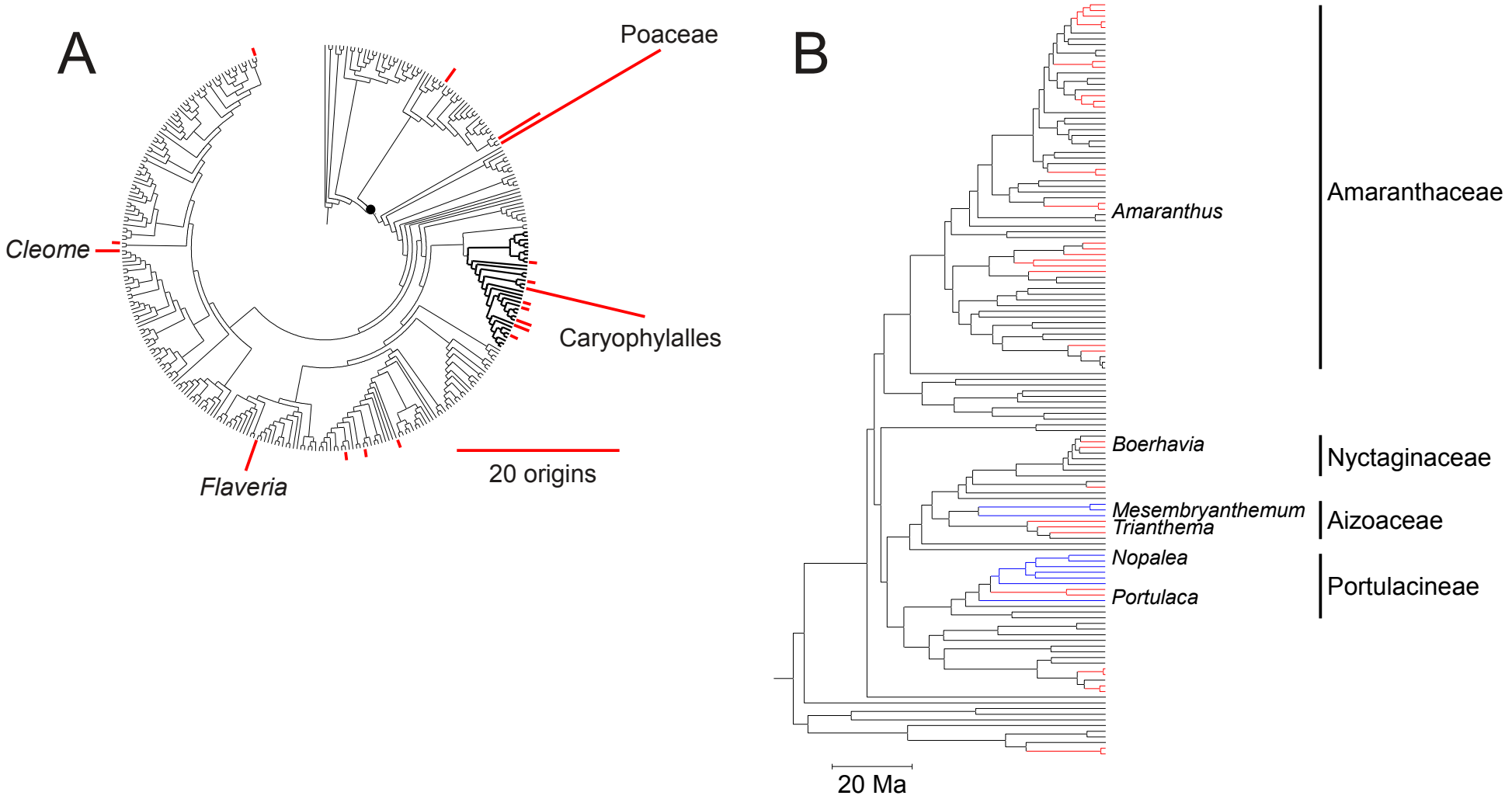

A
Poaceae
Cleome
Caryophylalles
Flaveria
20 origins
B
Amaranthus
Amaranthaceae
Boerhavia
Nyctaginaceae
Mesembryanthemum
Trianthema
Aizoaceae
Nopalea
Portulaca
Portulacineae
20 Ma

Figure 3

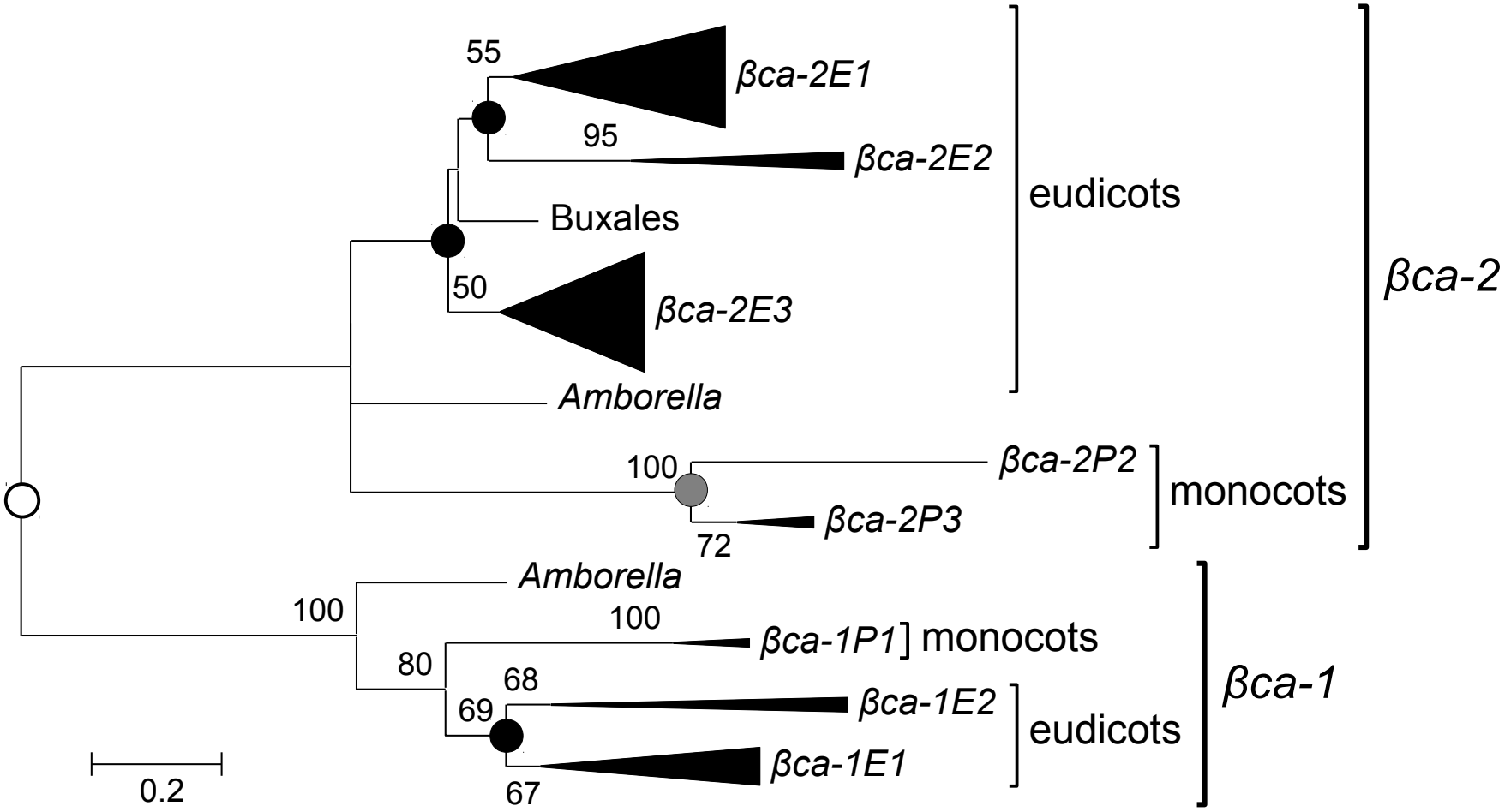
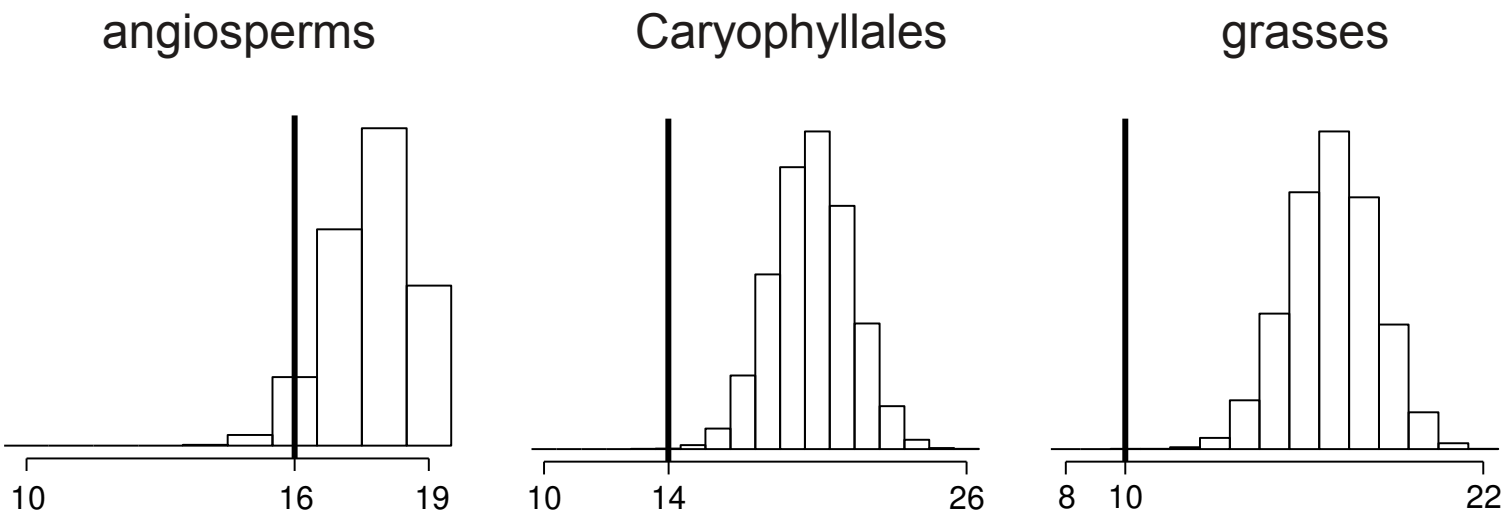

55
βca-2E1
95
βca-2E2
eudicots
Buxales
50
βca-2E3
βca-2
Amborella
100
βca-2P2
monocots
βca-2P3
72
Amborella
100
100
βca-1P1] monocots
80
68
βca-1E2
69
eudicots
βca-1
βca-1E1
0.2
67

Figure 4


angiosperms
Caryophyllales
grasses
10
16
19
10
14
26
8
10
22
Number of lineages co-opted at least once