

## OPINION

# The genetics of convergent evolution: insights from plant photosynthesis

Karolina Heyduk<sup>1</sup>, Jose J. Moreno-Villena, Ian S. Gilman, Pascal-Antoine Christin and Erika J. Edwards

**Abstract** | The tree of life is resplendent with examples of convergent evolution, whereby distinct species evolve the same trait independently. Many highly convergent adaptations are also complex, which makes their repeated emergence surprising. In plants, the evolutionary history of two carbon concentrating mechanisms (CCMs) —  $C_4$  and crassulacean acid metabolism (CAM) photosynthesis — presents such a paradox. Both of these modifications of ancestral  $C_3$  photosynthesis require the integration of multiple anatomical and biochemical components, yet together they have evolved more than one hundred times. The presence of CCM enzymes in all plants suggests that a rudimentary CCM might emerge via relatively few genetic changes in potentiated lineages. Here, we propose that many of the complexities often associated with  $C_4$  and CAM photosynthesis may have evolved during a post-emergence optimization phase. The ongoing development of new model clades for young, emerging CCMs is enabling the comparative studies needed to test these ideas.

Convergent evolution has been pervasive throughout the history of life. Even very complicated adaptations, such as camera eyes in animals<sup>1</sup>, sex determination systems in eukaryotes<sup>2</sup> and eusociality in insects<sup>3</sup>, have evolved multiple times. In plants, convergence has led to repeated transitions in flower colour<sup>4,5</sup> and scent profiles<sup>6</sup> to attract pollinators, multiple origins of parasitic lifestyles<sup>7</sup> and a multitude of other traits. Many convergent traits are genetically simple or perform secondary functions that are not linked to core metabolism. A notable exception is the repeated modification of one of the most fundamental processes on Earth, photosynthesis. Although the machinery involved in the sequestration of light energy is mostly conserved from cyanobacteria to flowering plants, the biochemical pathways involved in the capture of atmospheric carbon vary quite widely across photosynthetic organisms. In  $C_3$  photosynthesis, which is used by most plants, atmospheric  $CO_2$  is directly fixed by the Calvin cycle via the enzyme Rubisco. However, Rubisco has affinities for both

$CO_2$  and  $O_2$  (REF.<sup>8</sup>), and the processing of fixed  $O_2$  releases  $CO_2$  and results in energetically wasteful reactions<sup>9</sup>. To circumvent this problem, land plants have repeatedly evolved carbon concentrating mechanisms (CCMs) known as  $C_4$  photosynthesis and crassulacean acid metabolism (CAM), which consist of both anatomical and biochemical adaptations that internally concentrate  $CO_2$  before its fixation by Rubisco (FIG. 1), thereby making photosynthesis more efficient<sup>10,11</sup>.

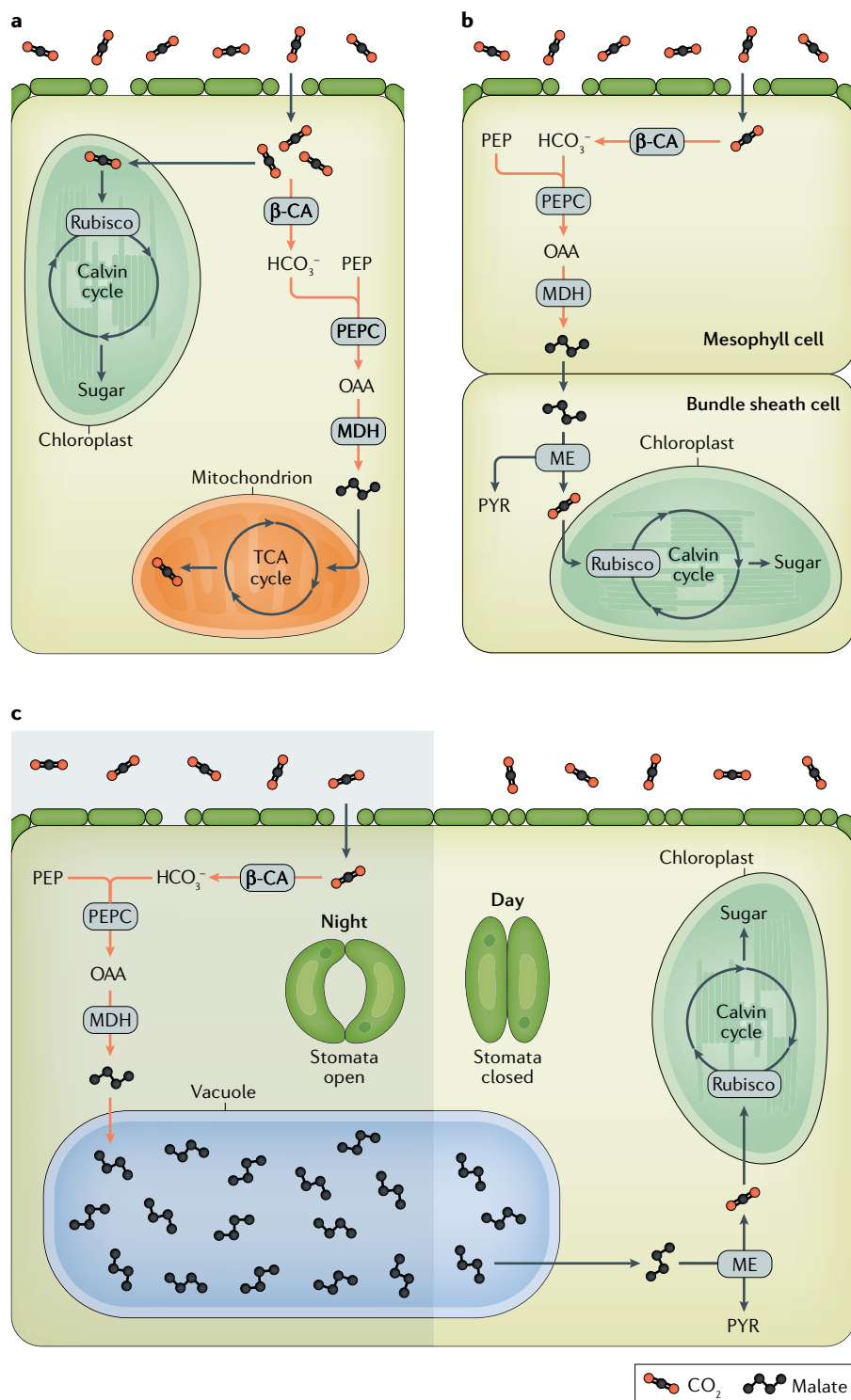
Over the past 25 years, improvements in our understanding of plant phylogenetic relationships have been instrumental in assessing the number and timing of CCM origins<sup>12,13</sup>, their relationship with environmental factors<sup>14–17</sup> and the history of associated phenotypic changes<sup>18–21</sup>. Both  $C_4$  and CAM photosynthesis represent textbook examples of convergent evolution, each having likely evolved independently more than 60 times<sup>22,23</sup>. On one hand, it is remarkable that CCMs could have evolved so frequently because of their seeming complexity and the fact that some relevant mutations would affect the primary

metabolism of a plant. In this context, it might be supposed that maladaptive mutations would be quite common and that the evolutionary trajectory of each CCM might be exceedingly narrow and rarely stumbled upon<sup>24,25</sup>. On the other hand, the sheer number of CCM origins argues precisely the opposite — these traits must be relatively simple to evolve and might be assembled via multiple and varied trajectories and, as such, are evolutionarily accessible (*sensu* Maynard Smith<sup>26,27</sup>).

In this Perspective, we highlight evidence that supports the view that CCMs are evolutionarily accessible, with a focus on the genetic elements that might give rise to their repeated origins. We first review shared key components of and differences between  $C_4$  and CAM photosynthesis, both of which use similar biochemical pathways. We highlight recent comparative studies in well-sampled clades that suggest that a newly emerging, rudimentary CCM may be less complex than many of the highly optimized  $C_4$  and CAM species that are commonly studied. We briefly review the roles of CCM enzymes in  $C_3$  species to emphasize that, at the broadest scale, all plants routinely express the essential biochemical building blocks of a functional CCM pathway. In this context, we discuss new evidence that some lineages may be genetically predisposed to evolve CCMs. Throughout the article, we emphasize how the growing field of comparative genomics is already catalysing discovery and improving our understanding of CCM evolution.

## $C_4$ and CAM photosynthesis

Both  $C_4$  and CAM photosynthesis work to reduce photorespiration, which occurs when Rubisco binds to  $O_2$  instead of  $CO_2$ . Plant photorespiration increases with temperature owing to the decreased solubility of  $CO_2$  and the reduced specificity of Rubisco at higher temperatures<sup>28</sup>. Photorespiration also increases with abiotic stresses, such as drought, that force stomata to close and prevent  $CO_2$  from entering the leaf and reaching Rubisco<sup>29</sup>. It is thought that photorespiratory stress is the major driver of  $C_4$  and CAM evolution<sup>25,30</sup>, and many plant species inhabiting hot and dry habitats use one of these CCMs. Both CCMs require that acquisition of atmospheric  $CO_2$  is separated from  $CO_2$  fixation via the Calvin cycle



**Fig. 1 | Biochemistry of  $\text{C}_3$  and CCM pathways.**  
**a** | The  $\text{CO}_2$  that  $\text{C}_3$  plants acquire from the atmosphere is largely fixed by Rubisco in the Calvin cycle. Small amounts of  $\text{CO}_2$ , mainly from respiration, can be used by anaplerotic pathways that replenish intermediates in the tricarboxylic acid (TCA) cycle. This process involves the conversion of  $\text{CO}_2$  to bicarbonate ( $\text{HCO}_3^-$ ) by a carbonic anhydrase ( $\beta\text{-CA}$ ) and then subsequent carboxylation of phosphoenolpyruvate (PEP) by a carboxylase (PEPC) to a four-carbon acid, oxaloacetate (OAA). The OAA is further converted to malate by malate dehydrogenase (MDH); finally, malate is fed into the TCA cycle.  
**b** | The  $\text{C}_4$  carbon concentrating mechanism (CCM) is largely composed of these same enzymes that are spatially separated to distinct compartments. In the most common type of  $\text{C}_4$  photosynthesis,  $\text{CO}_2$  diffuses into the mesophyll cells, where it is converted to malate via the same pathway ( $\beta\text{-CA}$ , PEPC and MDH). Malate is then transported into adjoining bundle sheath cells, where it is decarboxylated through one or two different malic enzymes (MEs), releasing  $\text{CO}_2$  for efficient carboxylation via Rubisco. This process also releases pyruvate (PYR), which can be used to regenerate the PEP substrate or for carbohydrate production. Although not shown here, in some  $\text{C}_4$  lineages, different intermediates are formed instead of malate (for example, aspartate), and other decarboxylases exist, which require slightly different biochemistry and transporters.  
**c** | Crassulacean acid metabolism (CAM) species are nearly identical to  $\text{C}_4$  plants in their biochemical pathway of carbon acquisition and fixation, although separation of uptake and fixation occurs temporally rather than spatially. At night,  $\text{CO}_2$  is taken up and converted to malate in the same manner as in  $\text{C}_4$  plants but then stored in the vacuoles. During the day, the stomata close and malate is decarboxylated in the cytosol, again flooding Rubisco with high concentrations of  $\text{CO}_2$ . Decarboxylation can occur in a variety of locations within the cell, including the cytoplasm, mitochondria and chloroplasts.

BS cells<sup>32,33</sup>. In  $\text{C}_4$  plants, spatial separation of reactions results in a tenfold increase in  $\text{CO}_2$  in the BS cells relative to M cells<sup>34,35</sup> and enables efficient carbon fixation in conditions where  $\text{CO}_2$  is limiting, such as warm, open habitats in the low- $\text{CO}_2$  atmosphere that has prevailed since the Oligocene<sup>12,36,37</sup>. Typically occupying high-light habitats,  $\text{C}_4$  plants have the highest measured photosynthetic rates of all plants owing to the extremely high carboxylation rates of the Calvin cycle when it is not limited by either  $\text{CO}_2$  or light reaction products<sup>38</sup>. Examples of  $\text{C}_4$  plant species include maize (*Zea mays*), sugarcane (*Saccharum officinarum*) and sorghum (*Sorghum bicolor*) in the grass family (Poaceae), as well as members of the sunflower family (*Flaveria*, Asteraceae) and amaranths (*Amaranthus*, Amaranthaceae).

and use the same set of enzymes to do so. However,  $\text{C}_4$  and CAM plants fundamentally differ in how this separation is achieved (FIG. 1).

**Spatial separation of  $\text{CO}_2$  uptake and fixation in  $\text{C}_4$  species.** In  $\text{C}_4$  plants,  $\text{CO}_2$  uptake and fixation occur in spatially distinct compartments within the leaf. In most  $\text{C}_4$  species, uptake and fixation occur in distinct mesophyll (M) cells and

bundle sheath (BS) cells, respectively (FIG. 1), although variations exist across independent origins of  $\text{C}_4$ , and in several cases  $\text{CO}_2$  assimilation and reduction take place in distinct regions of a single cell<sup>31</sup>. Specific leaf properties are required to sustain synchronized  $\text{C}_4$  reactions across two distinct cell types, including high leaf vein density, high BS:M cell volume ratio and the localization of most chloroplasts to

**Temporal separation of CO<sub>2</sub> uptake and fixation in CAM species.** In CAM species, the carbon uptake and fixation reactions are temporally distinct. CO<sub>2</sub> is primarily acquired at night when transpiration rates are low and is converted to malate and stored in vacuoles as malic acid. During the day, stomata close and malic acid is decarboxylated, resulting in increased CO<sub>2</sub> concentrations around Rubisco<sup>39</sup>. The large amount of malic acid stored in CAM plants demands bigger vacuoles and cells, and therefore CAM is generally associated with a succulent leaf phenotype. The temporal configuration of reactions, the inverted stomatal behaviour and the low transpirational demand at night help CAM plants achieve the highest water use efficiencies in the plant world<sup>40</sup>. As a result, many desert species are CAM plants, including cacti (Cactaceae), agaves (Asparagaceae), and euphorbs (Euphorbiaceae). Less intuitively, many tropical forest plants such as orchids (Orchidaceae) and bromeliads (Bromeliaceae) also use CAM; these are mostly epiphytes (plants that grow on other plants) and thus can also experience substantial drought stress.

#### Alternative and variant CCMs.

C<sub>4</sub> and CAM are quite distinct from C<sub>3</sub> photosynthesis, but a number of intermediate photosynthetic forms also exist. A third type of CCM, sometimes called C<sub>2</sub>, concentrates CO<sub>2</sub> in BS cells by restricting elements of the photorespiratory cycle to those cells; C<sub>2</sub> has long been supposed to be a precursor to C<sub>4</sub> (REFS<sup>30,41</sup>), although it is theoretically not necessary for C<sub>4</sub> evolution<sup>42</sup>. A variety of other phenotypes that are intermediate between C<sub>3</sub> and C<sub>4</sub>, termed C<sub>3</sub>+C<sub>4</sub>, have been identified, which have a limited C<sub>4</sub> photosynthetic cycle, sometimes in addition to a C<sub>2</sub> cycle<sup>41</sup>. CAM photosynthesis, unlike C<sub>4</sub>, is exceptionally phenotypically plastic because all photosynthetic cells in CAM plants still have a fully functional C<sub>3</sub> cycle. As a result, many CAM species can perform varying degrees of CAM or C<sub>3</sub> carbon fixation depending on their developmental stage or on environmental conditions<sup>43,44</sup>. For example, there are many so-called C<sub>3</sub>+CAM species, which predominantly rely on C<sub>3</sub> photosynthesis but fix a small amount of carbon via the CAM pathway nocturnally<sup>45–47</sup>. Some plant species can use both C<sub>4</sub> and CAM in a single individual<sup>48</sup> (FIG. 2a), although these instances are very rare. In general, intermediate C<sub>3</sub>+CAM, like C<sub>2</sub> or C<sub>3</sub>+C<sub>4</sub>, is thought to be an evolutionary precursor to a full CAM metabolism<sup>23,46</sup>.

#### Insights from comparative genomics

The regulation of cellular and temporal compartmentalization of CCM biochemistry is complicated, and, unsurprisingly, the genomics era has facilitated a new surge of research into the genetics of these complex traits. The past few years have seen the rapid production of genomic resources for an increasing number of species. The first genome of a C<sub>4</sub> plant (*S. bicolor*) was assembled in 2009 (REF<sup>49</sup>), and the first CAM genome (*Phalaenopsis equestris*, an orchid) was assembled in 2015 (REF<sup>50</sup>), and genomes and transcriptomes are now being sequenced for a variety of plants spanning CCM — including *Z. mays* (maize) and *Ananas comosus* (pineapple)<sup>50,51</sup> — and non-CCM species<sup>52</sup>. While fully sequenced reference genomes are still relatively cost-prohibitive for plants with large genomes, sequencing expressed genes via transcriptomics can be done at substantially lower cost and is much easier to perform in non-model systems. Although transcriptomes are unable to uncover regulatory regions and other non-coding sequences, they provide information on relevant changes in gene expression as well as any changes to gene sequences and possible selection across the genome. The growing taxonomic diversity of genomic resources will permit well-designed comparative analyses to better identify the genetic changes underlying the evolution of new phenotypes, such as a CCM.

**Broad comparisons likely overestimate CCM complexity.** Examination of only extant diversity, which is often an unavoidable constraint, will likely overestimate the number of changes required for an initial transition from C<sub>3</sub> photosynthesis to a CCM.

In general, comparisons between groups chosen because they differ in one particular aspect of their phenotype will also capture many unrelated differences, and the number of these irrelevant differences will increase with the evolutionary distance separating the groups being compared. Therefore, when traits are measured that differ between CCM and non-CCM lineages, the captured changes are not necessarily required for CCM evolution, as they may also include those that have occurred before or after the CCM origination (BOX 1; FIG. 2b). Although the changes that occur after the origin of the CCM are important for understanding CCM diversity, function and especially optimization, they may complicate an evaluation of the relative ease or difficulty with which CCMs have evolved.

Historically, many genomic studies of CCM evolution have relied on comparisons between distantly related C<sub>3</sub> and CCM taxa<sup>53,54</sup>, largely because genomic resources were only available for model systems. Such broad evolutionary comparisons between C<sub>3</sub> and CCM species have often highlighted hundreds to thousands of differentially expressed genes<sup>55–57</sup>. Although such studies have been foundational for developing comparative techniques and hypotheses, more recent work has focused on closely related C<sub>3</sub> and CCM taxa for finer-scale resolution of the genetic underpinnings of CCMs<sup>30,45,58,59</sup>. In *Flaveria* (Asteraceae), a lineage comprising closely related C<sub>3</sub>, C<sub>4</sub>, C<sub>2</sub> and C<sub>3</sub>+C<sub>4</sub> species, comparative studies found hundreds to over a thousand differentially expressed transcripts between different photosynthetic types<sup>51</sup>. Similarly, roughly 600 transcripts were differentially expressed in closely

#### Glossary

##### Anaplerotic

Chemical reactions that provide intermediates to various metabolic pathways, including the tricarboxylic acid (TCA) cycle.

##### Carboxylation

The addition of a carboxyl group to a substrate, often via a carboxylase enzyme.

##### Co-option

The recruitment of a gene, enzyme or other trait for an alternative function.

##### Decarboxylated

Pertaining to a molecule from which a carboxyl group has been removed by a decarboxylase enzyme in a process that releases CO<sub>2</sub>.

##### Duons

Portions of the genome that both code for amino acids and provide motifs that can regulate gene expression.

##### Gene flow

Movement of genetic information between populations.

##### Genome-wide association studies

Analyses that correlate genetic markers from across the genome with a phenotype of interest in order to find loci underlying traits.

##### Lateral gene transfer

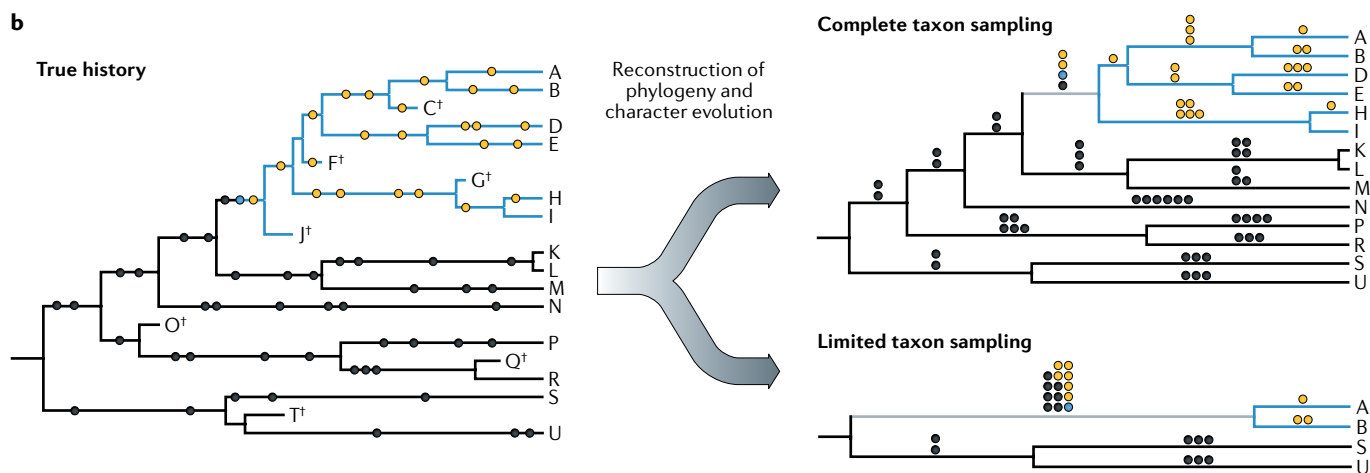
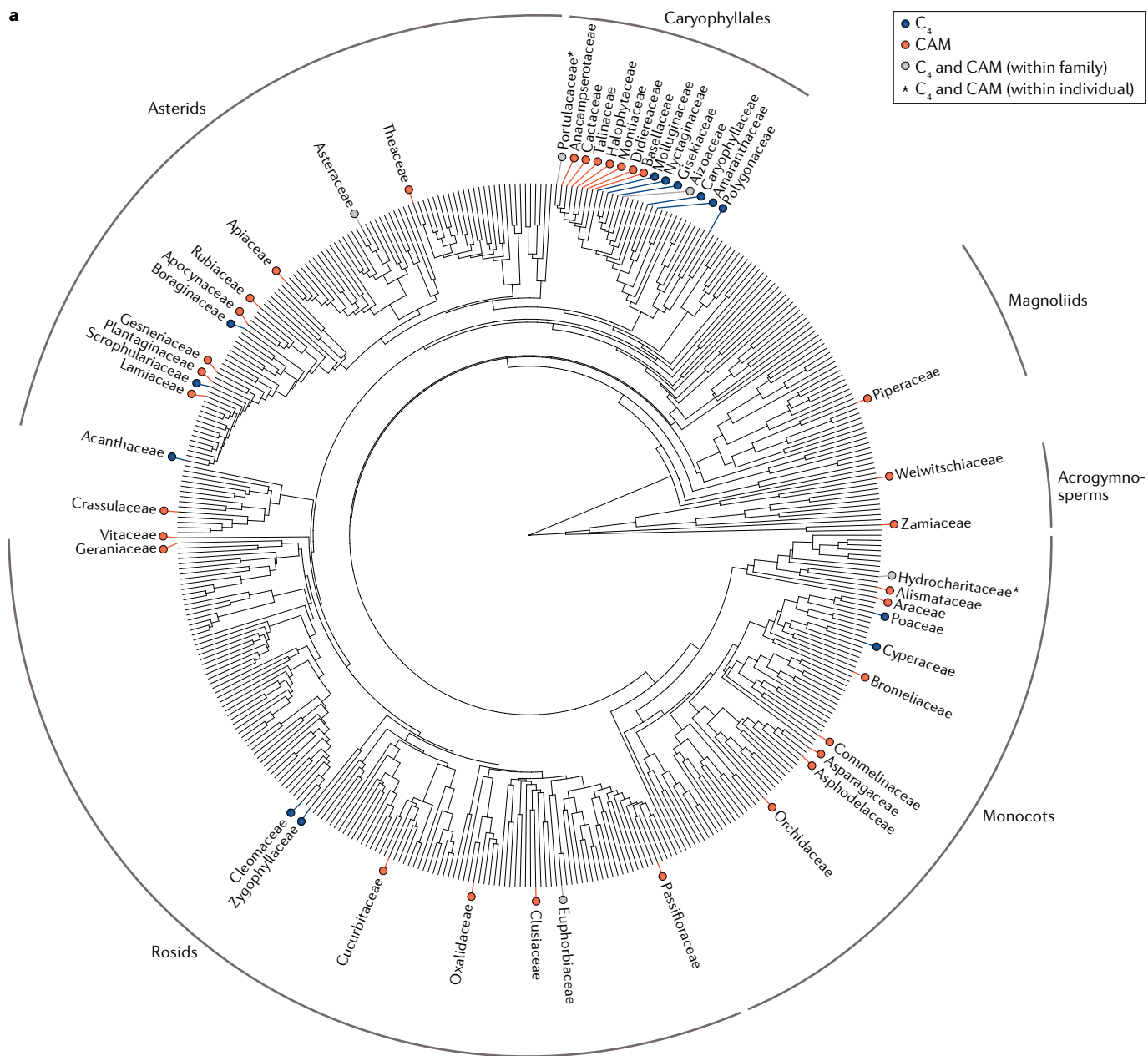
Movement of genes between individuals by mechanisms other than sexual reproduction.

##### Photorespiration

Fixation of oxygen by Rubisco, resulting in a loss of energy and a release of CO<sub>2</sub> but no net gain in carbohydrates.

##### Transpiration

The passive movement of water via stomata from the leaf intercellular airspace to the atmosphere along the water concentration gradient.





◀ **Fig. 2 | Evolutionary patterns of CCMs.** **a** | Lineages with carbon concentrating mechanisms (CCMs) are distributed across the phylogeny of vascular plants. In most cases,  $C_4$  and crassulacean acid metabolism (CAM) are found in distinct lineages (blue and orange family labels, respectively); however, families in which there are examples of both  $C_4$  and CAM photosynthesis exist (grey labels). Indeed, some individuals within some of these families (Portulacaceae and Hydrocharitaceae, indicated by asterisks) can use both  $C_4$  and CAM photosynthesis. Only CCM lineages are labelled. Figure created using phylogeny from REF.<sup>123</sup>. **b** | Even with phylogenetic information, comparative studies can still inflate the number of changes required for a CCM to evolve. For example, in a theoretical tree of extant and extinct (†) taxa labelled A to U, a single origin of a CCM occurred at the blue dot. All blue branches indicate portions of the tree where the CCM exists. Black dots represent changes in ancestral  $C_3$  species that pre-date the CCM origin, and yellow dots are changes that occurred after the CCM origin that may or may not be related to the CCM itself. If all extant taxa could be sampled, the number of changes inferred on the branch where the CCM occurred is relatively low; in a more realistic case, where only a subset of extant species are sampled, a greater number of changes are assigned to the branch with the CCM origin, even though many are unrelated to CCM evolution.

*Salsola divaricata*<sup>59</sup> and in the  $C_3$ +CAM intermediate hybrid *Yucca gloriosa*<sup>67</sup>. In addition, variation between accessions of the  $C_4$  species *Gynandropsis gynandra* has been proposed as a system to identify the genetic determinants of  $C_4$  photosynthesis<sup>68</sup>. The development of model systems in a greater number of species, particularly those with intermediate or variable CCM usage, will help determine whether patterns seen in *Alloteropsis* and *Flaveria* are representative of CCM evolution more generally. The generation of genomic resources for new and existing model species will enable genome-wide association studies and other approaches to detect the determinants of CCMs and potentially identify the precise genetic changes required for the initial emergence of a CCM and its subsequent adaptation.

related  $C_3$  and  $C_4$  members of Cleomaceae<sup>58</sup>. Even by reducing evolutionary distance, comparative transcriptomic studies between closely related species are still likely inflating the estimates of genetic changes required for CCM origins.

**Emerging model systems to study CCM evolution.** Model clades such as *Flaveria* will continue to be highly relevant to CCM evolution, but the problem of overestimating complexity can be reduced further. Ideally, a spectrum of photosynthetic phenotypes, all within a single species, would be studied to capture the emergence of a rudimentary CCM. Such a system is currently being developed in the grass species *Alloteropsis semialata*<sup>60</sup>. The existence of  $C_3$  and  $C_4$  genotypes of *A. semialata* has been known for some time, but recent work has demonstrated a diverse spectrum of physiology that spans  $C_3$  and  $C_4$  in important ways. Phenotypically intermediate populations of *A. semialata* that perform a weak  $C_4$  cycle have been identified<sup>61</sup>, and genome-wide analyses have provided evidence of genetic exchange between different photosynthetic types<sup>62</sup>.  $C_4$  populations of *A. semialata* show incomplete segregation of Rubisco into BS cells<sup>63</sup> and have a photosynthetic efficiency below that of other  $C_4$  grasses<sup>61</sup> and can, therefore, be considered as having a rudimentary CCM. Comparative work has indicated that the initial emergence of  $C_4$  photosynthesis in the group involved upregulation of only a handful of genes<sup>64</sup>. Subsequent refinement of the CCM in *Alloteropsis* is supported by the presence of genes better suited for the  $C_4$  context in only some of the  $C_4$  populations of *A. semialata* and by evidence of sustained positive selection within the  $C_4$  group<sup>64,65</sup>.

Although distinct  $C_3$  and  $C_4$  populations have not yet been reported in species other than *A. semialata*, we predict that new efforts to broadly screen wild populations of young CCM species will reveal subtle

but important variation. For example, it is already known that the strength of the  $C_4$  cycle varies between accessions of the  $C_3$ + $C_4$  intermediate *Mollugo verticillata*<sup>66</sup>, potentially between populations of

#### Box 1 | Refinement of a CCM pathway

Although all plant lineages possess the enzymatic machinery for a carbon concentrating mechanism (CCM), and some lineages appear to be potentiated for CCM evolution, phylogenetic analyses indicate that a subset of changes have occurred after the origin of the CCM pathway and are, therefore, likely to be involved in its refinement<sup>68</sup>.

##### Refinement of $C_4$ pathways

Much more is known about when key post-origin CCM traits evolved in  $C_4$  lineages than for crassulacean acid metabolism (CAM) lineages owing largely to more robust phylogenetic work in important  $C_4$  lineages, predominantly the grasses.

**Anatomical specialization.**  $C_4$  species arising from a single origin of the CCM show variation in anatomical traits important for  $C_4$  function, such as vein spacing and bundle sheath cell size<sup>19,106–108</sup>, which suggests that different lineages have different post-origin anatomical improvements for CCM activity.

**Gene evolution.** Although the core set of CCM enzymes is present in all plants, analyses of their amino acid sequence in  $C_4$  lineages suggest they undergo improvement after the origin of the CCM. For example, some core CCM enzymes are known to vary in their amino acid sequence within  $C_4$  lineages<sup>98</sup>, molecular evolution studies indicate that certain residues are under positive selection within  $C_4$  species<sup>47,109,110</sup>, and convergent substitutions have been identified that are restricted to some  $C_4$  species within each group<sup>111–113</sup>. Additionally, phylogenetic analyses indicate that a number of gene duplication events that allowed for enzyme adaptation occurred after a CCM origin<sup>114</sup>.

##### Refinement of CAM pathways

Post-origin changes are thought to be required for the refinement of constitutive CAM activity, but such changes are not necessarily required in  $C_3$ +CAM species or in taxa that have recently evolved a CCM. However, the timing of these changes is largely unknown owing to a lack of systematic comparisons between  $C_3$ ,  $C_3$ +CAM and taxa with strong CAM activity. Candidates for modifications that might occur after the origin of a CAM pathway include the following.

**Stomatal regulation.** Although some  $C_3$  species are known to open their stomata at night<sup>115,116</sup>, the inverse stomatal behaviour observed in CAM species (FIG. 1) likely requires large transcriptional reconfiguration. In particular, CAM species likely modify pathways involving abscisic acid and blue light sensing, two of the main pathways for stomatal signalling. Blue light in particular is a major signal for stomatal aperture, and some experiments on blue light sensitivity and transcriptomic analyses have suggested that guard cells in CAM species are less sensitive to blue light than guard cells in  $C_3$  species<sup>117–120</sup>.

**Circadian regulation.** Some CAM enzymes show clear circadian behaviour<sup>121</sup>, and, indeed, integration of carbon fixation and metabolism with an endogenous clock is thought to be critical for efficient CAM function<sup>121,122</sup>. Comparative genomics between pineapple (*Ananas comosus*, Bromeliaceae) and unrelated  $C_3$  and  $C_4$  species showed an increase in clock-regulated regulatory motifs upstream of canonical CAM genes (FIG. 1c), indicating circadian control of gene expression<sup>87</sup>. Although it is likely that fine-scaled circadian control of CAM gene expression occurs only with or after the origin of CAM, further work is needed to show circadian regulation as a refinement of the CAM cycle.

### All plants have key CCM elements

The known components of the CCM biochemical pathway are present in all vascular plants (FIG. 1), and the co-option of existing enzymes is a straightforward mechanism by which CCMs could initially develop. The core mechanism of carbon concentration — the fixation of CO<sub>2</sub> by the coupled action of the enzymes carbonic anhydrase and phosphoenolpyruvate carboxylase (PEPC) — is part of an anaplerotic pathway that supplements malate to the tricarboxylic acid (TCA) cycle and exists in the cytosol of all plants<sup>69</sup>. In a non-CCM plant, a rise in the concentration of cytosolic bicarbonate (HCO<sub>3</sub><sup>-</sup>), for example, from an increase in respired CO<sub>2</sub>, could result in increased malate production. If HCO<sub>3</sub><sup>-</sup> levels are high enough, PEPC may generate more malate than the maintenance pathways can accommodate. In this scenario, the high malate concentration would typically feed back to inhibit PEPC<sup>70</sup>. However, if excess malate instead became sequestered in the vacuole or diffused to adjacent cells along a concentration gradient, this feedback would not occur, and a rudimentary CCM cycle could be established. Thus, the upregulation of a few key enzymes might be sufficient to initiate CCM evolution.

The non-photosynthetic roles of some CCM enzymes in C<sub>3</sub> species may also support the view that CCMs are evolutionarily accessible. For example, non-photosynthetic CCM pathways that act in a tissue-specific manner have been identified in several C<sub>3</sub> species: low-level nocturnal CO<sub>2</sub> fixation without malic acid accumulation occurs in tobacco leaves<sup>71</sup> and in cotton ovules<sup>72</sup>, and a C<sub>4</sub>-like mechanism is found in cells adjacent to the vasculature of tobacco stems, whereby photosynthetic cells use CO<sub>2</sub> that has been respired by roots and transported through the xylem<sup>73</sup>. Genes that give identity to root endodermal cells have also been shown to pleiotropically affect differentiation of BS cells in the leaf, which are important for C<sub>4</sub>: SCARECROW and SHORTROOT mutants have deformed endodermal cell layers in the roots and a proliferation of BS cells in the leaves<sup>74,75</sup>. Perhaps most notably, *Camellia oleifera* (Theaceae, Ericales), a mesic forest tree, has been shown to respond to leaf fungal infection by strongly inducing tissue succulence and a fully functional CAM cycle<sup>76</sup>. The upregulation of a complete CAM cycle in a species so distantly related from any known CCM origin emphasizes both the ubiquitous presence of functional CCM enzymes in all plants and their

frequent co-option into a variety of cellular roles.

### Potential for CCM evolution

CCM origins seem to be phylogenetically clustered, with large regions of the land plant phylogeny completely lacking any known C<sub>4</sub> or CAM plants<sup>23</sup> (FIG. 2a). Thus, although all plants have the biochemical components for a CCM, some lineages may be more likely to evolve CCMs because of differential exposure to environmental pressures or the evolution of characteristics — either anatomical or genomic — that later facilitate the co-option of these enzymes into photosynthetic metabolic pathways.

**Anatomical potentiation.** The idea that some lineages may be potentiated (sensu Blount<sup>77</sup>) for CCM evolution has often been discussed<sup>28</sup>, but primarily in the context of leaf anatomy. The spatial separation of carbon assimilation and fixation via Rubisco in C<sub>4</sub> plants is generally realized across distinct cell types<sup>32,33</sup> (FIG. 1). The typical C<sub>4</sub> anatomical traits, such as large BS:M cell volume ratio and small distance between veins, have evolved before the C<sub>4</sub> pathway in a number of lineages including grasses<sup>19</sup>, *Flaveria*<sup>18</sup> and Cleomaceae<sup>78</sup>, suggesting that C<sub>4</sub> origins are clustered in certain regions of the plant phylogeny<sup>79</sup> (FIG. 2a) because some lineages happened to first evolve a C<sub>4</sub>-like anatomy. Anatomical potentiation could have arisen either from environmental pressures or stochastic processes; regardless, the establishment of a C<sub>4</sub>-like anatomy then allowed for frequent transitions to C<sub>4</sub> photosynthesis within these lineages<sup>64</sup>.

The phylogenetic distribution of CAM photosynthesis appears to be much less clustered, and the evidence of anatomical enabling is mixed<sup>46,80</sup> (FIG. 2a). Many known C<sub>3</sub>+CAM species are only mildly succulent, whereas species with strong CAM activity typically have large, tightly packed cells and thick photosynthetic tissues<sup>80,81</sup>. In the Agavoideae, tissue succulence seemingly evolved before the emergence of strong CAM photosynthesis<sup>82</sup>, but there are few studies that combine anatomy, CAM activity and a well-resolved and sampled phylogeny. Although more studies are needed, the lack of identifiable anatomical specialization in C<sub>3</sub>+CAM plants suggests that leaf anatomical changes are likely necessary for the development of strong CAM activity but not for the evolution of a weak CAM cycle.

**Genetic potentiation.** Similar to environment and anatomy, genetic characteristics could also predispose certain lineages to evolve a CCM. In C<sub>4</sub> and CAM lineages, CCM enzymes need to be expressed at specific locations<sup>83–85</sup>, times<sup>86,87</sup> and levels<sup>88,89</sup>. Non-CCM lineages that have regulatory pathways in place that could confer these properties on CCM enzymes might be predisposed to evolving CCMs. Indeed, genome-wide comparisons and transcription factor binding assays between C<sub>3</sub> and C<sub>4</sub> species have revealed shared regulatory motifs<sup>90,91</sup>. For example, a simple 59 bp deletion upstream of a gene encoding the P-subunit of glycine decarboxylase in the C<sub>3</sub> species *Arabidopsis thaliana* is sufficient to confer BS cell-specific expression, which is required for C<sub>2</sub> biochemistry<sup>92</sup>. Another *A. thaliana* gene, *NAD-me*, which encodes a CCM decarboxylating enzyme that also has roles in mitochondrial and chloroplast housekeeping, contains motifs within its coding sequence called *duons* that have regulatory functions. These sequences are also found in the C<sub>4</sub> species *G. gynandra*, in which they are necessary and sufficient for BS cell-specific expression<sup>93</sup>. Thus, these sequences confer C<sub>4</sub> activity through changes to gene regulation rather than via alterations to the coding sequence of the enzyme. Orthologues of C<sub>4</sub> genes in C<sub>3</sub> species have also been shown to be transcriptionally induced by light<sup>84,94</sup> and regulated by chloroplast-to-nucleus signalling<sup>95</sup>, two characteristics of many enzymes in the C<sub>4</sub> pathway. The prevalence of shared regulatory DNA sequences and transcriptional cascades in C<sub>3</sub> and CCM species suggests that pre-existing regulatory mechanisms may facilitate the repeated evolution of C<sub>4</sub> photosynthesis in certain lineages.

Genetic potentiation can also occur in certain groups as a result of genome-wide patterns and processes. In some cases, the ability of plant genomes to undergo dramatic reconfiguration may have facilitated the evolution of a CCM. For example, in C<sub>4</sub> maize, hundreds of DNA motifs associated with C<sub>4</sub> have apparently been moved around the genome by transposons<sup>96</sup>. Lateral gene transfer has even played a role in CCM evolution in certain cases; genes optimized for C<sub>4</sub> function were laterally transferred to *A. semialata* from distantly related C<sub>4</sub> grasses and are preferentially expressed relative to the native gene copies<sup>64</sup>. Hybridization between C<sub>3</sub> and CAM species in *Yucca* gave rise to a new C<sub>3</sub>+CAM species<sup>67</sup>, and population gene flow between

C<sub>4</sub> and non-C<sub>4</sub> genotypes in *A. semialata* facilitated movement of C<sub>4</sub>-adapted alleles across the range of the species<sup>62</sup>. Additionally, in some CCM lineages, duplicated genes are associated with co-option into CCM pathways<sup>97–100</sup> and subsequent positive selection<sup>101,102</sup>. By contrast, other studies have indicated that recruitment of genes into a CCM was not immediately preceded by a gene duplication event but instead relied on differentiation of *cis*-regulatory elements in ancient paralogues<sup>50,85,87</sup>. The evidence from such genomic comparative studies suggests that re-wiring of transcriptional cascades — whether through gene duplications, genomic rearrangements or molecular evolution — is critical for CCM evolution.

### Future perspectives

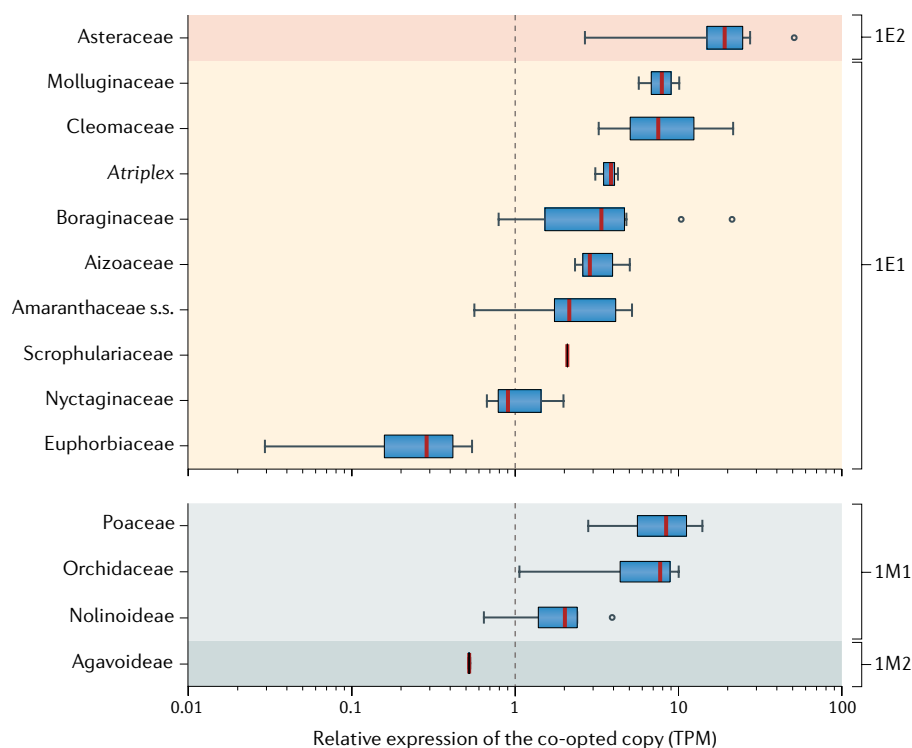
As sequencing costs decrease and protocols become more streamlined, gene expression data for non-model species are becoming increasingly available, which allows relative gene expression to be compared across hundreds or thousands of species in a single analysis. As a simple illustration of the types of studies we imagine will become increasingly common and insightful, we analysed the relative levels of expression of major PEPC gene copies in leaves of C<sub>3</sub> species across flowering plants (using 1KP data<sup>103,104</sup>, Supplementary Methods, Supplementary Figure 1 and Supplementary Table 1). PEPC is the best studied enzyme in the CCM pathway and is an exceptional system to explore the mechanics of gene recruitment into a novel function. Most flowering plants have at least three main copies of genes encoding PEPC, with two of those copies arising from independent duplication events in eudicots and monocots<sup>48,105</sup> (Supplementary Figure 1). Studies in grasses and orchids have suggested that the copy that is most highly expressed in C<sub>3</sub> relatives is the one recruited into the CCM<sup>88,89</sup>. We specifically focused on C<sub>3</sub> species in flowering plant lineages that have evolved CCMs, in which the copy recruited into the CCM is known from empirical studies. Because we relied on transcriptomic data rather than genomic data, we summed the expression of multiple assembled transcripts per PEPC paralogue per species. Even so, in 12 of the 14 CCM-evolving lineages, the copy recruited into the CCM is more highly expressed than other PEPC copies in the C<sub>3</sub> taxa (FIG. 3). While the recruitment bias is likely driven by expression levels of the two copies of PEPC, the differences in the expression

patterns could result from a variety of processes. Perhaps these copies are more highly expressed because of a dosage effect caused by local gene duplications (we did not quantify lineage-specific copy numbers per PEPC paralogue), which could then allow for subsequent specialization of paralogues<sup>100</sup>. Alternatively, the increased transcript abundance of one copy over the other might have been selected for because of a non-photosynthetic role of PEPC, which simultaneously made it amenable to co-option by a CCM. Although our analysis is based on a broadly sampled transcriptome data set that was not designed for this sort of question, the emergence of such a striking pattern reveals the great potential

of comparative genomics to identify the genetic potentiation of CCM evolution. Although the use of existing data sets such as 1KP for analysis of CCM evolution offers exciting possibilities, better phylogenetic resolution of CCM-evolving lineages will continue to expand the availability of model systems that are particularly powerful for assessing initial genetic changes associated with CCM evolution.

### Conclusions

In this Perspective, we have outlined evidence supporting the idea that relatively few modifications are required to reach a rudimentary CCM phenotype, at least in groups that are potentiated. If correct, this



**Fig. 3 | Expression of PEPC in the leaves of C<sub>3</sub> angiosperms belonging to CCM-evolving lineages.** We used the 1KP<sup>103</sup> database to assess whether phosphoenolpyruvate carboxylase (PEPC) copies upregulated in C<sub>3</sub> species predict which copy is co-opted in C<sub>4</sub> or crassulacean acid metabolism (CAM) origins within the same lineage. Gene expression for PEPC was assessed per species for the major clades of the gene family: PPC-1E1 and PPC-1E2 in eudicots (top panel) and PPC-1M1 and PPC-1M2 in monocots (bottom panel) (Supplementary Figure 1). Expression was summed across all contigs from the transcriptomes of species that, based on phylogenetic analysis, were within each of the major clades (Supplementary Methods and Supplementary Table 1). Expression of PPC-2, a separate paralogue of PEPC, was not considered here, as it is not commonly recruited to a carbon concentrating mechanism (CCM). The background colour indicates which copy is used by the C<sub>4</sub> or CAM species within a given lineage, and the box plots represent the log of the ratio of expression (in transcript per million (TPM)) of the co-opted copy to the other copy for all species within that lineage. Clades for which the box plot falls to the right of the dashed line co-opt the more highly expressed copy of PEPC, whereas box plots to the left of the dashed line indicate clades that have co-opted the lesser expressed copy of PEPC. For example, C<sub>4</sub> Asteraceae species use PPC-1E2 in the CCM pathway, and their C<sub>3</sub> relatives have higher expression of PPC-1E2 relative to PPC-1E1. In the majority of lineages assessed, the co-opted copy is more highly expressed than the other copy in C<sub>3</sub> species, suggesting that this quality is important for recruitment into a CCM and may actually facilitate CCM evolution in certain lineages.



hypothesis would help to explain how such a seemingly complex trait has evolved so frequently. Once a rudimentary CCM is operating, selection to improve its efficiency will be strong because it relates to the primary metabolism of plants. Over time, this selection will lead to the accumulation of additional traits that further optimize the new pathway and that strongly contrast with those of their non-CCM relatives. Although these specializations are important for understanding the nature of extant CCMs, they lead to an overestimation of the initial changes required to evolve a CCM and, therefore, to an underestimation of its evolutionary accessibility.

We envision that future comparative genomic studies will continue to refine our understanding of CCM evolution in two important ways. First, the accumulation of genomic data from an increasingly phylogenetically diverse species pool will facilitate robust comparative analyses across multiple CCM origins — in other words, we imagine a proliferation of refined versions of the preliminary 1KP analyses we presented here. Second, we predict that new data and analytical tools in population genomics will enable a renaissance of intraspecific studies of CCM variation and will uncover additional *A. semialata*-like systems with which to explore the evolutionary emergence of a nascent CCM. Taken together, these efforts will disentangle the genetic changes that are required for refinement of CCM evolution from those responsible for the origin of a CCM pathway. From a rich history of biochemical and genetic studies, our knowledge of plant CCM evolution may already be far ahead of other classical examples of convergence. In time, we predict that a growth of genomic resources in non-model systems has the potential to provide a fairly complete explanation of how  $C_4$  and CAM photosynthesis evolved so often in plants, and perhaps it is not so far away.

Karolina Heyduk<sup>1</sup>\*, Jose J. Moreno-Villena<sup>1</sup>, Ian S. Gilman<sup>1</sup>, Pascal-Antoine Christin<sup>2</sup> and Erika J. Edwards<sup>1</sup>

<sup>1</sup>Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA.

<sup>2</sup>Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, UK.

\*e-mail: karolina.heyduk@yale.edu

<https://doi.org/10.1038/s41576-019-0107-5>

Published online 18 March 2019

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## Acknowledgements

The authors thank members of the Edwards laboratory for their thoughtful discussions on this manuscript. K.H. is supported by a Donnelley Postdoctoral Fellowship through the Yale Institute of Biospheric Studies. Additional support came from US National Science Foundation awards DEB-1252901 and IOS-1754662 to E.J.E. P.-A.C. is supported by a Royal Society Research Fellowship (grant number URF120119).

## Author contributions

The authors contributed equally to all aspects of the article.

## Competing interests

The authors declare no competing interests.

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## Reviewer information

*Nature Reviews Genetics* thanks R. VanBuren and the other anonymous reviewer(s) for their contribution to the peer review of this work.

## Supplementary information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41576-019-0107-5>.