

EXPERT VIEW

Open questions on facultative C₄–CAM photosynthesis in *Portulaca*

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Abstract

Plants have evolved carbon-concentrating mechanisms (CCMs) to optimize carbon fixation under environmental conditions that increase photorespiratory and transpiration rates. C₄ and Crassulacean acid metabolism (CAM) photosynthesis are the two most widespread CCM examples across land plants and were typically considered to be incompatible due to biochemical, anatomical, evolutionary, and regulatory characteristics. However, *Portulaca oleracea* (purslane) was the first reported case to challenge this assumption >40 years ago. Although it remains one of the most characterized C₄–CAM species, many aspects of this dual system are still unresolved. This expert view summarizes recent advances in understanding C₄–CAM integration in *Portulaca*, highlighting key genomic, transcriptomic, biochemical, and physiological insights, and outlines the most pressing questions about the functioning and evolution of this combined CCM. Future directions for explaining how these convergent pathways emerged in the same lineage include functional studies on key regulatory elements, spatially resolved analyses for protein distribution and metabolic fluxes across leaves, an updated phylogeny of the family, and the integration of molecular and physiological data. So far, it is evident that expressing a weak CAM cycle in a C₄ crop might be possible and could entail increased survival, possibly contributing to higher productivity in a future with climate change.

Keywords: Abiotic stress, carbon-concentrating mechanism, C₄ photosynthesis, Crassulacean acid metabolism, drought stress, facultative CAM, PEPC, phosphoenolpyruvate carboxylase, photosynthesis, *Portulaca oleracea*.

Introduction

C₄ photosynthesis and Crassulacean acid metabolism (CAM) are two great examples of convergent evolution, repeatedly evolving at least 60 times for C₄ (~8100 species, Sage, 2017) and 66 for CAM (~23 900 species, Gilman *et al.*, 2023) in independent lineages. Together, these two carbon-concentrating

mechanisms (CCMs) occur in ~9% of all known vascular plant species (total number of species: 349 000 according to Plants of the World Online, <https://powo.science.kew.org/>). These numbers highlight that C₄ and CAM have a significant representativeness and have conferred specific adaptive advantages

Table 1. Characteristics of C₄ photosynthesis and CAM distinguishing these two CCMs

Aspect	C ₄ pathway	CAM pathway
Timing of CO ₂ fixation	Maximum CO ₂ fixation during the day corresponding to the illumination period.	Temporal separation: night-time CO ₂ fixation—stomata open; daytime CBBC and Rubisco activity—stomata closed for most of the day. However, transitional phases at dawn and dusk can vary according to environmental conditions.
Enzyme activity	PPC in mesophyll cells; Rubisco in bundle sheath cells; both active during the day.	PPC active at night; Rubisco active during the day; both activated in the same mesophyll cell.
Spatial organization	Initial fixation in mesophyll cells, CBBC and Rubisco in bundle sheath cells.	Carbon fixation in the mesophyll.
Anatomy	Requires spatial separation: either with Kranz anatomy (dual-cell C ₄ , with distinct mesophyll and bundle sheath cells), or the rare case of single-cell C ₄ (cell polarity).	Succulent tissues are common, also tight mesophyll packing with large vacuoles for malic acid storage.
Environment	Hot, high-irradiance climates with moderate drought.	Arid and hot climate (e.g. deserts).
Evolution (proposed models)	Stepwise, with the possibility of hybridization.	Phenotypes forming a continuum, with discrete distribution.

References for the information contained in the table are given in the text. CBBC, Calvin–Benson–Bassham cycle; PPC, phosphoenolpyruvate carboxylase.

on plants, leading to their repeated evolution. Still, C₄ and CAM biology has been historically analysed separately, resulting in an emphasis on their apparent biochemical, anatomical, and regulatory incompatibilities (Table 1). These incompatibilities arise from several well-established constraints. Without tight spatial or temporal control, nocturnal carboxylation and daytime decarboxylation could lead to repeated fixation and release of CO₂, resulting in energetically wasteful cycling. In addition, C₄ photosynthesis typically relies on a spatial division between mesophyll cells (MCs) and bundle sheath cells (BSCs), whereas CAM depends on large vacuoles and a clear diel separation of carboxylation and decarboxylation. These differences historically led to the assumption that C₄ and CAM could not operate together in the same leaf (Table 1). Classic works (e.g. Sage, 2002) mostly discuss these features and how they should hinder the stable co-existence of both mechanisms in a single cell. However, a few plant lineages challenge this notion of incompatibility, with recent evidence suggesting that some of these constraints can be at least partially circumvented under specific regulatory and anatomical configurations.

The first observation of C₄ and CAM operating in the same leaves was made in the common purslane (*Portulaca oleracea*) under water-limited conditions (Koch and Kennedy, 1980). After that seminal work, reports of newly discovered C₄–CAM species continued to emerge (Voznesenskaya *et al.*, 2010; Zhang *et al.*, 2014; Winter *et al.*, 2021; Siadjeu and Kadereit, 2024), but the mechanism and specifics enabling the C₄–CAM photosynthetic system still remain unclear. Although few, the available studies in the field focused mostly on *P. oleracea* and, to a lesser extent, *P. grandiflora* (Guralnick *et al.*, 2002, 2020). Until 2021, progress in understanding how C₄ and CAM co-exist was based mostly on enzyme activity and kinetics of the whole leaf (Ku *et al.*, 1981; Lara *et al.*, 2003, 2004), total leaf transcriptomics (Christin *et al.*, 2014;

Ferrari *et al.*, 2020a), or on defining whether the activation of the CAM pathway occurred in additional members of *Portulaca* (Holtum *et al.*, 2017; Winter *et al.*, 2019).

In the last 5 years, advances in genomics (Gilman *et al.*, 2022; Wang *et al.*, 2023), modulation of gene expression (Ferrari *et al.*, 2022), spatial transcriptomics, and the inclusion of modelling approaches (Moreno-Villena *et al.*, 2022) have added to our understanding of metabolic flexibility in response to stress and into how two complex traits can co-exist within *Portulaca*, yet many key questions remain. In this Expert view article, we cover recent developments and current knowledge about C₄–CAM plants, specifically focusing on the case of *P. oleracea*. We discuss the following questions. (i) How do C₄ and CAM pathways co-exist within the same leaf at the biochemical and regulatory levels? (ii) What evolutionary processes enabled the convergence of two CCMs within the same genus? (iii) Can this knowledge inform efforts to engineer metabolic flexibility in crops? (Table 2). These questions are the next steps in moving forward not only with understanding of C₄–CAM but also contributing directly to the individual fields of C₄ and CAM. We argue that the C₄–CAM system challenges more traditional categories of photosynthetic types and deserves more attention to improve our understanding.

To be or not to be: why plants perform C₄ or CAM

C₃ photosynthesis relies on the Calvin–Benson–Bassham cycle (CBBC) that incorporates CO₂ into three-carbon skeletons through the activity of Rubisco. This process yields photoassimilates essential for both plant metabolism and the broader ecological food chain. However, due to the oxygenase activity of Rubisco, inhibitory by-products are generated and need to

Table 2. Summary of the most pressing questions to understand the complex photosynthesis of *Portulaca oleracea*, merging C₄ and CAM in a single leaf

Main topics	Specific questions
How do C ₄ and CAM pathways co-exist within the same leaf at the biochemical and regulatory levels?	<ul style="list-style-type: none">• What role do enzymes such as phosphoenolpyruvate carboxylase kinase (PPCK) play in pathway regulation?• What transcriptional and post-transcriptional mechanisms regulate the switch between pathways?• How are metabolic fluxes coordinated?
What evolutionary processes enabled the convergence of two CCMs within the same genus?	<ul style="list-style-type: none">• What selective pressures favoured this convergence?• What genomic and regulatory elements enabled the integration of these pathways?• What insights can comparative genomics provide?
Can this knowledge inform efforts to engineer metabolic flexibility in crops?	<ul style="list-style-type: none">• How might synthetic biology approaches exploit this system for enhanced stress resilience?• Can aspects of this dual pathway be introduced into other species?

be metabolized through photorespiration. While this process evolved as a solution to recover carbon and detoxify the cell environment, there is an associated energetic and nutritional cost (Walker et al., 2024). Over the past 35 million years, declining atmospheric CO₂ levels have driven the evolution of two CCMs, C₄ and CAM, becoming the most widespread among land plants (Edwards and Ogburn, 2012). CCMs overcome the shortcomings of Rubisco under conditions that favour high rates of photorespiration, such as increased heat, drought, and salinity, which have become more prevalent over the same period.

Overall, C₄ and CAM biochemistry rely on similar molecular components, recruited from ancestral C₃ enzymes, reflecting on how complex traits often evolve by repurposing pre-existing molecular elements (Bräutigam et al., 2017; Sage, 2017). Both pathways use β-carbonic anhydrase (βCA), which catalyses the rapid interconversion of CO₂ to HCO₃⁻, providing substrate to phosphoenolpyruvate carboxylase (PPC). In CAM species, however, the temporal contribution of βCA is less well defined, with expression patterns varying across lineages. HCO₃⁻ serves as the substrate for PPC during the initial fixation step that carboxylates phosphoenolpyruvate (PEP), forming oxaloacetate (OAA). In the case of CAM, OAA is always converted to malate, but in C₄, either malate or aspartate (Asp) can be formed (Winter and Smith, 1996; Kanai and Edwards, 1999). Subsequently, three decarboxylases, namely NADP-malic enzyme (NADP-ME, chloroplastic), NAD-malic enzyme (NAD-ME, mitochondrial), or phosphoenolpyruvate carboxykinase (PEPCK, cytosolic) will release CO₂ close to Rubisco, ensuring carboxylation and reducing photorespiration. Different lineages use distinct decarboxylases, resulting in varied intermediate metabolites and sometimes combining multiple decarboxylation pathways, especially known in C₄ (Furbank, 2011).

Despite the shared biochemistry, the spatial and temporal organization of these processes differ between C₄ and CAM plants (Table 1). Although there are known examples of single-cell C₄ (Edwards et al., 2004), in most C₄ species the enzymatic

components of the pathway are divided between the leaf MCs and BSCs (Hatch and Slack, 1966; Hatch, 1971). On the other hand, CAM is a temporal process, taking place at different times of the day but in the same MCs (Osmond, 1978). Four phases have been traditionally reported for the physiology of CAM: malic acid formation and PPC activity with open stomata at night (Phase I); malic acid consumption with Rubisco activation under closed stomata during the day (Phase III); and transitional stages in between (Phases II and IV) (Osmond, 1978; Winter and Smith, 1996).

While the two pathways increase CO₂ availability close to Rubisco, the selection pressures driving their evolution are thought to be slightly different (Table 1). C₄ is usually attributed to a reduction of photorespiratory rates, and thus it occurs in habitats with high irradiance, for instance grasslands. CAM in terrestrial plants, possibly also as an adaptation to reduce photorespiratory rates, is commonly found in habitats with limited water availability such as deserts or the epiphytic environment, since reduced transpiration and a co-evolution with succulence resulted in a water-saving strategy (Edwards and Ogburn, 2012). Nevertheless, in aquatic environments, CAM evolution is usually associated with limited CO₂ availability (Keeley, 1998).

Some of the current models for C₄ evolution consider a step-wise trajectory including C₃–C₄ intermediates, with gradual changes in the MC to BSC ratio and the segregation of glycine decarboxylase and Rubisco into BSCs, while PPC is only found in MCs (Sage, 2004; Sage et al., 2012). However, more recently, C₃–C₄ intermediates have also been considered stable evolutionary stages on their own (Lundgren, 2020), and the contribution of hybridization for C₄ evolution has been discussed (Morales-Briones and Kadereit, 2023).

In contrast, the evolutionary path to CAM remains under debate, with a need to reconcile whether discrete and intermediate phenotypes can be found as proposed for C₄, or whether CAM species would show different degrees of activation forming a more inconspicuous continuum (reviewed in Edwards, 2023). In this case, CAM evolution would have

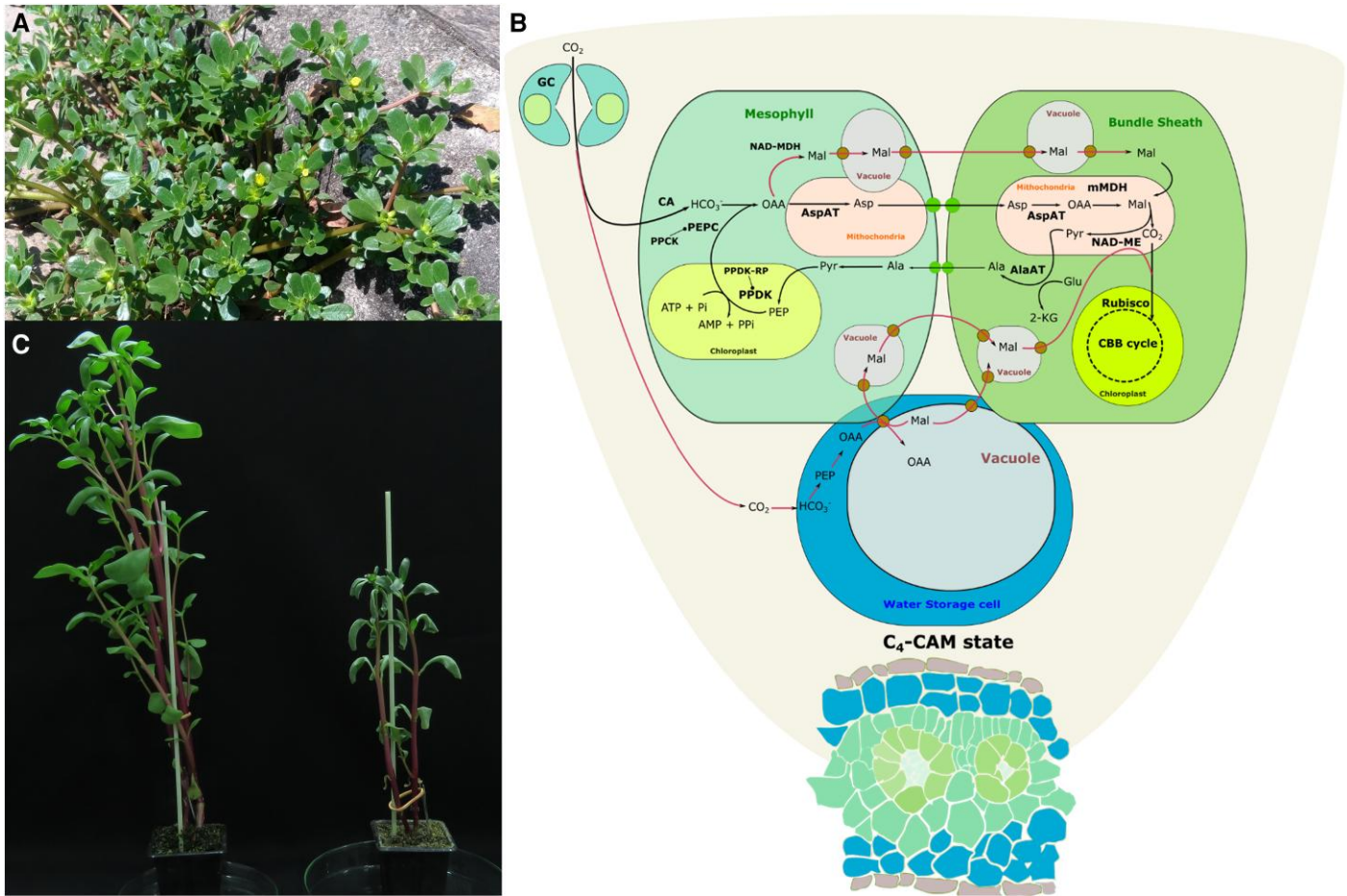


Fig. 1. *Portulaca oleracea* has been the most studied species in the genus in efforts to understand the co-existence of C₄ and CAM in a single leaf. (A) A wild accession of *P. oleracea* growing in an urban environment in Sao Paulo, Brazil, with detail of its small leaves and prostrate habit. (B) Hypothetical schematic of the dual CCM co-existence in the leaves of *P. oleracea* based on the literature (Lara *et al.*, 2004; Moreno-Villena *et al.*, 2022). The contribution of the water storage cells is still uncertain. Metabolite and enzyme abbreviations are defined in the text, except for alpha-ketoglutarate (2-KG), PPDK-regulatory protein (PPDK-RP), and Rubisco (RBS). Bold font indicates enzyme names. Black arrows indicate the constitutive C₄ pathway taking place under well-watered conditions. Red arrows indicate the metabolic switch that happens when drought is established and the hybrid C₄–CAM system is activated. (C) Cultivar accessions of *P. oleracea* cultivated under controlled conditions highlighting its erect habit, robust morphology, and big leaves. To the left, a plant kept under well-watered conditions and, to the right, a plant drought stressed for 2 weeks.

relied on increasing flow capacities already present in C₃ (Bräutigam *et al.*, 2017). In any case, weak and strong CAM phenotypes exist, which can be distinguished on the basis of how much CO₂ is assimilated at night or during the day, with weak CAM having <5% of its total carbon input originating from dark CO₂ assimilation (Winter, 2019). CAM might also be expressed constitutively, as the plant develops, or triggered by environmental conditions (i.e. facultative CAM), in a reversible way (Winter and Smith, 1996).

The best of both worlds: the C₄–CAM system

While C₄ and CAM photosynthesis have been extensively characterized individually, the *Portulaca* genus stands out as

a unique biological system to dissect the co-regulation and co-evolution of these two pathways. *Portulaca oleracea* is the most studied C₄–CAM plant (Fig. 1A) and, beyond this photosynthetic adaptation, *P. oleracea* has diverse uses and properties. Purslane is consumed as a neglected crop (Hernández-Bermejo and León, 1994), rich in omega-3, antioxidants, and anti-inflammatory and immunomodulatory compounds (reviewed in Kumar *et al.*, 2022). At the same time, it has long been regarded as a noxious weed and invasive species in several regions (Singh and Singh, 1967; Matthews *et al.*, 1993). Cultivar varieties have bigger leaves (~8 cm²), as opposed to wild accessions, which have smaller leaves (~1–5 cm²) (Fig. 1; Ferrari *et al.*, 2020b). In addition, the species has been shown to be extremely tolerant to drought, temperature, photoperiod, soil, and light

intensity (Zimmerman, 1976, 1977, Ren *et al.*, 2011; D'Andrea *et al.*, 2015). *Portulaca oleracea* possesses valuable molecular resources, including a published genome (Wang *et al.*, 2023), and is amenable to *Agrobacterium*-mediated transformation (Ferrari *et al.*, 2020c), reproducing rapidly and producing thousands of seeds (Zimmermann, 1977). Overall, *P. oleracea* has served as an excellent model for studying C₄–CAM photosynthesis.

How do C₄ and CAM pathways co-exist within the same leaf at the biochemical and regulatory levels?

Although incompatibilities between C₄ and CAM exist (Table 1), Koch and Kennedy (1980, 1982) studying *P. oleracea* showed that, under drought stress, a circadian pattern emerged for higher acidification in the morning and lower in the afternoon, together with low values of dark CO₂ assimilation, indicating CAM induction. At the time, the finding was particularly surprising since *P. oleracea* presents a typical C₄ Kranz anatomy of the Atriplicoid type (in which vascular bundles are in a zig–zag pattern in the middle region of the mesophyll) and, although its mesophyll shows water storage cells (WSCs), the leaves are not very succulent (Voznesenskaya *et al.*, 2010). Fifteen years later, studies on PPC under varying water availability in *P. oleracea* showed that total PPC activity increased as drought intensified (Mazen, 1996), and the kinetic and regulatory properties of PPC differed between the C₄ and CAM modes (Mazen, 2000). Subsequent research focused on enzyme kinetics and regulation (e.g. electrophoretic mobility, isoelectric point, and *in vivo* phosphorylation assays, sensitivity to L-malate inhibition, and affinity in PEP assays), indicating that PPC is biochemically optimized for the demands of each CCM (Lara *et al.*, 2003).

Studies on leaf development in *Portulaca* have shown that cotyledons of *P. grandiflora* initially display Atriplicoid anatomy, but later transition to a Pilosoid arrangement, where the vascular bundles form a ring in the mesophyll. These cotyledons can also induce CAM, although the CAM cycle develops more slowly than the C₄ cycle (Guralnick *et al.*, 2020). A key advance was the identification of multiple PPC gene lineages in *Portulaca*, namely *PPC-1E1*, *PPC-1E2*, and *PPC-2*, which encode distinct isoforms with different biological roles in *P. oleracea* (Christin *et al.*, 2014). Photosynthetic PPC copies usually contain a Ser residue at position 780, similar to *Zea mays*, while anaplerotic copies have an Ala at this position. In *P. oleracea*, *PPC-1E1a'* was shown to be mostly expressed under abundant watering, whereas *PPC-1E1c* was up-regulated when water was scarce, and the products of both genes have the Ser780 residue. These results suggested that PPC gene duplication and subfunctionalization provided *P. oleracea* with enough elements for adjusting carbon fixation in response to the environment (Christin *et al.*, 2014).

What role do regulatory enzymes such as phosphoenolpyruvate carboxylase kinase (PPCK) play in this pathway regulation?

In C₄ or CAM plants, one essential component regulating the activity of PPC is PPCK, which phosphorylates PPC and controls the sensitivity of the enzyme to L-malate as a key inhibitor (Carter *et al.*, 1991). As a result of PPCK phosphorylation, PPC tolerates the higher levels of malic acid produced during the first carboxylation reaction. It is known that the transcription of PPCK is modulated by light in C₄ plants and controlled by the circadian clock in CAM plants (Hartwell *et al.*, 1996, 1999; Table 1). In the case of *Portulaca*, it was observed that although there exists a *PPC* orthologue for C₄ (*PPC-1E1a'*) and CAM (*PPC-1E1c*), respectively, there is only one *PPCK* copy that regulates both PPCs by changing its circadian pattern of expression according to water availability. Interestingly, PPCK transcript abundance peaks during the day in C₄ and during the night in CAM (Ferrari *et al.*, 2020b; Wang *et al.*, 2023).

A first approach in understanding PPCK function in *Portulaca* examined its diel expression patterns during light/dark cycles or under constant light and temperature conditions (LL) in *P. oleracea* (Box 1; Ferrari *et al.*, 2022). When CAM was induced, changes in expression of the molecular clock components resembled those reported in other CAM plants; however, PPCK showed an arrhythmic pattern under LL. Two genes involved in the molecular clock mechanism, *GIGANTEA* (*GI*) and *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*), showed the same pattern as PPCK, suggesting a specific cross-talk point between the clock and PPCK (Ferrari *et al.*, 2022).

Still, the extent of PPCK regulation in C₄ and CAM functioning is not fully elucidated. Controversially, a change from an Asp to Glu residue in position D509 of the product of the C₄ *PPC* gene copy (*PPC-1E1a'*) in *P. oleracea* has been suggested to be able to reduce the sensitivity to malate even without PPCK activity, as seen in *Kalanchoë fedtschenkoi* (Yang *et al.*, 2017; Gilman *et al.*, 2022). At the same time, RNAi lines targeting PPCK in *K. fedtschenkoi* revealed a loss of 66% of nocturnal CO₂ assimilation capacity in comparison with the wild-type control (Boxall *et al.*, 2017). Hence, although 33% of CAM activity could be retained without PPCK, it still plays a significant role in fine-tuning CAM metabolism to its full capacity. This highlights that there is still a need for further investigation of PPCK regulation not only in C₄–CAM plants, but also in plants with each CCM individually.

What transcriptional and post-transcriptional mechanisms regulate the switch between pathways?

Unlike the two photosynthetically active *PPC* genes recruited to act either in C₄ or in CAM, the genes involved in the remaining steps of the pathway (i.e. malate dehydrogenases,

Box 1. Recent key developments in the last 5 years in understanding the coupling of C₄ and CAM photosynthesis in *Portulaca oleracea*.

(A) Gilman *et al.* (2022) generated a chromosome-level genome assembly for *Portulaca amilis* and conducted transcriptomic profiling in both *P. amilis* and *P. oleracea* during CAM induction and drought. Their choice to include two species with different C₄ biochemical types (NADP-ME in *P. amilis* and NAD-ME in *P. oleracea*) was unprecedented in C₄–CAM transcriptomic studies. C₄ and CAM primarily relied on different gene sets for carbon fixation, and low-level metabolite cycling between the two pathways was suggested. C₄ evolution in *Portulaca* probably involved co-opting redundant gene modules and integrating daytime CAM functions within the ancestral CAM network, posing little constraint on C₄ acquisition due to largely independent regulatory architectures that avoid pleiotropic interference. Overall, this work supported the hypothesis that C₄–CAM co-existence in *Portulaca* is enabled by modular regulation and gene duplication, and clarified how photosynthetic plasticity evolved through network integration and redundancy.

(B) Moreno-Villena *et al.* (2022) performed spatial transcriptomics using two different methodological approaches, and included flux balance modelling (FBM) applied to C₄–CAM for the first time. Their findings provided the first empirical evidence for an integrated C₄–CAM cycle under drought, in which malate would accumulate in MCs and be stored overnight either locally or in BSCs, followed by decarboxylation in BSCs during the day. They also used tissue-specific transcriptomics to confirm that most genes forming part of the CCM machinery are shared between the two photosynthetic types across the different cell types, except for phosphoenolpyruvate carboxylase (PPC) and possibly beta-carbonic anhydrase (β CA) and NAD-malate dehydrogenase (NAD-MDH).

(C) Ferrari *et al.* (2022) provided the first insights into the circadian and hormonal regulation of C₄–CAM photosynthesis in *P. oleracea*. At the transcriptional level, PPCK was suggested as a key point of connection to the molecular clock, and the expression patterns of several transcription factor genes were investigated, highlighting *HB7*, *NFYA7*, *NFYC9*, *TT8*, and *ARR12* as likely candidate regulators of CAM induction, and *NFYC4* and *ARR9* were connected to C₄ expression. Under varying water availability, the exogenous application of abscisic acid was able to induce the C₄ gene homologue of PPC, whereas the application of cytokinins (both zeatin and 6-benzylaminopurine, BAP) was able to repress the CAM homologues of PPC.

(D) Wang *et al.* (2023), through whole-genome sequencing of *P. oleracea*, revealed two rounds of whole-genome duplication (WGD): one ancient (P- β , ~66 Mya, shared with close relatives) and one lineage-specific (Po- α , ~7.7 Mya), resulting in numerous duplicate gene copies related to C₄ and CAM pathways. This work successfully confirmed that these duplications provided four C₄-specific and two CAM-specific PPC genes, with additional duplicated β CA genes supporting both C₄ and CAM functionalities. Such genetic redundancy facilitated biochemical compatibility and regulatory independence for each photosynthetic mode in purslane. This study offered a fresh insight into the evolutionary plasticity in *Portulaca*, supporting the existence of an integrated C₄–CAM function at the genomic level, with tandem and WGD event-driven diversification targeting key metabolic genes required for optimal CCMs under water limitation.

decarboxylation enzymes, and substrate regeneration) did not seem to be recruited in pairs in *P. oleracea* (Christin *et al.*, 2015; Ferrari *et al.*, 2020a). In general, genes encoding enzymes that metabolize C₄ acids during the day are down-regulated under drought but remain expressed and thus are probably still able to function in CAM (Ferrari *et al.*, 2020a). Exceptions include the transporter genes *ALMT-9E*, and β CA, which show a mild nocturnal up-regulation during drought (Ferrari *et al.*, 2020a). Building on this, Moreno-Villena *et al.* (2022) proposed a CAM-recruited NAD-malate dehydrogenase, and more recent genomic analyses have corroborated CAM recruitment of different gene copies of β CA, *ALMT*, *PPDK*, and *PPDK-RP* (Wang *et al.*, 2023).

The transcriptional modulation of the core CCM genes seems to be conserved at least across two different *Portulaca* species with different C₄ subtypes (Gilman *et al.*, 2022; Box 1). Transcriptomic analyses of well-watered and water-deprived

P. oleracea (NAD-ME type) and *P. amilis* (NADP-ME type) showed a significant overlap in gene expression changes between the two species (Gilman *et al.*, 2022). Gene co-expression network analyses (GCNA) in the same work also linked modules containing carboxylation, starch metabolism and catabolism, the circadian rhythm, and light responses (Gilman *et al.*, 2022; Box 1). At least at the transcriptional level, it was also suggested that a constitutively weak CAM cycle could be operating in the two species (Gilman *et al.*, 2022). Constitutive CAM had also been suggested for well-watered stems of *P. oleracea*, but in this case supported by titratable acidity data (Ferrari *et al.*, 2020a). Overall, further analyses of metabolites, enzyme activity, and the phosphorylation status of PEPC during the daytime and night-time are needed to confirm constitutive CAM in leaves of *Portulaca*. So far, most studies showed a clear facultative component in leaves (Koch and Kennedy, 1980; Ku *et al.*, 1981; Mazen, 1996, 2000;

Guralnick *et al.*, 2002, 2020; Lara *et al.*, 2003, 2004; Ferrari *et al.*, 2020a, b, c).

In addition to phosphorylation of PPC by PPCK as a key post-transcriptional regulatory step, the role of post-translational modifications (PTMs) in regulating other key enzymes which form part of the CCM cycle is still not known. Schiller *et al.* (2025) searched for PTM sites in CAM orthologues present in *C₃* species. The authors identified 78 homologues for *K. laxiflora* CAM genes in Arabidopsis and checked the occurrence and position of these PTMs. Overall, they found dozens of new sites for phosphorylation, acetylation, and S-nitrosylation in several enzymes of the CAM pathway, highlighting that the role of regulatory enzymes is definitely not restricted to PPCK (Schiller *et al.*, 2025). This could be especially relevant in *C₄*-CAM systems, which share many enzymes but rely on differential regulation.

How are metabolic fluxes coordinated?

Perhaps one of the most pressing questions in *C₄*-CAM is the localization of each step of the pathway across the different leaf cell types. Few studies have addressed the localization of each cycle empirically. In *P. grandiflora*, *in situ* immunolabelling of total PPC and Rubisco were performed together with tissue prints of PPC, which involve pressing leaves and stem pieces cut with a razor blade against a nitrocellulose membrane and subsequent incubation with specific antibodies for protein localization (Guralnick *et al.*, 2002). These assays revealed an increase in protein content under drought but did not fully resolve metabolite localization for *P. grandiflora* (Guralnick *et al.*, 2002).

In *P. oleracea*, in addition to MCs and BSCs, a hypodermis composed of WSCs is also present (Vonzenskaya *et al.*, 2010; Ocampo *et al.*, 2013). Enzymatic activity quantification of Rubisco, PPC, orthophosphate, dikinase (PPDK), and NAD-ME in leaves was used to draw the first scheme for *C₄*-CAM as an integrated pathway, suggesting a hybrid system (Lara *et al.*, 2004; Fig. 1C). Under well-watered conditions, *P. oleracea* would operate *C₄* through the NAD-ME type between MCs and BSCs. Once drought stress was established, malate would then be produced and stored in the MCs overnight, then shuttled for decarboxylation in BSCs during the day. This would represent an innovative dual-cell CAM cycle so far exclusive to *Portulaca* when compared with other plant lineages. We present in Fig. 1B the overview of a possible metabolic pathway for the hybrid *C₄*-CAM system based on previously published data (Lara *et al.*, 2003, 2004; Moreno-Villena *et al.*, 2022).

Transcriptomic approaches so far have supported the integration of the two pathways (Ferrari *et al.*, 2020a; Gilman *et al.*, 2022; Box 1). The first spatial resolution study using a high-throughput spatial transcriptomics platform for *P. oleracea* leaves grown under abundant or restricted watering conditions revealed that there are transcripts for the two PPC copies being

expressed in the same MCs, further reinforcing the previous hybrid model for the integration of CAM into *C₄* using data from flux balance model (FBM) analysis (Moreno-Villena *et al.*, 2022; Box 1). In this model, steady-state fluxes in metabolic networks are considered, and here the authors sought a parsimonious integration for *C₄* and CAM based on first principles of general plant metabolism, but using the dual-cell arrangement also for CAM and considering diel flux and charge balance in each cellular compartment (Moreno-Villena *et al.*, 2022). Not only should new modelling approaches be pursued (Burgos *et al.*, 2022), but new research should also focus on empirically resolving the localization in terms of protein and metabolite distribution, which will hopefully shed light on the necessary tissue specificity to allow the hybrid *C₄*-CAM to be expressed.

The partially opposite regulation (e.g. nocturnal versus diurnal PPC carboxylation) and partially shared enzymatic activity (e.g. common use of the decarboxylation system from *C₄* in CAM) seem to enable the dual operation of both CCMs in the same leaf of *P. oleracea*. Although the identity of the metabolites being used in these CCMs is known, namely malate, Asp, Ala, and OAA, among others (Winter and Smith, 1996; Kanai and Edwards, 1999), the exact dynamics and fluxes between cell types are still to be assessed. In fact, the identity of vacuolar transporters involved in CAM, especially malate efflux transporters during the daytime, remains unknown not only in *P. oleracea* but also in strong CAM species such as *K. fedtschenkoi*. This will be an essential finding for subsequent engineering initiatives (Ceusters *et al.*, 2021).

In terms of metabolite fluxes, NAD-ME-type *C₄* relies on Asp as a transport molecule between MCs and BSCs, and it is not yet clear whether, during drought, the malate formed as a result of CAM (i) would be synthesized and stored in MCs, and later remobilized from the vacuoles and shuttled to BSCs; (ii) intermediate steps for its conversion to Asp, for instance, exist for the transport between MCs and BSCs; or (iii) could be mostly formed and stored in BSCs, raising questions as to the capacity of these cells for storage, and if this could act as a limiting step to their CAM capacity. The contribution of WSCs is also still not fully understood (Lara *et al.*, 2003; Moreno-Villena *et al.*, 2022; Box 1; Fig. 1B), and thus to clarify metabolite fluxes, metabolic modelling and networks will contribute greatly.

What evolutionary processes enabled the convergence of two CCMs within the same genus?

The *Portulacaceae* comprise ~150 species and are currently subdivided into five clades (African-Asian, Australian, Oleracea, Umbraticola, and Pilosa) (Ocampo and Columbus, 2012; according to Plants of the World Online, <https://powo.science.kew.org/>). There is a pressing need for a new phylogenetic analysis of the family, including more species from the family to revise the division into five clades, and most

specifically with a broader sampling in diversity hot spots such as South America. Previously, *Portulaca* was grouped together with *Anacampseros*, *Portulacaria*, *Talinum*, *Calandrinia*, and *Montia*, among others, that have also been shown to perform facultative CAM (Guralnick and Jackson, 2001). Now these genera have been separated into different families (Thorne and Reveal, 2007) and, although close to *Portulaca*, none is C₄ (Guralnick *et al.*, 2008). Closest to *Portulacaceae* are *Anacampserotaceae*, *Cactaceae*, and *Talinaceae*, all of which perform only CAM, either constitutively or facultatively (Nyffeler and Eggli, 2010; Moore *et al.*, 2018).

Being cosmopolitan and showing considerable morphological variation in terms of leaf and plant size and area (Fig. 1), *P. oleracea* genotypes originating from places with contrasting climate conditions were all able to perform CAM reversibly (Ferrari *et al.*, 2020b). In fact, the species is referred to as the *P. oleracea* complex and includes ~20 subspecies, although it was shown to be paraphyletic in at least two branches (Danin and Raus, 2012; Ocampo and Columbus, 2012). Not only can hybrids form between previously classified *P. oleracea* subspecies, but also different chromosomal counts are found across genotypes, with variation encompassing hexaploidy in most accessions to diploidy in *P. oleracea* subsp. *nicaraguensis* (Walter *et al.*, 2015). It is likely that historical increases in trade and plant dispersal facilitated the widespread distribution of *P. oleracea*, which has become invasive in several regions. However, its original centres of diversification—whether in South America or Africa—remain uncertain (Ocampo and Columbus, 2012). All in all, a new phylogeny with a broad sampling for *P. oleracea* genotypes might be able to address the taxonomic complexity of this clade.

What selective pressures favoured this convergence?

It is now clear that gene duplications enabled the modularity of C₄ and CAM in *P. oleracea*, but the question still remains as to the exact selective pressure which was favouring this unusual combination. The phylogenetic proximity to the *Cactaceae* and other genera performing facultative CAM, in addition to studies on PPC evolution, suggested that the common ancestor for *Portulaca* was able to perform CAM (Christin *et al.*, 2014), and the biochemical and anatomical diversity of C₄ subtypes must have evolved independently in the different clades (Kadereit *et al.*, 2003; Ocampo *et al.*, 2013). The adaptive significance of weak, facultative CAM and its contribution to species fitness still need to be tested. Nevertheless, increased survivability in C₃–CAM species may be attributed to photo-protection, water saving, and carbon reutilization from respiratory losses, which have been shown to be able to contribute to source–sink balance and reproduction to some extent (reviewed in Herrera, 2009).

C₄ and CAM species usually have different niches, which would favour photosynthetic efficiency in the case of C₄, or resilience in the case of CAM (Ehleringer and Monson, 1993). It

could be that selective pressures in these habitats are usually exclusive and, once one CCM evolved, there was no need to evolve a second one, or that there was simply not enough time to evolve a second CCM once one had already developed (Sage, 2002; Arakaki *et al.*, 2011). However, in general, C₄ eudicots have been shown to colonize and diversify in drier regions in comparison with C₄ grasses (Berasategui *et al.*, 2023), which could increase the chances of C₄ evolving in a lineage performing CAM ancestrally such as *Portulaca*. In fact, more C₄–CAM species are expected to be found in broader future surveys for CAM expression in families such as *Amaranthaceae* and *Aizoaceae*, for example, since they comprise C₄ species with some level of succulence in leaves.

Another aspect that remains underexplored in the CAM literature is the variability across and within populations and how that contributes to phenotypic plasticity or genotypic differences between populations. Overall, more field studies in species performing facultative CAM are needed for better inferring the effect of this photosynthetic switch on fitness (Herrera, 2009). In C₃–CAM *Portulacaria afra*, although reproductive tissue was formed using carbon assimilated via CAM, a high variability in stable carbon isotope ratios ($\delta^{13}\text{C}$) composition across several populations in the Eastern Cape was found, revealing that the microclimate plays a significant role (Guralnick and Gladsky, 2017). More specifically, understanding population genetics and trait plasticity in *Portulaca* species can possibly contribute to understanding pre-conditions and selective pressures allowing C₄–CAM co-evolution.

What genomic and regulatory elements enabled the integration of these pathways?

Currently, the evolutionary gene enablers and core enzymes responsible for the operation of C₄–CAM in *P. oleracea* are known (Christin *et al.*, 2014, 2015). A chromosome-level genome for *P. oleracea* was recently published, with $2n = 52$ chromosomes (Wang *et al.*, 2023; Box 1). It was shown that two rounds of whole-genome duplication enabled the differential recruitment of PPC and βCA . Wang *et al.* (2023) also highlighted a residue change from Arg to Met at position 890 according to *Z. mays* (Zm00001d046170) in C₄-specific PPC-1E1a'-encoded proteins, and correlated it to higher efficiency in carbon fixation. This work showed for the first time that *cis*-elements, which are evening specific and involved in drought responses mediated by abscisic acid (ABA), are enriched in the promoter regions of CAM-specific genes (Box 1). Still, the specifics of hormonal regulation in C₄–CAM transcription are as yet poorly understood. In *P. oleracea*, positive and negative effector roles have been suggested for ABA and cytokinin (CK) during CAM induction when the exogenous application of both hormones was able to modulate the expression of C₄- and CAM-specific PPC transcripts (Ferrari *et al.*, 2022; Box 1).

Sequencing and annotating genomes from additional species—spanning the major sub-clades and representing different

C₄–CAM subtypes—will allow researchers to pinpoint conserved gene modules, regulatory elements, and duplication events linked to photosynthetic plasticity. For example, comparing *P. cryptopetala* (C₃–C₄, Cryptopetala clade), *P. oleracea* (Atriplicoid anatomy, Oleracea clade), and *P. grandiflora* (Piloid anatomy, NADP-ME subtype) can reveal both lineage-specific adaptations and shared genetic mechanisms underlying the co-existence of C₄ and CAM. Despite this potential, genomic resources are limited to *P. oleracea* and *P. amilis* (Gilman *et al.*, 2022; Wang *et al.*, 2023). In summary, expanding genomic comparisons across various *Portulaca* species, which vary in the deployment of C₄ and CAM, will allow better insights into the genetic modules and regulatory networks underlying CCM integration—an endeavour not feasible when studying these pathways in isolation.

Can this knowledge inform efforts to engineer metabolic flexibility in crops?

As well as being a C₄–CAM species, *P. oleracea* stands out for its versatile photosynthetic physiology, showing resistance to drought, salinity, as well as variations in temperature, photoperiod, soil, and light intensity (Zimmerman, 1977; Yazici *et al.*, 2007; Ren *et al.*, 2011; Jin *et al.*, 2015). Considering that these features might be connected to C₄–CAM, it inspires further research to engineer crops for their metabolic flexibility (Ferrari and Freschi, 2019). Given current knowledge of gene modules and signalling, along with an established transformation protocol (Ferrari *et al.*, 2020c), synthetic biology approaches could be used to induce weak CAM expression in C₄ crops, enhancing water-saving strategies and drought survival (Table 2).

How might synthetic biology approaches exploit this system for enhanced stress resilience?

CRISPR/Cas9 [clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9] brings an exciting possibility of performing loss-of-function studies that can cast light on the specific contribution of each core enzyme involved in C₄ and CAM mechanisms. Functional studies can also shed light on the signalling of the transition to CAM and its fast reversibility to C₄ upon rewatering (Ferrari *et al.*, 2022), although the genotypic complexity of the taxon could still prove transformation experiments challenging. *Portulaca oleracea* is characterized by a high degree of polyploidy, which could complicate genome editing and functional genomics due to the presence of multiple gene copies and potential redundancy, although successful transformation efforts have already been reported (Ferrari *et al.*, 2020c). Perhaps to move the field forward faster, other species with lower genomic complexity should be explored in parallel. For instance, *P. oleracea* subsp. *nicaraguensis* exhibits lower ploidy levels (2n=26) and is currently considered a subspecies of *P. oleracea* (Ocampo and Columbus,

2012). This species could make genetic manipulation and interpretation of gene function potentially more straightforward. Overall, comparative approaches and generating genomic data for multiple *Portulaca* species will prove helpful to unravelling C₄–CAM.

Can aspects of this dual pathway be introduced into other species?

C₄ and CAM are two traits that increase plant fitness in dry and marginal environments. Because both mechanisms have evolved independently in >60 lineages each, and many of the underlying enzymes and transporters are already present in C₃ plants, there is a strong rationale for introducing either of the CCMs into C₃ crops (Heckmann *et al.*, 2013; Reyna-Llorens and Hibberd, 2017; Sage, 2017; Gilman *et al.*, 2023). Engineering efforts along these lines are already underway, including attempts at C₄ installation in rice (Furbank *et al.*, 2023) and conceptual frameworks for C₃ to CAM biodesign (Lim *et al.*, 2019; Yang *et al.*, 2024). In this context, combining aspects of both pathways could be particularly valuable if it mirrors what is observed in *Portulaca*: a predominance of C₄ to sustain yield, coupled with a facultative CAM cycle that enhances survival under severe drought (Ferrari and Freschi, 2019). Achieving such a dual CCM in crops is a formidable challenge, but it highlights how mechanistic insights from *Portulaca* can provide a concrete blueprint for designing metabolically flexible and climate-resilient crops.

Conclusion

In this Expert view, we have discussed the most pressing questions to advance the C₄–CAM field and all that has been achieved using *P. oleracea* as a model. Despite decades of separate research on C₄ and CAM pathways, the molecular basis for their co-existence in a single organism remains largely unresolved, and a better understanding of C₄–CAM can also contribute to the C₄ and CAM fields. *Portulaca oleracea* provides a blueprint for this integration, positioned to accelerate understanding of complex trait convergence. Moreover, an in-depth understanding of the role PPCK plays in both CCMs at the same time is needed, and this goes hand in hand with generating more data for PTMs. A clear picture of the metabolites involved in each CCM, in which cells they are produced and transported to, and their diel dynamics will also be essential. From the evolutionary point of view, addressing the selective pressures that allowed C₄ and CAM to converge in a single organism will rely on assessing physiological advantages of this metabolic plasticity in terms of species fitness. Future research should focus on direct measurements of enzyme activity and analysis of protein regulation, especially with tissue- or cell-specific resolution. In addition, studies should expand these multidimensional analyses to additional species within the *Portulaca* lineage, which will enable a deeper perspective on

the diversity and evolution of C₄–CAM traits across the genus. Another promising approach is to investigate the promoter regions for core C₄–CAM genes, which will provide insight into regulatory elements and crosstalk with other pathways such as those involved in hormonal signalling. Various modelling approaches applied to C₄–CAM specifically will also contribute to advancing the field. Lastly, synthetic biology initiatives should start exploring how to engineer C₄ crops to perform weak CAM using *Portulaca* as a model, since expanding research on the C₄–CAM system will also aid efforts to enhance water use efficiency and stress resilience.

Author contribution

RCF and IRL conceptualized the manuscript and wrote it together.

Conflict of interest

The authors declare no conflicts of interest.

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