Open questions on facultative C_4-CAM photosynthesis in *Portulaca*

Renata Callegari Ferrari[1]*; Ivan Reyna-Llorens[2]*

[1] Prinzessin Therese von Bayern Lehrstuhl für Systematik, Biodiversität und Evolution der Pflanzen, Menzinger Straße 67, D-80638 Munich, Germany.

[2] Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTAUAB-UB, Campus UAB, Bellaterra, Barcelona, Spain.

*Corresponding authors; both authors contributed equally to this manuscript.

Renata.callefe@gmail.com; r.ferrari@lmu.de; ivan.reyna-llorens@cragenomica.es

ORCID

Renata Callegari Ferrari

0000-0002-9497-8442

Ivan Reyna-Llorens

0000-0001-7964-7306

ABSTRACT

Plants have evolved carbon concentrating mechanisms (CCMs) to optimize carbon fixation under environmental conditions that increase photorespiratory and transpiration rates. C_4 and 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 more than 40 years ago. Although it remains as one of the most characterized C_4-CAM species, many aspects of this dual system are still unresolved. This expert review summarizes recent advances in understanding C_4-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 C_4 crop might be possible and could entail increased survival, possibly contributing to higher productivity in a future with climate change.

HIGHLIGHT

C_4-CAM photosynthesis confers high resilience under abiotic stress, yet little is known about the mechanisms that facilitate this integration. We highlight innovations and opportunities to unravel this system in *Portulaca*.

KEYWORDS

C_4 photosynthesis - crassulacean acid metabolism - facultative CAM - Portulaca oleracea - carbon concentrating mechanism - abiotic stress - drought stress - phosphoenolpyruvate carboxylase - photosynthesis - PEPC

INTRODUCTION

C_4 photosynthesis and the Crassulacean acid metabolism (CAM) are two great examples of convergent evolution, repeatedly evolving at least 60 times for C_4 (ca. 8100 species, Sage, 2017) and 66 for CAM (ca. 23900 species, Gilman *et al*., 2023) in independent lineages. Together, these two carbon concentrating mechanisms (CCMs) occur in roughly 9% of all known vascular plant species (total number of species: 349000 according to POWO, 2025). These numbers highlight that C_4 and CAM have a significant representativeness and have conferred plants specific adaptive advantages leading to their repeated evolution. Still, C_4 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 CO_2, resulting in energetically wasteful cycling. In addition, C_4 photosynthesis typically relies on a spatial division between mesophyll and bundle sheath cells, whereas CAM depends on large vacuoles and a clear diel separation of carboxylation and decarboxylation. These differences historically led to the assumption that C_4 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 coexistence 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 C_4 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 C_4-CAM species continued to emerge (Zhang *et al*., 2014; Winter *et al*., 2021, Siadjeu and Kadereit, 2024), but the mechanism and specifics enabling the C_4-CAM photosynthetic system still remain unclear. Although few, the available studies in the field focused mostly on *P. oleracea*, and to a less extent, *P. grandiflora* (Guralnick *et al*., 2002, 2020). Until 2021, progress in understanding how C_4 and CAM coexist 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 five 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 C_4-CAM plants, specifically focusing on the case of *P. oleracea*. We discuss the following questions: (1) How do C_4 and CAM pathways coexist within the same leaf at the biochemical and regulatory levels?; (2) What evolutionary processes enabled the convergence of two CCMs within the same genus?; (3) 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 the C_4-CAM understanding but also contributing directly to C_4 and CAM individual fields. We argue that the C_4-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 C_4 or CAM?

C_3 photosynthesis relies on the Calvin-Benson-Bassham cycle (CBBC) that incorporates CO_2 into three-carbon skeletons through the activity of ribulose-1,5-biphosphate carboxylase oxygenase (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 be metabolised through photorespiration. While this process evolved as a solution to recover carbon (C) and detoxify the cell environment, there is an energetic and nutritional cost associated (Walker *et al*., 2016). Over the past 35 million years, declining atmospheric CO_2 levels have driven the evolution of two CCMs, C_4 and CAM, becoming the most widespread among land plants (Edwards and Ogburn, 2012). CCMs overcome Rubisco's shortcomings 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, C_4 and CAM biochemistry rely on similar molecular components, recruited from ancestral C_3 enzymes, reflecting on how complex traits often evolve by repurposing pre-existing molecular elements (Sage, 2016; Bräutigam *et al*., 2017). Both pathways use β-carbonic anhydrase (βCA), which catalyses the rapid interconversion of CO_2 to HCO_3^-, 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. HCO_3^- 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 (MAL), but in C_4, either MAL or aspartate (ASP) can be formed (Winter and Smith, 1996; Kanai and Edwards, 1999). Subsequently, three decarboxylases including NADP-malic enzyme (NADP-ME, chloroplastic), NAD-malic enzyme (NAD-ME, mitochondrial) or phosphoenolpyruvate carboxykinase (PEPCK, cytosolic) will release CO_2 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 C_4 (Furbank, 2011).

Despite the shared biochemistry, the spatial and temporal organization of these processes differ between C_4 and CAM plants (Table 1). Although there are known examples of single-cell C_4 (Edwards *et al*., 2004), in most C_4 species the enzymatic components of the pathway are divided between the leaf mesophyll cells (MCs) and bundle sheath cells (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, including 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 CO_2 availability close to Rubisco, the selection pressures driving their evolution are thought to be slightly different (Table 1). C_4 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 coevolution with succulence resulted in a water saving strategy (Edwards and Ogburn, 2012). Nevertheless, in aquatic environments, CAM evolution is usually associated with limited CO_2 availability (Keeley, 1998).

Some of the current models for C_4 evolution consider a stepwise trajectory including C_3-C_4 intermediates, with gradual changes in 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, C_3-C_4 intermediates have also been considered stable evolutionary stages on their own (Lundgren, 2020), and the contribution of hybridization for C_4 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 C_4, 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 relied on increasing existing flow capacities already present in C_3 (Bräutigam *et al.,* 2017). In any case, weak and strong CAM phenotypes exist, which can be distinguished on the basis of how much CO_2 is assimilated at night or during the day, with weak CAM having less than 5% of its total C input originating from dark CO_2 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 C_4-CAM system

While C_4 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 C_4-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, anti-inflammatory and immunomodulatory compounds (reviewed in Kumar *et al*., 2022). At the same time, it has since 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 (circa 8cm^2), as opposed to wild accessions, which have smaller leaves (circa 1-5 cm^2) (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 C_4-CAM photosynthesis.

How do C_4 and CAM pathways coexist within the same leaf at the biochemical and regulatory levels?

Although incompatibilities between C_4 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 CO_2 assimilation, indicating CAM induction. At the time, the finding

was particularly surprising since *P. oleracea* presents a typical C_4 Kranz anatomy of the Atriplicoid type (in which vascular bundles are in zig-zag in the middle region of the mesophyll), and although its mesophyll shows water storage cells (WSCs), the leaves are not very succulent. 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 C_4 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 to 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 C_4 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 serine residue at position 780, similar to *Zea mays*, while anaplerotic copies have an alanine at this position. In *Portulaca oleracea*, *PPC-1E1a'* was shown to be mostly expressed under abundant watering, whereas *PPC-1E1c* was upregulated when water was scarce, and both genes have the Ser780 residue. These results suggested that PPC gene duplication and subfunctionalization provided *P. oleracea* with enough elements for adjusting C 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 C_4 or CAM plants, one essential component regulating the activity of PPC is PPC kinase (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 C_4 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 C_4 (*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 C_4 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 a 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 C_4 and CAM functioning is not fully elucidated. Controversially, a change from an aspartic acid to glutamic acid residue in position D509 of the C_4-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 CO_2 assimilation capacity in comparison to the wild type control (Boxall et al., 2017). Hence, although 33% of CAM activity could be kept 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 the PPCK regulation not only in C_4-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 C_4 or in CAM, the genes involved in the remaining steps of the pathway (i.e., malate dehydrogenases, decarboxylation enzymes, 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 C_4 acids during the day are downregulated under drought but remain expressed and thus are likely still able to function in CAM (Ferrari *et al*., 2020a). Exceptions include the transporter *ALMT-9E*, and *βCA*, which show a mild nocturnal upregulation 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 *βCA*, *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 C_4 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 transcriptional level, it was also suggested that a constitutive 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 PPC during day and nighttime 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, Lara *et al*. 2003, 2004, Guralnick *et al*., 2002, 2021, Ferrari *et al*., 2020).

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 part of the CCM cycle is still not known. Schiller *et al*., (2025) searched for PTM sites in CAM orthologues present in C_3 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 C_4-CAM systems, which share many enzymes but rely on differential regulation.

How are metabolic fluxes coordinated?

Perhaps one of the most pressing questions in C_4-CAM is to resolve 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* immuno-labelling 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 (Vonznenskaya *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 C_4-CAM as an integrated pathway, suggesting a hybrid system (Lara *et al*., 2004, Fig. 1c). Under well-watered conditions, *P. oleracea* would operate C_4 through the NAD-ME-type between MCs and BSCs. Once drought-

stress would be 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 exclusive to *Portulaca* so far when compared to other plant lineages. We present in Fig. 1c the overview of a possible metabolic pathway for the hybrid C_4-CAM system based on previously published data (Lara *et al*., 2003, Lara *et al.,* 2004, Moreno-Villena *et al*., 2022, Fig 2).

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 to *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, and further reinforcing the previous hybrid model for the integration of CAM into C_4 using data from flux balance model 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 C_4 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 new modelling approaches should 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 C_4-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 C_4 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, i.e., MAL, ASP, alanine (ALA), OAA, among others (Winter and Smith 1996; Kanai and Edwards 1999), the exact dynamic and fluxes between cell types is still to be assessed. In fact, the identity of vacuolar transporters involved in CAM, especially malate efflux transporters during daytime, remains unknown not only in *P. oleracea* but also in strong CAM species like *K. fedtschenkoi*. This will be an essential finding for subsequent engineering initiatives (Ceusters *et al*., 2021).

In terms of metabolite fluxes, NAD-ME type C_4 relies on ASP as a transport molecule between MCs and BSCs, and it is not yet clear whether, during drought, the MAL formed as a result of CAM would: (1) be synthesized and stored in MCs, later remobilized from the vacuoles and shuttled to BSCs; (2) there could be intermediate conversion steps to ASP, for instance, for the transport between MCs and BSCs; (3) or MAL 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. 1c), 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?

Portulacaceae comprises ca. 100 species and is currently subdivided into five clades (African-Asian, Australian, Oleracea, Umbraticola, and Pilosa) (Ocampo and Columbus, 2012). 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, Montia,* among others that have also been shown to perform facultative CAM (Guralnick and Jackson, 2001). Now these genera were separated into different families (Thorne and Reveal, 2007) and, although close to *Portulaca*, none are C_4 (Guralnick *et al*., 2008). Closest to Portulacacae 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 ca. 20 subspecies, although it was shown to be paraphyletic in at least two branches (Danin and Raus, 2012; Ocampo and Columbus, 2012). Not only hybrids can 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, Africa, or Australia 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 C_4 and CAM in *P. oleracea*, but the question still remains as to what the exact selective pressure 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 ancestral for *Portulaca* was able to perform CAM (Christin *et al*., 2014), and the biochemical and anatomical diversity of C_4 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 needs to be tested. Nevertheless, increased survivability in C_3-CAM species may be attributed to photoprotection, water saving, and C 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).

C_4 and CAM species usually have different niches, which would favour photosynthetic efficiency in the case of C_4, or resilience in the case of CAM (Erlehringer and Monsoon, 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, C_4 eudicots have been shown to colonize and diversify in drier regions in comparison to C_4 grasses (Berasategui *et al*., 2023), which could increase the chances of C_4 evolving in a lineage performing CAM ancestrally such as *Portulaca*. In fact, more C_4-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 C_4 species with some level of succulence in leaves.

Another aspect that remains underexplored in CAM literature is the variability across and within populations and how does that contribute to phenotypic plasticity or genotypic differences between populations. Overall, more field studies in species performing facultative CAM are needed for better inferring the role of this photosynthetic switch on fitness (Herrera, 2009). In C_3-CAM *Portulacaria afra*, although reproductive tissue was formed using C assimilated via CAM, a high variability in $\delta^{13}C$ 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 preconditions and selective pressures allowing the C_4-CAM coevolution.

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

Currently, the evolutionary gene enablers and core enzymes responsible for the operation of C_4-CAM in *P. oleracea* are known (Christin *et al*., 2014, 2015). A chromosome level genome for *P. oleracea* was recently published, with a number of 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 arginine to methionine at position 890 according to *Z. mays* (Zm00001d046170) in C_4-specific *PPC1E1a'* proteins, and correlated it to higher efficiency in carbon fixation. This work presented for the first time that cis-elements, which are evening specific and involved in drought responses mediated by ABA, are enriched in the promoter regions of CAM specific genes (Box 1). Still, the specifics of hormonal regulation in C_4-CAM transcription are still poorly understood. In *P. oleracea*, positive and negative effector roles have been suggested for abscisic acid (ABA) and cytokinin (CK) during CAM induction when the exogenous application of both hormones was able to modulate the expression of C_4- 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 C_4–CAM subtypes—will allow researchers to pinpoint conserved gene modules, regulatory elements, and duplication events linked to photosynthetic plasticity. For example, comparing *P. cryptopetala* (C_3–C_4, Cryptopetala clade), *P. oleracea* (Atriplicoid anatomy, Oleracea clade) and *P. grandiflora* (Pilosoid anatomy, NADP-ME subtype) can reveal both lineage-specific adaptations and shared genetic mechanisms underlying the coexistence of C_4 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 C_4 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?

Aside from being a C_4-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; Jin *et al*., 2015; Ren *et al*, 2011). Considering these features might be connected to C_4-CAM, it inspires further research to engineer crops for their metabolic flexibility as well (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 C_4 crops, enhancing water-saving strategies and drought survival (Table 2).

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

CRISPR-Cas9 brings an exciting possibility of performing loss-of-function studies that can enlighten the specific contribution of each core enzyme involved in C_4 and CAM mechanisms. Functional studies can also shed light into the signalling of the transition to CAM and its fast reversibility to C_4 upon rewatering (Ferrari *et al*., 2022), although the genotypic complexity of the taxon could still prove transformation experiments challenging. *P. 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 achieved (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 C_4-CAM.

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

C_4 and CAM are two traits that increase plant fitness in dry and marginal environments. Because both mechanisms have evolved independently in more than 60 lineages each, and many of the underlying enzymes and transporters are already present in C_3 plants, there is a strong rationale for introducing either CCMs into C_3 crops (Reyna-Llorens and Hibberd, 2017; Heckmann *et al*., 2013; Sage, 2017; Gilman *et al*., 2023). Engineering efforts along these lines are already underway, including C_4 installation attempts in rice (Furbank *et al*., 2023) and conceptual frameworks for C_3 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 C_4 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.

CONCLUSIONS

In this expert review, we discussed the most pressing questions to move the C_4-CAM field and all that has been achieved using *P. oleracea* as a model. Despite decades

of separate research on C_4 and CAM pathways alone, the molecular basis for their coexistence in a single organism remains largely unresolved, and a better understanding C_4-CAM can contribute to C_4 and CAM fields as well. *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 in line with generating more data for post-translational mechanisms. A clear picture of the metabolites involved in each CCM, in which cells they are produced and transported to, and their diel dynamic will also be essential. On the evolutionary side, addressing the selective pressures that allowed C_4 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 C_4–CAM traits across the genus. Another promising approach is to investigate the promoter regions for core C_4-CAM genes, which will give insight into regulatory elements and crosstalk with other pathways such as those involved in hormonal signalling. Various modelling approaches applied to C_4-CAM specifically will also contribute to advancing the field. Lastly, synthetic biology initiatives should start exploring how to engineer C_4 crops to perform weak CAM using *Portulaca* as a model, since expanding research on the C_4-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.

FUNDING STATEMENT

RCF thanks the Faculty of Biology from the Ludwig-Maximilians-Universität München for the funds provided via the Bio Mentoring program. IRL was funded by PID2022-142613NA-I00 funded by MICIU/AEI/ 10.13039/501100011033 and by ERDF/EU

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Arakaki M, Christin PA, Nyffeler R, Lendel A, Eggli U, Ogburn RM, Spriggs E, Moore MJ, Edwards EJ. 2011. Contemporaneous and recent radiations of the world's major succulent plant lineages. Proceedings of the National Academy of Sciences, **108**, 8379-8384. https://doi.org/10.1073/pnas.110062810

Berasategui JA, Žerdoner Čalasan A, Zizka A, Kadereit G, 2023. Global distribution, climatic preferences and photosynthesis-related traits of C_4 eudicots and how they differ from those of C_4 grasses. Ecology and Evolution, **13**, e10720. https://doi.org/10.1002/ece3.10720

Bräutigam A, Schlüter U, Eisenhut M, Gowik U. 2017. On the evolutionary origin of CAM photosynthesis. Plant physiology, **174**, 473-477. https://doi.org/10.1104/pp.17.00195

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB. 1991. Circadian rhythms in the activity of a plant protein kinase. The EMBO journal, **10**, 2063-2068. https://doi.org/10.1002/j.1460-2075.1991.tb07737.x

Burgos A, Miranda E, Vilaprinyo E, Meza-Canales ID, Alves R. 2022. CAM models: lessons and implications for CAM evolution. *Frontiers in plant science*, *13*, p.893095. https://doi.org/10.3389/fpls.2022.893095

Ceusters N, Borland AM, Ceusters J. 2021. How to resolve the enigma of diurnal malate remobilisation from the vacuole in plants with crassulacean acid metabolism?. New Phytologist, **229**, 3116-3124. https://doi.org/10.1111/nph.17070

Christin PA, Arakaki M, Osborne CP, Edwards EJ. 2015. Genetic enablers underlying the clustered evolutionary origins of C_4 photosynthesis in angiosperms. Molecular biology and evolution, **32**, 846-858. https://doi.org/10.1093/molbev/msu410

Christin PA, Arakaki M, Osborne CP, Bräutigam A, Sage RF, Hibberd JM, Kelly S, Covshoff S, Wong GKS, Hancock L, Edwards EJ. 2014. Shared origins of a key enzyme during the evolution of C_4 and CAM metabolism. Journal of Experimental Botany, **65**, 3609-3621. https://doi.org/10.1093/jxb/eru087

Danin A, Raus T. 2012 A key to 19 microspecies of the *Portulaca oleracea* aggregate. In: Caryophyllales Simposium 2012, Anals. Moskov: Lomonosov State University, 70–83.

D'Andrea RM, Triassi A, Casas MI, Andreo CS, Lara MV. 2015. Identification of genes involved in the drought adaptation and recovery in *Portulaca oleracea* by differential display. *Plant Physiology and Biochemistry*, **90**, 38-49. https://doi.org/10.1016/j.plaphy.2015.02.023

Edwards EJ, Ogburn RM. 2012. Angiosperm responses to a low-CO_2 world: CAM and C_4 photosynthesis as parallel evolutionary trajectories. International journal of plant sciences, 173(6), pp.724-733. https://doi.org/10.1086/666098

Edwards EJ. 2023. Reconciling continuous and discrete models of C_4 and CAM evolution. Annals of Botany, **132**, 717-725. https://doi.org/10.1093/aob/mcad125

Edwards GE, Franceschi VR, Voznesenskaya EV. 2004. Single-cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annual Review Plant Biology, **55**, 173-196. https://doi.org/10.1146/annurev.arplant.55.031903.141725

Ehleringer JR, Monson RK. 1993. Evolutionary and ecological aspects of photosynthetic pathway variation. Annual Review of Ecology and Systematics, **24**, 411-439. https://www.jstor.org/stable/2097185

Ferrari RC, Freschi L. 2019. C_4/CAM facultative photosynthesis as a means to improve plant sustainable productivity under abiotic-stressed conditions: Regulatory mechanisms and biotechnological implications. In: Khan, M. I. R, Reddy, P- S., Ferrante, A, Khan, N. A. Plant signaling molecules. Woodhead Publishing, 517-532. https://doi.org/10.1016/B978-0-12-816451-8.00032-0.

Ferrari RC, Bittencourt PP, Rodrigues MA, Moreno-Villena JJ, Alves FR, Gastaldi VD, Boxall SF, Dever LV, Demarco D, Andrade SC, Edwards EJ, Hartwell J, Freschi L. 2020a. C_4 and crassulacean acid metabolism within a single leaf: deciphering key components behind a rare photosynthetic adaptation. New Phytologist, **225**, 1699-1714. https://doi.org/10.1111/nph.16265

Ferrari RC, Cruz BC, Gastaldi VD, Storl T, Ferrari EC, Boxall SF, Hartwell J, Freschi L. 2020b. Exploring C_4–CAM plasticity within the *Portulaca oleracea* complex. Scientific Reports, 10(1), p.14237. https://doi.org/10.1038/s41598-020-71012-y

Ferrari RC, Kawabata AB, Ferreira SS, Hartwell J, Freschi L. 2022. A matter of time: regulatory events behind the synchronization of C_4 and crassulacean acid metabolism in *Portulaca oleracea*. Journal of Experimental Botany, **73**, 4867-4885. https://doi.org/10.1093/jxb/erac163

Ferrari RC, Bittencourt PP, Nagumo PY, Oliveira WS, Rodrigues AM, Hartwell J, Freschi L. 2020c. Developing *Portulaca oleracea* as a model system for functional genomics analysis of C_4/CAM photosynthesis. Functional Plant Biology, **48**, 666-682. https://doi.org/10.1071/FP20202

Furbank R, Kelly S, von Caemmerer S. 2023. Photosynthesis and food security: the evolving story of C_4 rice. Photosynthesis Research, **158**, 121-130. https://doi.org/10.1007/s11120-023-01014-0

Furbank RT. 2011. Evolution of the C_4 photosynthetic mechanism: are there really three C_4 acid decarboxylation types? Journal of experimental botany, **62**, 3103-3108. https://doi.org/10.1093/jxb/err080

Gilman IS, Moreno-Villena JJ, Lewis ZR, Goolsby EW, Edwards EJ. 2022. Gene co-expression reveals the modularity and integration of C_4 and CAM in *Portulaca*. Plant Physiology, **189**, 735-753. https://doi.org/10.1093/plphys/kiac116

Gilman IS, Smith JAC, Holtum JA, Sage RF, Silvera K, Winter K, Edwards EJ, 2023. The CAM lineages of planet Earth. Annals of Botany, **132**, 627-654. https://doi.org/10.1093/aob/mcad135

Guralnick LJ, Jackson MD. 2001. The occurrence and phylogenetics of crassulacean acid metabolism in the Portulacaceae. *International Journal of Plant Sciences*, *162*, 257-262. https://doi.org/10.1086/319569

Guralnick LJ, Edwards G, Ku MS, Hockema B, Franceschi, V. 2002. Photosynthetic and anatomical characteristics in the C_4 crassulacean acid metabolism-cycling plant *Portulaca grandiflora*. Australian Journal of Plant Physiology, **29**, 763-773. https://doi.org/10.1071/PP01176

Guralnick LJ, Cline A, Smith M, Sage RF. 2008. Evolutionary physiology: the extent of C_4 and CAM photosynthesis in the genera *Anacampseros* and *Grahamia* of the Portulacaceae, *Journal of Experimental Botany*, **59**, 1735–1742. https://doi.org/10.1093/jxb/ern081

Guralnick L J Gladsky K. 2017. Crassulacean acid metabolism as a continuous trait: variability in the contribution of Crassulacean acid metabolism (CAM) in populations of Portulacaria afra. *Heliyon*, *3, e00293*. http://dx.doi.org/10.1016/j.heliyon.2017.e00293

Guralnick LJ, Gilbert KE, Denio, D, Antico, N. 2020. The development of crassulacean acid metabolism (CAM) photosynthesis in cotyledons of the C_4 species, *Portulaca grandiflora* (Portulacaceae). Plants, **9**, 55. https://doi.org/10.3390/plants9010055

Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI, Nimmo, HG. 1999. Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. *The Plant* Journal, **20**, 333-342. https://doi.org/10.1046/j.1365-313X.1999.00609.x

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo, HG. 1996. Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. The Plant Journal, **10**, 1071-1078. https://doi.org/10.1046/j.1365-313X.1996.10061071.x

Hatch., MD, Slack CR. 1966. Photosynthesis by sugar-cane leaves: a new carboxylation reaction and the pathway of sugar formation. Biochemical Journal, **101**, 103. https://doi.org/10.1042/bj1010103

Hatch MD. 1971. The C_4-pathway of photosynthesis. Evidence for an intermediate pool of carbon dioxide and the identity of the donor C_4-dicarboxylic acid. Biochemical Journal, **125**, 425-432. https://doi.org/10.1042/bj1250425

Heckmann D, Schulze S, Denton A, Gowik U, Westhoff P, Weber AP, Lercher MJ. 2013. Predicting C_4 photosynthesis evolution: modular, individually adaptive steps on a Mount Fuji fitness landscape. Cell, **153**, 1579-1588. https://doi.org/10.1016/j.cell.2013.04.058

Hernández-Bermejo J E, León J. 1994. Neglected Crops - 1492 from a different perspective. FAO Plant Production and Protection Series, n. 26. https://doi.org/10.1017/CBO9781107415324.004.

Herrera A. 2009. Crassulacean acid metabolism and fitness under water deficit stress: if not for carbon gain, what is facultative CAM good for? Annals of botany, **103**, 645-653. https://doi.org/10.1093/aob/mcn145

Holtum JA, Hancock LP, Edwards EJ, Winter K. 2017. Optional use of CAM photosynthesis in two C_4 species, *Portulaca cyclophylla* and *Portulaca digyna*. Journal of Plant Physiology, **214**, 91-96. https://doi.org/10.1016/j.jplph.2017.01.010

Jin R, Shi H, Han C, Zhong B, Wang Q, Chan Z. 2015. Physiological changes of purslane (*Portulaca oleracea* L.) after progressive drought stress and rehydration. Scientia Horticulturae, **194**, 215–221. https://doi.org/10.1016/j.scienta.2015.08.023.

Kadereit G, Borsch T, Weising K, Freitag H. 2003. Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of C_4 photosynthesis. International Journal of Plant Sciences, **164**, 959-986. https://doi.org/10.1086/378649

Kanai R, Edwards GE. The Biochemistry of C_4 photosynthesis. In: **Sage R F, Monson RK.** C_4 Plant Biology. San Diego: Academic Press, 49–87.

Keeley JE. 1998. CAM photosynthesis in submerged aquatic plants. *The Botanical Review*, **64**, 121–175. https://doi.org/10.1007/BF02856581

Koch K, Kennedy RA. 1980. Characteristics of crassulacean acid metabolism in the succulent C_4 dicot, *Portulaca oleracea* L. Plant physiology, **65**, 193-197. https://doi.org/10.1104/pp.65.2.193

Koch KE, Kennedy RA. 1982. Crassulacean acid metabolism in the succulent C_4 dicot, *Portulaca oleracea* L under natural environmental conditions. Plant Physiology, **69**, 757-761. https://doi.org/10.1104/pp.69.4.757

Ku SB, Shieh YJ, Reger BJ, Black CC. 1981. Photosynthetic characteristics of *Portulaca grandiflora*, a succulent C_4 dicot: Cellular compartmentation of enzymes and acid metabolism. *Plant Physiology*, **68**, 1073-1080. https://doi.org/10.1104/pp.68.5.1073

Kumar A, Sreedharan S, Kashyap AK, Singh P, Ramchiary N. 2022. A review on bioactive phytochemicals and ethnopharmacological potential of purslane (*Portulaca oleracea* L.). Heliyon, **8**, e08669. https://doi.org/10.1016/j.heliyon.2021.e08669

Lara MV, Disante KB, Podestá FE, Andreo, CS, Drincovich MF. 2003. Induction of a Crassulacean acid like metabolism in the C_4 succulent plant, *Portulaca oleracea* L.: physiological and morphological changes are accompanied by specific modifications in phosphoenolpyruvate carboxylase. Photosynthesis Research, **77**, 241-254. https://doi.org/10.1023/A:1025834120499

Lara MV, Drincovich MF, Andreo CS. 2004. Induction of a crassulacean acid-like metabolism in the C_4 succulent plant, *Portulaca oleracea* L.: study of enzymes involved in carbon fixation and carbohydrate metabolism. Plant and Cell Physiology, **45**, 618-626. https://doi.org/10.1093/pcp/pch073

Lim SD, Lee S, Choi WG, Yim WC, Cushman JC. 2019. Laying the foundation for crassulacean acid metabolism (CAM) biodesign: expression of the C_4 metabolism cycle genes of CAM in Arabidopsis. Frontiers in Plant Science, 10, p.101. https://doi.org/10.3389/fpls.2019.00101

Lundgren MR. 2020. C_2 photosynthesis: a promising route towards crop improvement?. New Phytologist, **228**, 1734-1740. https://doi.org/10.1111/nph.16494

Matthews JF, Ketron DW, Zane, SF. 1993. The biology and taxonomy of the *Portulaca oleracea* L. (Portulacaceae) complex in North America. Rhodora, 166-183. http://www.jstor.org/stable/23312973

Mazen AMA. 1996. Changes in levels of phosphoenolpyruvate carboxylase with induction of Crassulacean acid metabolism (CAM)-like behavior in the C_4 plant *Portulaca oleracea*. Physiologia plantarum, **98**, 111-116. https://doi.org/10.1111/j.1399-3054.1996.tb00681.x

Mazen AMA. 2000. Changes in properties of phosphoenolpyruvate carboxylase with induction of Crassulacean Acid Metabolism (CAM) in the C_4 plant *Portulaca oleracea*. Photosynthetica, **38**, 385-391. https://doi.org/10.1023/A:1010969419962

Moore, AJ, Vos, JMD, Hancock, LP, Goolsby, E and Edwards, EJ, 2018. Targeted enrichment of large gene families for phylogenetic inference: phylogeny and molecular evolution of photosynthesis genes in the Portullugo clade (Caryophyllales). Systematic Biology, **67**, 367-383. https://doi.org/10.1093/sysbio/syx078

Morales-Briones, DF and Kadereit, G, 2023. Exploring the possible role of hybridization in the evolution of photosynthetic pathways in Flaveria (Asteraceae), the prime model of C4 photosynthesis evolution. Bulletin of the Society of Systematic Biologists **2**, 1-16. https://doi.org/10.18061/bssb.v2i3.8992

Moreno-Villena JJ, Zhou H, Gilman IS, Tausta SL, Cheung CM, Edwards EJ. 2022. Spatial resolution of an integrated C_4+ CAM photosynthetic metabolism. Science Advances, **8**, p.eabn2349. https://doi.org/10.1126/sciadv.abn2349

Nyffeler R, Eggli U. 2010 Disintegrating Portulacaceae: a new familial classification of the suborder Portulacineae (Caryophyllales) based on molecular and morphological data. Taxon, **59**, 227-240. https://doi.org/10.1002/tax.591021

Ocampo G, Columbus JT. 2012. Molecular phylogenetics, historical biogeography, and chromosome number evolution of *Portulaca* (Portulacaceae). Molecular phylogenetics and evolution, **63**, 97-112. https://doi.org/10.1016/j.ympev.2011.12.017

Ocampo G, Koteyeva NK, Voznesenskaya EV, Edwards GE, SageTL, Sage, RF, Columbus JT. 2013. Evolution of leaf anatomy and photosynthetic pathways in Portulacaceae. American Journal of Botany, **100**, 2388-2402. https://doi.org/10.3732/ajb.1300094

Osmond CB. 1978. Crassulacean acid metabolism: a curiosity in context. Annual review of plant physiology, 29, 379-414. https://doi.org/10.1146/annurev.pp.29.060178.002115

POWO. 2025. Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; https://powo.science.kew.org/.

Retrieved 05 December 2025."

Ren S, Weeda S, Akande O, Guo Y, Rutto L, Mebrahtu T. 2011. Drought tolerance and AFLP-based genetic diversity in purslane (*Portulaca oleracea* L.). *Journal of Biotech Research*, **3**, 51–61.

Reyna-Llorens I, Hibberd JM. 2017. Recruitment of pre-existing networks during the evolution of C_4 photosynthesis. Philosophical Transactions of the Royal Society B: Biological Sciences, **372**, p.20160386. https://doi.org/10.1098/rstb.2016.0386

Sage RF. 2002. Are crassulacean acid metabolism and C_4 photosynthesis incompatible? Functional Plant Biology, **29**, 775-785. https://doi.org/10.1071/PP01217

Sage RF. 2004. The evolution of C_4 photosynthesis. New phytologist, **161**, 341-370. https://doi.org/10.1111/j.1469-8137.2004.00974.x

Sage RF. 2017. A portrait of the C_4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. Journal of experimental botany, **68**, e11-e28. https://doi.org/10.1093/jxb/erx005

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C_4 photosynthesis. Annual review of plant biology, **63**, 19-47. https://doi.org/10.1146/annurev-arplant-042811-105511

Schiller K, Janshoff S, Zenker S, Viehöver P, Hartwell J, Eirich J, Finkemeier I, Bräutigam A. 2025. Regulation of Crassulacean acid metabolism at the protein level in *Kalanchoë laxiflora*. Plant Physiology, **197**, kiaf095. https://doi.org/10.1093/plphys/kiaf095

Siadjeu C, Kadereit G. 2024. C_4-like *Sesuvium sesuvioides* (Aizoaceae) exhibits CAM in cotyledons and putative C_4-like+ CAM metabolism in adult leaves as revealed by transcriptome analysis. BMC genomics, 25, 688. https://doi.org/10.1186/s12864-024-10553-2

Singh JS, Singh KP. 1967. Contribution to the ecology of ten noxious weeds. Journal of the Indian Botanical Society, b, 440-451.

Thorne RF, Reveal JL. 2007. An updated classification of the class Magnoliopsida ("Angiospermae"). *The Botanical Review,* **73**, 67-182. https://doi.org/10.1663/0006-8101(2007)73[67:AUCOTC]2.0.CO;2

Voznesenskaya EV, Koteyeva NK, Edwards GE, Ocampo G. 2010. Revealing diversity in structural and biochemical forms of C_4 photosynthesis and a C_3–C_4 intermediate in genus *Portulaca* L. (Portulacaceae). Journal of Experimental Botany, **61**, 3647-3662. https://doi.org/10.1093/jxb/erq178

Walker B, Schmiege, SC and Sharkey, TD, 2024. Re-evaluating the energy balance of the many routes of carbon flow through and from photorespiration. Plant, Cell & Environment, **47**, 3365-3374. https://doi.org/10.1146/annurev-arplant-043015-111709

Wang X, Ma X, Yan G, Hua L, Liu H, Huang W, Liang Z, Chao Q, Hibberd JM, Jiao Y, Zhang M. 2023. Gene duplications facilitate C_4-CAM compatibility in common purslane. Plant Physiology, **193**, 2622-2639. https://doi.org/10.1093/plphys/kiad451

Winter K. 2019. Ecophysiology of constitutive and facultative CAM photosynthesis. Journal of experimental botany, **70**, 6495-6508. https://doi.org/10.1093/jxb/erz002

Winter K, Garcia M, Virgo A, Ceballos J, Holtum JA. 2020. Does the C_4 plant *Trianthema portulacastrum* (Aizoaceae) exhibit weakly expressed crassulacean acid metabolism (CAM)? Functional Plant Biology, **48**, 655-665. https://doi.org/10.1071/FP20247

Winter K, Sage RF, Edwards EJ, Virgo A, Holtum JA. 2019. Facultative crassulacean acid metabolism in a C_3–C_4 intermediate. Journal of Experimental Botany, **70**, 6571-6579. https://doi.org/10.1093/jxb/erz085

Winter K, Smith JAC. An introduction to crassulacean acid metabolism. Biochemical principles and ecological diversity. 1996. In: **Winter K, Smith JAC.**: Crassulacean Acid Metabolism Biochemistry, Ecophysiology and Evolution. Berlin, Heidelberg: Springer-Verlag, 1–10. https://doi.org/10.1007/978-3-642-79060-7_1

Yang X, Hu R, Yin H, Jenkins J, Shu S, Tang H, Liu D, Weighill DA, Cheol Yim W, Ha J, Heyduk, K, et al. 2017. The *Kalanchoë* genome provides insights into convergent evolution and building blocks of crassulacean acid metabolism. Nature communications, **8**, 1899. https://doi.org/10.1038/s41467-017-01491-7

Yang X, Liu Y, Yuan G, Weston DJ, Tuskan GA. 2024. Engineering crassulacean acid metabolism in C_3 and C_4 plants. Cold Spring Harbor perspectives in biology, **16**, a041674. https://doi.org/10.1101/cshperspect.a041674

Yazici I, Ismail T, Sekmen AH, Demiral T. 2007. Salinity tolerance of purslane (*Portulaca oleracea* L.) is achieved by enhanced antioxidative system, lower level of lipid peroxidation and proline accumulation. Environmental and Experimental Botany, **61**, 49–57, 2007. https://doi.org/10.1016/j.envexpbot.2007.02.010.

Zhang Y, Yin L, Jiang HS, Li W, Gontero B, Maberly SC. 2014. Biochemical and biophysical CO_2 concentrating mechanisms in two species of freshwater macrophyte within the genus *Ottelia* (Hydrocharitaceae). Photosynthesis research, **121**, 285-297. https://doi.org/10.1007/s11120-013-9950-y

Zimmerman CA. 1976. Growth characteristics of weediness in *Portulaca oleracea* L. *Ecology*, **57**, 964-974. https://doi.org/10.2307/1941061

Zimmerman CA. A comparison of breeding systems and seed physiologies in three species of *Portulaca* L. *Ecology*, **58**, 4, 860–868, 1977. https://doi.org/10.2307/1936221

Box 1. Recent key developments in the last five years to understanding the coupling of C_4 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 C_4 biochemical types (NADP-ME in *P. amilis* and NAD-ME in *P. oleracea*) was unprecedented in C_4-CAM transcriptomic studies. C_4 and CAM primarily relied on different gene sets for carbon fixation, and low level metabolite cycling between the two pathways was suggested. C_4 evolution in *Portulaca* likely involved co-opting redundant gene modules and integrating daytime CAM functions with the ancestral CAM network, posing little constraint on C_4 acquisition due to largely independent regulatory architectures that avoid pleiotropic interference. Overall, this work supported the hypothesis that C_4-CAM coexistence 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 a flux balance modelling (FBM) applied to C_4-CAM for the first time. Their findings provided the first empirical evidence for an integrated C_4-CAM cycle under drought, in which malate would accumulate in MCs and be stored overnight either locally or in BSCs, followed by decarboxylation in BCSs during the day. They also used tissue-specific transcriptomics to confirm that most genes 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 the C_4-CAM photosynthesis in *P. oleracea*. At transcriptional level, PPCK was suggested as a key point of connection to the molecular clock, and the expression patterns of several transcription factors were investigated, highlighting *HB7*, *NFYA7*, *NFYC9*, *TT8*, and *ARR12* as likely candidate regulators of CAM induction, and *NFYC4* and *ARR9* were connected to a C_4 expression. Under varying water availability, the exogenous application of abscisic acid was able to induce the C_4 gene homolog of PPC, whereas the application of cytokinins (both as zeatin and 6-Benzylaminopurine, BAP) was able to repress the CAM homologs 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 C_4 and CAM pathways. This work successfully confirmed that tthese duplications provided four C_4-specific and two CAM-specific *PPC* genes, with additional duplicated *β-CA* genes supporting both C_4 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 C_4-CAM function at the genomic level, with tandem and WGD event-driven diversification targeting key metabolic genes required for optimal CO_2-concentrating mechanisms under water limitation.

Table 1. Characteristics of C_4 photosynthesis and crassulacean acid metabolism (CAM) distinguishing these two carbon concentrating mechanisms (CCMs). References for the information contained in the table are given in the text. CBBC = Calvin-Benson-Bassham cycle; PPC = phosphoenolpyruvate carboxylase.

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: nighttime CO_2 fixation - stomata open; daytime CBBC and Rubisco activity - stomata closed for most part 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.

Table 2. Summary of the most pressing questions to understand the complex photosynthesis of *Portulaca oleracea*, merging C_4 and CAM in a single leaf.

Main topics	Specific questions
How do C_4 and CAM pathways coexist within the same leaf at the biochemical and regulatory levels?	○ What role do enzymes like 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?	○ What selective pressures favored 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?	○ How might synthetic biology approaches exploit this system for enhanced stress resilience? ○ Can aspects of this dual pathway be introduced into other species?

Figure caption

Fig. 1 *Portulaca oleracea* has been the most studied species in the genus in efforts to understand the coexistence of C_4 and CAM in a single leaf. (A) Wild accession of *Portulaca 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 coexistence 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), glutamate (Glu), PPDK-regulatory protein (PPDK-RP). and Rubisco (RBS). Bold font indicates enzyme names. Black arrows indicate the constitutive C_4 pathway taking place under well-watered conditions. Red arrows indicate the metabolic switch that happens when drought is established and the hybrid C_4-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 drought-stressed plant for two weeks.

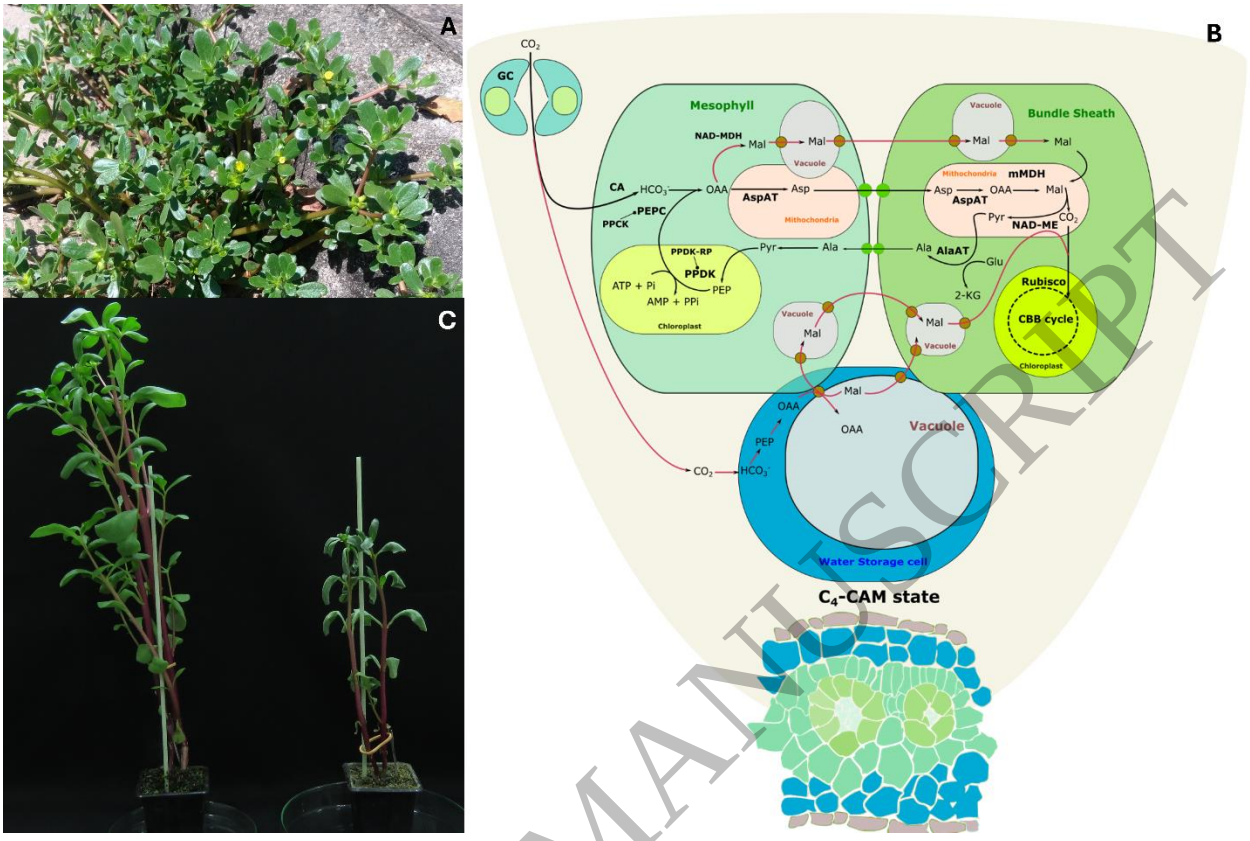

Figure 1
559x370 mm (x DPI)