**C4 and crassulacean acid metabolism within a single leaf: deciphering key components behind a rare photosynthetic adaptation**

Renata C. Ferrari1, Priscila P. Bittencourt1, Maria A. Rodrigues1, Jose J. Moreno-Villena2, Frederico R. R. Alves1, Vinícius D. Gastaldi3, Susanna F. Boxall4, Louisa V. Dever4, Diego Demarco1, Sónia C. S. Andrade5, Erika J. Edwards2, James Hartwell4, Luciano Freschi1

1Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, 05508-090, Brasil;

2Department of Ecology and Evolutionary Biology, Yale University, P.O. Box 208105, New Haven, Connecticut 06520, USA;

3Departamento e Instituto de Psiquiatria, Hospital das Clínicas (HCFMUSP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, 05403-903, Brasil;

4Department of Functional and Comparative Genomics, Institute of Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK*.*

5Departamento de Genética e Biologia Evolutiva,Instituto de Biociências, Universidade de São Paulo, São Paulo, 05508-090, Brasil;

Authors for correspondence:

Luciano Freschi

Tel: +55 11 3091 8068

Email: [freschi@usp.br](mailto:freschi@usp.br)

James Hartwell

Tel: +44 0 151 795 4561

Email: james.hartwell@liverpool.ac.uk

**Word count:**

Main body of the text (Introduction, Materials and Methods, Results, Discussion): 6,500 words

Introduction: 1,023 words

Materials and Methods: 715 words

Results: 2,987 words

Discussion: 1,775 words

Number of figures: 7 figures (all figures should be published in colour)

Supporting information: 14 figures, 8 tables and 1 extended method.

**ORCiD record:**

Renata C. Ferrari: https://orcid.org/0000-0002-9497-8442

Frederico R. R. Alves: https://orcid.org/0000-0002-2540-4699

Vinícius D. Gastaldi: https://orcid.org/0000-0002-6249-6035

Susanna F. Boxall: https://orcid.org/0000-0002-8753-101X

Louisa V. Dever: https://orcid.org/0000-0001-7801-5622

Diego Demarco: https://orcid.org/0000-0002-8244-2608

Sónia C. S. Andrade: https://orcid.org/0000-0002-1302-5261

James Hartwell: https://orcid.org/0000-0001-5000-223X

Luciano Freschi: https://orcid.org/0000-0002-0737-3438

**Brief heading:** Deciphering the key components behind C4 and CAM in *Portulaca oleracea*

**SUMMARY**

* Though biochemically related, C4 and crassulacean acid metabolism (CAM) systems are expected to be incompatible. However, *Portulaca* species, including *P. oleracea*, operate C4 and CAM within a single leaf, and the mechanisms behind this unique photosynthetic arrangement remain largely unknown.
* Here, we employed RNA-seq to identify candidate genes involved exclusively or shared by C4 or CAM, and provided an in-depth characterization of their transcript abundance patterns during the drought-induced photosynthetic transitions in *P. oleracea*. Data revealed fewer candidate CAM-specific genes than those recruited to function in C4. The putative CAM-specific genes were predominantly involved in nighttime primary carboxylation reactions and malate movement across the tonoplast. Analysis of gene transcript-level regulation and photosynthetic physiology indicated that C4 and CAM co-exist within a single *P. oleracea* leaf under mild drought conditions.
* Developmental and environmental cues were shown to regulate CAM expression in stems, whereas the shift from C4 to C4-CAM hybrid photosynthesis in leaves was strictly under environmental control. Moreover, efficient starch turnover was identified as part of the metabolic adjustments required for CAM operation in both organs.
* These findings provide insights into C4/CAM connectivity and compatibility, contributing to a deeper understanding of alternative ways to engineer CAM into C4 crop species.

**Keywords**: C4 photosynthesis, crassulacean acid metabolism, drought stress, facultative CAM, *Portulaca oleracea*, RNA-seq, transcriptome.

# INTRODUCTION

C4 photosynthesis and crassulacean acid metabolism (CAM) are two major carbon concentrating mechanisms (CCMs) in higher plants (Hatch 1987; Griffiths 1989; Keeley & Rundel, 2003). C4 occurs in ca. 19 families, mostly distributed in hot and high-light environments (Sage, 2017). It relies on the spatial separation between primary CO2 assimilation by phosho*enol*pyruvate carboxylase (PPC) in mesophyll cells (MCs) and secondary refixation by ribulose 1,5-biphosphate carboxylase/oxygenase (RuBisCO) in bundle sheath cells (BSCs) (Laetsch, 1968; Kanai & Edwards, 1999), being usually associated with Kranz anatomy (Voznesenskaya *et al.,* 2001, 2002). Besides increasing photosynthetic rates and suppressing photorespiration, C4 can increase water use efficiency (WUE) relative to C3 as lower stomatal conductance is required for a given assimilation rate (Taylor *et al.,* 2014).

CAM is found in at least 35 families mainly distributed in arid environments (Winter, 2019), and is also commonly, but not exclusively, associated with higher leaf succulence (Nelson *et al.,* 2005; Silvera *et al.,* 2010; Edwards 2019). In contrast to C4, CAM occurs in each individual MC and relies on temporal coordination of primary and secondary CO2 fixation by the circadian clock (Hartwell, 2006). In strong CAM plants, stomatal opening and consequently atmospheric CO2 uptake and C4 acid formation are rescheduled to the dark period, when transpiration rates are lower, whereas stomata close for much of the light period, preventing water loss and increasing WUE (Osmond, 1978; Borland *et al*., 2009). The dark CO2 fixation by PPC and the daytime refixation of CO2 by RuBisCO characterize CAM phases I and III, respectively, with additional transitional phases at dawn and dusk (phases II and IV, respectively) where stomata can also be open allowing direct fixation of atmospheric CO2 by RuBisCO (Winter & Smith, 1996). CAM also differs from C4 in its variability of expression, as many facultative plants operate CAM under stress (e.g., drought), but perform C3 photosynthesis whenever conditions allow (Winter & Holtum, 2014). CAM is weakly expressed in many facultative species as nocturnal CO2 fixation under stress conditions contributes less than 5% compared with daytime CO2 fixation via C3 under optimal growth conditions (Winter, 2019).

A set of biochemical reactions are shared by C4 and CAM, including many involved in carboxylation and decarboxylation steps (Bräutigam *et al*., 2017), since both CCMs depend on the formation of four-carbon organic acids to store, transport and release CO2 in the vicinity of RuBisCO (Edwards & Ogburn, 2012).). Post-translational regulatory steps are also shared in C4 and CAM species, including the phosphorylation-dependent control of PPC and PPDK (pyruvate, orthophosphate dikinase) activities by PPC kinase (PPCK) and PPDK-regulatory protein (PPDKRP), respectively (Nimmo *et al*., 2001; Chastain & Chollet, 2003; Dever *et al.,* 2015). However, while C4 requires specific metabolites, transporters and decarboxylases (Kanai & Edwards, 1999; Schluter *et al.,* 2016), CAM relies on strict temporal coordination of primary and secondary CO2 fixation and appropriate transporters for acid import and export from the vacuole (Winter & Smith, 1996; Hartwell *et al*., 2016). Differently from C4 species, a dedicated pool of carbohydrates (starch or soluble sugars, depending on the species) is converted into PEP via glycolysis each night to sustain PPC activity in CAM (Black *et al.,* 1996; Weise *et al.,* 2011). Moreover, a change from the hydrolytic to the phosphorolytic starch breakdown pathway is usually observed during CAM induction in facultative species (Borland *et al*., 2016).

C4 and CAM are believed to be incompatible to occur in the same cell, as each CCM requires potentially conflicting regulatory processes, metabolic fluxes and structural arrangements (Sage, 2002). However, the occurrence of both CCMs within a single leaf is rare, but possible, as observed in *Portulaca* species (Sage, 2002; Winter, 2019). Species from all main *Portulaca* lineages have been reported to induce CAM in response to drought (Koch & Kennedy, 1980, 1982; Ku *et al.,* 1981; Guralnick & Jackson, 2001; Holtum *et al.,* 2017; Winter & Holtum, 2017; Winter *et al.,* 2019). Among them, *P. oleracea* L. displays rapid growth, high seed production (up to 200,000 seeds/plant), a cosmopolitan distribution, and it is economically important as food and medicine (Zimmerman, 1976; Miyanishi & Cavers, 1980; Gonnella *et al.,* 2010). *P. oleracea*, therefore, represents an attractive model for the study of the C4-CAM compatibility in leaves (Hartwell *et al*., 2016; Ferrari & Freschi, 2019). Moreover, stems are also photosynthetic in this species, and can accumulate acids overnight under drought (Koch & Kennedy, 1980), but are devoid of C4 anatomical attributes (Voznesenskaya *et al.,* 2010).

Both C4 and CAM are relevant targets for forward genetic engineering into C3 crop species due to their natural ability to outperform C3 plants in hotter and drier environments. Attempts are underway to engineer the C4 pathway into rice, such as the C4 Rice Initiative (Kajala *et al.,* 2011; Covshoff & Hibberd, 2012), and to transfer CAM into C3 food and bioenergy crops (Borland *et al.,* 2009, Yang *et al.,* 2015; Hartwell *et al.,* 2016). From this perspective, *P. oleracea* provides a potentially unique genetic blueprint of how C4 and CAM can occur within a single leaf. As such, unraveling the components behind this rare photosynthetic adaptation may provide valuable insights that can guide and inform the challenge of bioengineering facultative CAM into C4 crops, combining high productivity under optimum growth conditions and increased survival during intermittent drought events (Ferrari & Freschi, 2019).

In this study, we report the gene transcript level and metabolic changes associated with the modulation of C4 and CAM expression in *P. oleracea* plants challenged by alterations in water availability. We focused on the identification of components that were either exclusive to or shared by C4 and/ or CAM pathways. Our results revealed that inducible CAM in *P. oleracea* requires the up-regulation of relatively few CCM genes, and that the induction of these genes can be controlled either by the environment (drought) or by developmental cues, depending on the organ (leaves or stems). Simultaneous C4 and CAM functioning were observed soon after drought exposure, indicating that both CCMs can co-exist within a single leaf. Novel insights are also provided into the additional metabolic adjustments (e.g., sugar metabolism rewiring) and regulatory mechanisms (e.g., transcriptional regulation of key genes) involved in this remarkable photosynthetic adaptation.

**MATERIAL AND METHODS**

**Plant material, growth conditions and sampling**

Plants were grown in 25-ml square pots containing commercial substrate and vermiculite at a photosynthetic flux density (PFD) at plant height of 250-300 μmol m-2 s-1, 12h photoperiod, temperature of 27 ± 1ºC day and 22 ± 1ºC night, and air humidity of 60 ± 10% day and 80 ± 10% night. After 20-30 days of initial growth, plants were separated into three experimental groups, each one submitted to a different watering regime as follows: (1) plants watered daily (well-watered), (2) plants subjected to water withholding until soil volumetric water content (SVWC) reached 10-20% and subsequently maintained at this level until sampling (drought-stressed), (3) plants irrigated to field capacity after 22 and 34 days of drought (re-watered). SVWC was continuously monitored (every 30 minutes) using Decagon soil moisture meter EC-5 coupled to the Em5b datalogger (Decagon Devices, Pullman, WA, USA). Leaf and stem osmotic potential (Ψs) were monitored at dawn, as described in Methods S1.

Sampling of leaves and stems took place after 0, 10, 22 and 34 days of drought treatment (D0, D1, D2 and D3, respectively), and also 4-days after re-watering events that were initiated at D2 and D3 (R1 and R2, respectively). Samples were harvested one hour after the onset of illumination (dawn samples) and one hour before the end of the light period (dusk samples). At D3, leaf and stem samples were also harvested every four hours over a light/dark cycle starting two hours after the onset of the light period (8h, 12h, 16h, 20h, 24h, 4h, 8h). Three to four biological replicates, each composed of at least three individual plants per replicate, were harvested at each sampling time. All fully-expanded and non-senescent leaves (leaf samples) and whole stems (stem samples) were frozen in liquid N2, powdered and stored at -80°C until use.

**Leaf gas exchange, chlorophyll *a* fluorescence and metabolite analysis**

Net CO2 uptake (*A*), stomatal conductance (*gs*) and transpiration rate (*E*) were determined for the third fully expanded leaf (counting from the top) of at least three individuals using an infrared gas analyzer (Li 6400XT, Li-Color, Lincoln, USA). Measurements were performed between the 2nd and the 5th hour after the onset of illumination, under controlled conditions of CO2 concentration (380 ppm CO2), PFD approx. 300 μmol m-2 s-1 and chamber temperature of 25ºC. Whenever relevant, continuous monitoring of net CO2 uptake and transpiration rate over the 24h cycle was also performed as described in Boxall *et al.* (2019). Chlorophyll *a* fluorescence parameters were measured using a portable Pulse Amplitude Modulation fluorometer (PAM-2500, Walz, Germany) following the protocols and equations described by Alves *et al.* (2016). Values of minimal (Fo) and maximal (Fm) fluorescence were obtained from leaves dark-adapted for 30 min before receiving a saturating pulse of light (3000 μmol photons m-2 s-1 for 1 s).

Titratable acidity analysis and organic acid and soluble carbohydrate profiling were performed as described in Freschi *et al.* (2010), with modifications described in Methods S1. Starch content was determined from dried pellets as described in Amaral *et al.* (2007). Whenever applicable, malate content was also quantified enzymatically as described in Boxall *et al.* (2019).

**Anatomical analyses**

Leaves and stems were analyzed by light microscopy and scanning electron microscopy as described in Methods S1.

**RNA extraction and** **qPCR analysis**

Total RNA was extracted using the ReliaPrep™ RNA Tissue Miniprep System (Promega), with modifications described in Methods S1. Complementary DNA (cDNA) synthesis and reverse-transcriptase-quantitative PCR (qPCR) analysis were performed according to Cruz *et al.,* (2018), with modifications described in Methods S1. All primer sequences used are listed in Table S1.

**RNA-seq** ***de novo* generation, assembly, annotation and analysis**

Total RNA was extracted as described above from leaf and stem samples harvested in biological triplicates from well-watered and drought-stressed plants at D3 (8h, 16h and 24h, corresponding to 2h and 10h after the onset of light, and 6h into the 12 h dark period, respectively). Library preparation and sequencing using the Illumina HiSeq4000 platform was detailed in Methods S1. RNA-seq assembly, annotation and all statistical analysis were performed as described in Methods S1. Fastq sequence files were deposited at NCBI under BioProject PRJNA576481.

**Identification of** **homologs and co-orthologs of CCM genes**

Homologous and co-orthologous sequences related to genes encoding known CCM-related enzymes were identified as described in Moreno-Villena *et al.* (2017) with modifications described in Methods S1.

**RESULTS**

**Assessing CAM induction in *P. oleracea* leaves and stems** **during progressive drought treatment**

Well-watered *P. oleracea* leaves showed atriplicoid-type anatomy, high daytime CO2 uptake rates, and did not accumulate acids overnight (Fig. 1), confirming the use of C4 whenever water was not limiting. In contrast, 34 days of water withholding promoted significant leaf nocturnal malic acid accumulation (ΔH+) as detected via either enzymatic (Fig. 1b) or chromatographic (Fig. S1a) malate quantification and titratable acidity analysis (Fig. S1b), which was associated with no diel leaf gas exchange (Fig. 1c), thereby characterizing a CAM idling state (Winter, 2019). CAM expression was up-regulated rather than induced in *P. oleracea* stems in response to drought, likely relying on internal refixation of respiratory CO2 as the stems lacked stomata (Fig. S2c-d). Kranz anatomy traits were absent in stems, in which ΔH+ detected under well-watered conditions was significantly intensified upon drought (Fig. 1a-b), and chloroplasts are mainly distributed in cells of the inner cortex (Fig. S2g-h).

**Global transcriptional changes in response to drought**

To gain insight into the transcriptional changes associated with C4 and CAM functioning in *P. oleracea*, RNA-seq was performed using leaf and stem RNA samples isolated from well-watered and drought-stressed plants harvested at 8h, 16h and 24h. A total of 1,061,191,351 reads were obtained from all 36 libraries. Our *de novo* assembly for *P. oleracea* yielded 452,522 contigs, of which 84,494 were kept after ORF identification and redundancy elimination (Table S2). 52,514 contigs were successfully annotated against the UniProt database and 80.35% of trimmed reads of all samples were mapped against our *de novo* assembly. Only the longest isoform for each annotated contig was kept (see Methods S1 for details), resulting in a total of 32,306 contigs.

Differentially expressed (DE) contigs in response to drought were identified at each sampling time-point, revealing that approximately 6 and 14% of the whole transcriptome were down-regulated upon drought in leaves and stems, respectively (Fig. S3a; Table S3). Enrichment analysis for gene ontology (GO) terms revealed that photosynthesis and sugar metabolism-related terms were particularly down-regulated in drought-stressed leaves (Table S4), which agrees with the negative influence of drought on leaf CO2 assimilation (Fig. 1c). Photosynthesis- and carbohydrate metabolism-related GO terms were also enriched in well-watered leaves compared to well-watered stems (Table S4), reinforcing that, although photosynthetically active, *P. oleracea* stems play a minor role in the overall shoot carbon fixation compared to the leaves.

The mean correlation coefficients obtained by comparing log2 fold-change (logFC) values from RNA-seq and log2 ratio of mRNA relative abundance from qPCR analysis revealed adequate correspondence between RNA-seq and qPCR data in both leaf and stem samples (Fig. S4).

**Using transcriptomic data to identify CCM-related genes**

Multiple approaches were employed to identify key candidate genes involved in *P. oleracea* CCM reactions. First, genes encoding major C4/CAM-related genes were filtered according to annotation (Table S5), and the most representative contig for each gene was selected based on transcript abundance and logFC (Figs. 2-3, S5-S6). Second, gene nomenclature was established according to Christin *et al.* (2014), based on phylogenetic trees inferred using data from C3, C4 and CAM plants. Groups of co-orthologs for selected CCM genes (Figs. S7, S8) matched those previously identified for *Portulaca* (Christin *et al.,* 2014, 2015). Third, CCM genes differentially regulated by drought were further analyzed via qPCR for a detailed characterization of their diel cycles of transcript abundance (Figs. 3, 5).

A typical C4-like transcript abundance pattern was observed for CCM genes down-regulated by drought (Fig. 2a), as they were more abundant in leaves (Fig. 2b) and accumulated overnight reaching a peak level phased to dawn (Fig. 3a), thereby preceding the start of C4 reactions. In contrast, fewer CCM genes were significantly up-regulated following drought in both organs (Fig. 2a) and peaked in the dark (Fig. 3b), coinciding with the nocturnal acid accumulation promoted by drought in *P. oleracea* leaves and stems (Fig. 1b).

In both CCMs, CO2 can be converted to HCO3- passively or enzymatically by a beta-carbonic anhydrase (βCA). HCO3- is combined with phospho*enol*pyruvate (PEP) by PPC to generate oxaloacetate (OAA) (Hatch, 1987). Although shared by both CCMs, these reactions occur at different times over the 24 h light/ dark cycle in C4 and CAM, namely in the light and the dark, respectively. Among *P. oleracea* βCA- and PPC-encoding genes, *βCA-2E3* and *PPC-1E1a’* exhibited a C4-like expression pattern, whereas *βCA-1E1* and *PPC-1E1c* were expressed in a CAM-like fashion (Fig. 2), reinforcing previous evidence that specific gene family members may have been recruited to fulfill the C4 and CAM carboxylation reactions according to the prevailing environmental conditions (Christin *et al.,* 2014).

*PPCK* is induced by high-light in C4 leaves (Carter *et al*., 1991). In contrast, *PPCK* expression is mainly controlled by the endogenous circadian clock in CAM species, leading to a dark phased peak of PPCK activity, which promotes PPC phosphorylation and consequently increases the Ki of PPC for feedback inhibition by L-malate (Hartwell *et al.,* 1996; Hartwell *et al*., 1999). In *P. oleracea*, RNA-seq data revealed a single *PPCK* ortholog (*PPCK-1E*), which showed transcript levels that peaked 2 h into the 12-h-light period (08:00) in C4-performing leaves and peaked 16 h later (at 24:00) in CAM-performing leaves and stems (Figs. 3c, S5).

C4 species can use either NAD-malic enzyme (NAD-ME) or NADP-ME as primary decarboxylation enzymes with or without the additional involvement of PEP carboxykinase (PCK) (Furbank, 2011). *P. oleracea* has been classified as a NAD-ME-type species (Lara *et al*., 2004; Voznesenskaya *et al*., 2010), implying that most CO2 incorporated as OAA in this species is converted to aspartate (ASP) in MCs via aspartate aminotransferase (AspAT) activity. The ASP formed diffuses to BSCs via plasmodesmata and enters the mitochondria, where AspAT reverts ASP to OAA, and NAD-malate dehydrogenase (MDH) converts OAA to malate, which is decarboxylated by NAD-ME (Kanai & Edwards, 1999). To sustain these reactions, ASP and malate movements across mitochondria are facilitated by Uncoupling protein 2 (UCP2) and Dicarboxylate transport 1 (DIC1) proteins, respectively (Vozza *et al.,* 2014; Monné *et al.,* 2018; Palmieri *et al.,* 2008). Transcripts encoding enzymes and transporters characteristic of NAD-ME-type photosynthesis (*ASPAT-1E1*, *NADMDH-2E*, *NADMDH-3C1a*, *NADME-1E, NADME-2E.1*, *UCP-2* and *DIC-1.2*) were abundant in well-watered leaves and exhibited C4-like diel regulation, with peak levels phased to 08:00, 2h into the 12-h-light period (Figs. 2-5). In contrast, *ASPAT-3C2* was up-regulated significantly by drought in both leaves and stems, with a transcript peak phased to the second half of the dark period (Fig. 3b). However, ASP to OAA interconversion via AspAT is not part of the canonical CAM cycle, and *ASPAT-3C2* is phylogenetically closest to Arabidopsis *ASP3* (AT5G11520) (Fig. S7), which encodes a peroxisomal and chloroplastic protein implicated in photorespiration and senescence processes (Fukao *et al.,* 2002, Schultz & Coruzzi 1995; Wilkie & Warren 1998). Therefore, it seems unlikely that *ASPAT-3C2*-encoded protein is directly involved in *P. oleracea* CAM machinery.

Other genes, including *NADMDH-1C2*, *NADMDH-6E1* and *NADME-2E.2*, could be either shared by both CCMs or responsible for anaplerotic reactions since they did not show a clear pattern of modulation by drought (Figs. 3c, S5). Transcripts of a NADP-ME-encoding gene (*NADPME-1E1b*) were abundant in both C4- and CAM-performing tissues (Fig. S5), but *NADPME-1E1b* is phylogenetically closest toArabidopsis *NADP-ME4* (AT1G79750) (Fig. S7), which is implicated in fatty acids biosynthesis (Wheeler *et al.,* 2005). The only PCK-encoding gene identified (*PCK-1E1*) was a low abundance transcript compared to other decarboxylases (Fig. S5), suggesting a limited, if any, contribution of PCK for decarboxylation reactions in this species.

In C4 plants such as *P. oleracea*, after CO2 is released by NAD-ME, the residual pyruvate (PYR) is transported to the cytosol by an unknown transporter, converted to alanine (ALA) by ALA aminotransferase (AlaAT) to flow back to MCs and be reverted to PYR again finally entering the chloroplast (Kanai & Edwards, 1999). PYR is imported into the chloroplast in exchange of sodium by the Bile acid:sodium symporter family protein (BASS), functioning in concert with the sodium:hydrogen antiporter (NHD) (Furumoto *et al.,* 2011). PEP supply is restored via PPDK, releasing pyrophosphate (PPi) and adenosine monophosphate (AMP), which are further metabolized via pyrophosphorylase (PPa) and AMP kinase (AK) activities, respectively (Matsuoka, 1995). PEP is then exported to the cytosol by PEP/phosphate translocator (PPT) (Thompson *et al.,* 1987). Genes encoding this suite of enzymes (*ALAAT-1E1, PPDK-1C1b.1, PPA-1E, AK-1*) and transporters (*BASS-1* and *PPT-1E2*) were abundant transcripts that displayed a C4-like temporal pattern in well-watered leaves (Figs. 2-5). In response to drought, *BASS-1*, *PPT-1E2*, *ALAAT-1E1* and *AK-1* were down-regulated in leaves, whereas *PPA-4C* was upregulated by drought in leaves (Fig 2a) and *PPA-1E* and *PPDK-1C1b.2* were up-regulated by drought in stems (Fig. 3c). As PPDK activity is required at daytime in both C4 and CAM systems (Chastain *et al.,* 2002; Dever *et al.,* 2015), the *PPDKRP-1C1* transcript peak detected at the end of the light period (Fig. 3a) is consistent with the fact that PPDK needs to be activated in the light and inactivated in the dark in both C4 and CAM. Similar diel transcript abundance patterns have also been reported previously for *PPDKRP*-encoding genes in C4 and CAM species (Chastain & Chollet, 2003; Dever *et al.,* 2015).

In CAM plants, malate accumulates as vacuolar malic acid due to nocturnal CO2 fixation, and is subsequently released from the vacuole in the light period. The most likely candidates for nocturnal voltage-gated uptake of malate are the tonoplast-localized aluminum-activated malate transporters (ALMT) (Kovermann *et al.,* 2007; Borland *et al*., 2009). Also, the tonoplast dicarboxylate transporter (tDT) may function in the transport of malate out of the vacuole for decarboxylation in the light period (Borland *et al*., 2009). In *P. oleracea*, *ALMT-12E.1* and *ALMT-9E* were up-regulated by drought in both leaf and stem tissues, whereas the opposite was observed for *ALMT-12E.2*, which was most abundant at the start of the light period in leaves under well-watered conditions (Figs. 4-5, S6). Among *ALMT* genes, *ALMT-9E* is closely related to Arabidopsis *ALMT9* (AT3G18440), a voltage-gated chloride channel in the tonoplast of guard cells (Zhang *et al.,* 2013), that has also been reported to act as a vacuolar malate channel in MCs (Kovermann *et al.,* 2007; De Angeli *et al.,* 2012). In addition to *ALMT* genes, *TDT-1E* was markedly up-regulated by drought in stems, with a peak phased to 2 h before dawn (Figs. 3, S6), which may suggest involvement in CAM.

Malate import into vacuoles in the dark period during CAM is mediated by a voltage-gated, inward-rectifying anion channel (Hafke *et al*., 2003). The membrane potential difference across the tonoplast membrane that energizes this import is generated by the V-ATPase (also known as Vacuolar-type proton ATPase – VHA) and/ or the VPPase (Pyrophosphate-energized membrane proton pump – AVP) (Smith *et al.,* 1996). Drought promoted up- and down-regulation of *VHAB2-1E1* and *AVP1-2E*, respectively (Figs. 4-5), and slightly increased transcript abundance for a range of other VHA subunit genes, including *VHAA3-3E*, *VHAF-1E* and *VHAG1-1E* (Fig. S6). Taken together, these results suggest apreference for VHA over AVP for the nocturnal accumulation of protons in the vacuole, which in turn energize nocturnal malate import via the putative tonoplast ALMT in CAM-performing *P. oleracea* tissues. It was noteworthy that *VHAB2-1E1* transcripts peaked in the middle of the night in drought-stressed leaves of *P. oleracea* (Fig. 3c), consistent with the proposed regulation of V-ATPase activity via the level of the transcript abundance of the associated subunit genes (Chen *et al*., 2012).

**Temporal dynamics of photosynthetic transitions in response to water availability**

Following the identification of signature C4 and CAM genes (Figs. 2-5), we next performed comprehensive analysis of their temporal expression dynamics, aiming to determine (1) whether C4 and CAM co-occur as drought conditions intensify, and (2) whether CAM is reverted after re-watering events applied at distinct points of the drought treatment. Therefore, transcript abundance of CCM-marker genes (Table S6), fluctuations in *Ψs*, ΔH+, leaf gas exchange and chlorophyll *a* fluorescence were monitored in samples harvested after 0-, 10-, 22- and 34-days of drought treatment (named D0, D1, D2 and D3, respectively) and during re-watering events initiated after 22- and 34-days of drought (R1 and R2, respectively).

Drought reduced plant growth, resulting in shorter shoots with fewer branches in comparison to well-watered plants (Fig. 6a). The prolonged maintenance of SVWC below 20% field capacity (Fig. 6b) resulted in a significant decrease in both leaf and stem *Ψs*(Fig. 6c), which was associated with the reduction of *A*, *gs* and *E* values close to or below zero, the latter signifying the respiratory loss of CO2 from the leaf (Fig. 6d). In agreement, leaf mRNA levels of genes encoding RuBisCO small subunit (*RBCS*), RuBisCO activase (*RCA*) and most photorespiration-related enzymes (Table S7), which were named according to phylogenetic analysis (Fig. S9), declined progressively in response to lengthening drought (Fig. S10).

Leaf transcript abundance of C4-marker genes and daytime CO2 uptake were progressively reduced as drought intensified over time, whereas CAM-marker genes and ΔH+ exhibited the opposite trend (Figs. 6e-h, S11), indicating a gradual, rather than abrupt, intensification of CAM in leaves. Consequently, both physiological and gene transcript abundance data suggested that both CCMs may co-exist in *P. oleracea* leaves under mild water deficit (D1). Furthermore, transcript levels of C4-related genes in leaves were still detected at about 10 to 40% of their original abundance when maximum CAM expression had already been achieved (D2).

In contrast, drought-induced ΔH+ and CAM-related transcript accumulation were rapidly and completely reversed upon re-watering (Fig. 6), regardless of the duration of the preceding drought treatment (R1 and R2). Within 1 and 2 days after re-watering, leaf *Ψs*, daytime CO2 uptake and C4-related gene expression were fully re-established to levels similar to well-watered plants (Fig. 6). Therefore, CAM seems to be entirely replaced by C4 in leaves of *P. oleracea* soon after the water supply is re-established. In contrast, stems showed a progressive increase in both ΔH+ and *PPC-1E1c* mRNA levels during plant growth under well-watered conditions, which was intensified by drought (Fig. 6e,g), reinforcing that CAM expression in stem tissues is under both ontogenetic and environmental regulation.

The progressive decline in *A*, *gs*, *E* and transcript levels of *RBCS*, *RCA* and C4-signature genes in *P. oleracea* droughted leaves was not accompanied by significant changes in chlorophyll *a* fluorescence parameters such Fv/Fm, Fq′/Fm, ETR and NPQ (Fig. S12). This suggests that linear electron transport within chloroplasts may have been maintained despite the drought-induced decline in atmospheric CO2 uptake (Fig. 1c).

**Starch turnover intensified during CAM in *P. oleracea***

Transitory leaf starch metabolism is not as important for C4 as it is for starch-storing CAM species, which require a sufficient pool size of starch at the start of each night in order to supply PEP to PPC throughout the dark period (Weise *et al*., 2011). In line with these differential metabolic demands, a significant rewiring of carbohydrate-associated metabolism was observed during CAM induction and intensification in *P. oleracea* leaves and stems, respectively (Fig. 7; Table S8). Drought-promoted diel fluctuation in starch levels was exclusively observed in stem tissues, achieving levels up to 2-fold higher in drought-stressed compared to well-watered plants at the end of the light period, and declining to similar levels in both watering conditions during the first half of the dark period (Fig. 7a). Consequently, the drought-triggered increment in stem ΔH+ coincided with an intensification in diel starch fluctuation. CAM induction in leaves was instead associated with lower starch accumulation in leaves, which correlates with the progressive reduction in leaf photosynthetic carbon assimilation caused by drought-induced stomatal closure (Figs. 1b, 6d).

Transcript levels of contigs encoding both the small (*ADG1* and *ADG2*) and large (*APL3*) subunits of ADP-glucose pyrophosphorylase (AGPase), the first committed step in starch biosynthesis, as well as Granule-bound starch synthase (*GBSS1*), responsible for amylose synthesis, peaked at 2 h into the light period, regardless of the tissue or watering condition (Fig. S13; Table S8). At this temporal peak, drought promoted transcript accumulation of *ADG1*, *APL3* and *GBSS1* in stems, but not in leaves (Figs 7b, S13). Transcript abundance of genes encoding enzymes involved in both the hydrolytic (amylolytic) and the phosphorolytic pathways of transitory starch degradation, including Isoamylase 3 (*ISA3*), Limit Dextrinase or Pullulase (*LDA*), Disproportionating enzyme 1 (*DPE1*) and Alpha-glucan water dikinase dikinase (*GWD*) were up-regulated in both organs in response to drought (Fig. 7b; Table S8). Moreover, genes encoding enzymes associated with hydrolytic starch degradation – Phosphoglucan phosphatase (*SEX4*), Alpha-amylase (*AMY3*) and Beta-amylase (*BAM1*) – as well as those required for the phosphorolytic pathway – Starch phosphorylase1 (*PHS1*) – were also up-regulated upon drought (Figs. 7b, S13). Besides, chloroplast maltose exporter-encoding gene *MEX1* was predominantly down-regulated upon drought, whereas *GPT2.2*, which encodes a G6P/phosphate translocator, was up-regulated in both organs in response to drought (Fig. 7b, S14).

Maltose can be converted to glucose via cytosolic Disproportionating enzyme 2 (*DPE2*), Starch phosphorylase 2 (*PHS2*) and Phosphoglucomutase 2 (*PGM2*) (Brilhaus *et al.,* 2016). Transcripts encoding these three cytosolic enzymes remained unchanged or were down-regulated in response to drought, whereas *PGM1*, which encodes a chloroplasticPGM*,* was up-regulated by drought at dawn in both leaves and stems (Fig. 7b, S13). The abundance of transcripts encoding cytosolic Hexokinase 2 (HXK2), responsible for catalyzing the conversion of glucose to G6P (Brilhaus *et al.,* 2016), remained unchanged, whereas other *HXK* genes, such as *HXK1* (AT4G29130, nuclear localization) and *HXK3* (AT1G47840, chloroplastic) were only slightly modulated by drought. Glucose-6-phosphate isomerase (PGI) can be either chloroplastic (*PGI1*) or cytosolic (*SIS.1* and *SIS.2*) and only the cytosolic forms were slightly up-regulated in our transcriptome.

Despite the significant reduction in leaf and stem soluble sugar content following drought (Fig. 7a), genes encoding tonoplast sugar transporters, such as Tonoplast monosaccharide transporters (*TMT*), Early responsive to dehydration-like 6 (*ERDL6*) and *SWEET* (Sugars will eventually be exported transporters), were predominantly up-regulated upon drought, with the exception of *TMT1* and *SWEET16*. Variable transcript abundance patterns were also observed for genes encoding enzymes involved in glycolytic PEP production, with Phosphofructokinase (*PFK6*), Fructose-bisphosphate aldolase(*FBA6*) and Triosephosphate isomerase (*TPI.1*) up-regulated significantly in drought-stressed leaves 2 h before dusk (16:00) and in the middle of the dark period (24:00) (Figs. 7b, S13; Table S8).

**DISCUSSION**

Species across the Caryophyllales comprisea hot-spot for CCM evolution, including C3, C3-C4 intermediates, variations of C4 or CAM and even C4-CAM facultative species, the latter occurring exclusively in *Portulaca,* (Edwards & Ogburn, 2012). In *Portulaca,* CAM is believed to be ancestral to C4 considering its position relative to CAM-performing clades (e.g., cacti, *Ancampseros* and *Talinum*), and the evolution of biochemically and anatomically distinct C4 sub-types within the lineage (Voznesenskaya *et al.,* 2010; Christin *et al.,* 2014). Previous reports for *P. oleracea*, (Koch & Kennedy, 1980, Mazen 1996, Lara *et al.,* 2003), as well as our current data, indicate that well-watered leaves perform C4, whereas weak CAM is induced in both leaves and stems in response to drought.

Adding to the identification of two *P. oleracea* *PPC* genes recruited to function in the C4 and CAM cycles (Christin *et al.,* 2014), here we demonstrated contrasting diel cycling of transcript abundance for *PPC-1E1a’* compared to *PPC-1E1c*. Hence, the light/dark synchronization of the PPC-mediated primary carboxylation reactions in C4- and CAM-performing leaves may, at least partially, rely on the opposite diel transcriptional regulation of these two *PPC* genes (Fig. 3). However, PPC is also post-translationally regulated, with PPCK-mediated PPC phosphorylation during daytime (C4) or nighttime (CAM) adjusting the enzyme’s sensitivity to its allosteric inhibitor L-malate over the diel cycle (Nimmo *et al*., 2001; Hibberd & Covshoff, 2010; Boxall *et al.,* 2017). Previously published results demonstrated that PPC kinetic properties, including its sensitivity to feedback inhibition by malate, activation by G6P, and affinity for PEP, were inverted relative to one another within the diel cycle as C4 was replaced by CAM in *P. oleracea* leaves (Mazen 1996, 2000). Our findings revealed that a single PPCK-encoding gene is shared by both CCMs, since *PPCK-1E1* mRNA accumulation coincided with C4 *PPC-1E1a’* accumulationin well-watered and with CAM *PPC-1E1c* accumulationin droughted leaves.

Putative C4 signature genes were identified, including those implicated in carboxylation (*βCA-2E3*, *PPC-1E1a’*), acid formation(*ASPAT-1E1, NADMDH-2E, NADMDH-3C1a, ALAAT-1E1*), decarboxylation (*NADME-2E.1)*,and PEP regeneration reactions(*AK-1, PPDK-1C1b.1)*,as well as in the transport of PEP, ASP and PYR between sub-cellular compartments(*UCP-2, BASS-1, PPT-1E2*). Most reactions and processes associated with CAM functioning in *P. oleracea*, except nocturnal carboxylation reactions (*PPC-1E1c*, *βCA-1E1)* and malate transport across the tonoplast (*ALMT-9E*, *VHAB2-1E1, TDT-1E*), were able to utilize genes shared by both CCMs. Since drought-stressed *P. oleracea* display low-level diel acid fluctuation (weak CAM; Winter 2019), it probably demands reduced steady-state levels of dedicated enzymes to fulfill decarboxylation, PEP regeneration and other CAM cycle-related reactions.

The connectivity between C4 and CAM pathways in *P. oleracea* remains enigmatic and poorly investigated (Lara *et al.*, 2004; Christin *et al.,* 2014; Ferrari & Freschi, 2019). At one extreme, C4 and CAM pathways could be disconnected, never occurring at the same time and/or region of the mesophyll. Alternatively, an interconnected C4/CAM hybrid system may exist, in which several components and reactions would be shared between both CCMs. Within these extremes, any level of C4/CAM interconnection seems to be possible. Our findings support the temporal coexistence of both CCMs within *P. oleracea* leaves whenever water supply is restricted. We demonstrated that prolonged drought exposure promoted a gradual, rather than abrupt, down- and up-regulation of leaf C4 and CAM central components, respectively. Significant ΔH+ and mRNA levels of CAM marker genes were observed as early as 10 d after water withholding, when diurnal CO2 uptake and C4 gene expression were only slightly reduced. Moreover, mRNA levels of C4 signature genes were still detectable when daytime gas exchange had ceased.

However, gathering reliable information about the tissue, cell type and sub-cellular distribution of C4 and CAM machineries within the *P. oleracea* leaf mesophyll remains critical for a full understanding of the interconnections between both CCMs. Therefore, the identification of CAM and C4 signature genes provided by this study provides a comprehensive set of candidates for future *in situ* hybridization and immunolocalization studies. Only one *in situ* immunolocalization study has been performed on drought-stressed *P. oleracea* leaves, which revealed that both RuBisCO and NAD-ME were localized to the BSCs, whereas PPC was found in MCs and water storage cells (WSCs)(Lara *et al.*, 2004). Thus, instead of all CAM-reactions taking place within a single cell (Winter & Smith 1996), an alternative two-cell C4/CAM hybrid system has been proposed to operate in droughted *P. oleracea*. This hypothetical model proposes that malate accumulated overnight in vacuoles of MCs and WSCs would be transferred to BSCs during the light period for decarboxylation, providing CO2 to sustain RuBisCO activity behind closed stomata (Lara *et al.*, 2004).

Although malate is typically transported from MCs to BSCs via plasmodesmata in C4 (Kanai & Edwards 1999), the C4/CAM hybrid system would implicate additional metabolic fluxes, including the daily transport of malate stored at MCs, and possibly WSCs, to BSCs (Lara *et al.*, 2004). Interestingly, drought stress significantly impacted the transcript abundance of *P. oleracea* *ALMT-12E.1* and *ALMT-12E.2*, both closely related to Arabidopsis *ALMT12*, which encodes a plasma membrane malate transporter predominantly found in guard cells (Meyer *et al.,* 2010). As *P. oleracea* stems are devoid of stomata, the predominance of *ALMT-12E.1* transcripts in leaves suggests a functionally conserved role for this gene in controlling stomatal movements (Meyer *et al.,* 2010). In contrast, *ALMT-12E.2* transcripts were equally abundant in tissues containing or lacking stomata (leaves and stems, respectively), which may indicate functional divergence compared to Arabidopsis *ALMT12*. As *ALMT-12E.2* was highly induced by drought, it could suggest their involvement in the daytime transport of malate between WSCs, MCs and BSCs in *P. oleracea* leaves engaged in the C4/CAM hybrid system.

Our data revealed completely reversible and environmentally-controlled CAM in *P. oleracea* leaves, which agrees with previous findings for the species (D’Andrea *et al.,* 2014, Winter & Holtum, 2014). As no ΔH+ or CAM-marker transcript accumulation was observed after re-watering events applied following different lengths of the drought period, the ability of *P. oleracea* to revert from the C4/CAM hybrid system to C4 was not influenced by the intensity or duration of CAM prior to re-watering. By contrast, both developmental and environmental cues were shown to regulate the occurrence and intensity of CAM in stems. As a consequence, multiple photosynthetic CO2 fixation systems were detected in *P. oleracea*, including: (1) C3 stems and C4 leaves in young, well-watered plants, (2) CAM stems and C4 leaves in adult well-watered and re-watered plants, and (3) CAM stems and C4/CAM hybrid leaves in adult, drought-stressed plants.

As stems represent up to 50% of *P. oleracea* total biomass (Zimmerman 1976) and display ΔH+ values as high as those found in droughted leaves (Fig. 6e), the presence of CAM in stem tissues may facilitate recycling the nighttime respiratory CO2 produced by its large chlorenchymatous cells. On the other hand, the adaptive value of facultative CAM in leaves is presumably similar for both C4-CAM and C3-CAM facultative plants. As other weak CAM facultative species, dark CO2 fixation in drought-stressed, CAM-performing *P. oleracea* plants is negligible compared to daytime C4-mediated CO2 assimilation under well-watered conditions (Winter and Holtum, 2014; Winter 2019). Instead of contributing to carbon gain, weak and inducible CAM may promote plant fitness by offering other adaptive advantages (reviewed by Herrera, 2009). Our findings connect CAM induction to a photoprotective role in *P. oleracea* as the daytime CO2 release from organic acids behind closed stomata may have supported linear electron transport within chloroplasts, maintaining the integrity of the photosynthetic apparatus under extreme drought as evidenced by chlorophyll *a* fluorescence data (Fig. S12). The rapid recovery of photosynthetic rates once sufficient water supply becomes available may also be seen as an indicator of this photoprotective role of weak CAM (Adams & Osmond, 1988; Herrera, 2009).

Compared to CAM plants, PEP generation represents a much less significant sink for carbohydrates in C4 plants due to the continuous daytime conversion of PYR to PEP by PPDK or its direct generation via PCK (Weise *et al*., 2011; Borland *et al*., 2016). In *Mesembryanthemum crystallinum*, where ΔH+  contributes to plant carbon gain under stressful conditions (Bohnert & Cushman, 2000), incremental accumulation of starch during the C3-to-CAM switch seems essential for the supply of PEP (Haider *et al.,* 2012, Cushman *et al.,* 2008). In contrast, leaf starch levels either remained unchanged, or were slightly reduced, upon drought in weak CAM facultative species such as *Tallinum triangulare* (Brilhaus *et al.,* 2016) and *P. oleracea* (Fig. 7, D’Andrea *et al.,* 2014), accompanied by lower soluble sugar content, particularly sucrose and glucose, in both cases. In *P. oleracea* leaves, CAM induction was accompanied by the up-regulation of most, but not all, starch metabolism genes (*ADG1, APL3, GBSS1, BE3, ISA3, GWD1, SEX4* and *BAM1*), as also observed for both *M. crystallinum* and *T. triangulare* (Cushman *et al.,* 2008; Brilhaus *et al.,* 2016). Therefore, an increased starch turnover, rather than a higher accumulation of this carbohydrate, seems to be sufficient to sustain the dark CO2 fixation and ΔH+ observed in *P. oleracea*.

It has been hypothesized that a transition to phosphorolytic starch turnover favors the energetic balance for the nocturnal production of PEP from starch, requiring increased levels of GPT2, which transports G6P across the chloroplast envelope in exchange for Pi (Neuhaus & Schulte, 1996; Cushman *et al.,* 2008; Borland *et al*., 2009; Weise *et al*., 2011). In *P. oleracea*, an overall increment in transcript abundance of *GPT2* as well as genes encoding enzymes either shared by both (*LDA*, *DPE1*, *GWD1*),or exclusively involved in the hydrolytic (*SEX4, BAM1*) and phosphorylytic (*PHS1*) starch degradation pathways was observed in response to drought. Therefore, both pathways seem to contribute to starch degradation in CAM-performing *P. oleracea* tissues. Additionally, the up-regulation of *GPT2.2* may connect with the up-regulation of cytosolic *SIS.1* and *SIS.2*, by transporting cytosolic G6P formed by SISs into the chloroplast to increase PEP regeneration via starch formation (Wai *et al.,* 2019).

Overall, our findings provide novel insights into the gene transcript level and metabolic adjustments required to accommodate both the C4 and CAM cycles within a single leaf. In particular, our work builds on previous findings by identifying transcripts that are likely to play an exclusive role in drought-induced CAM reactions, as well as components that are shared by both the C4 and CAM. By demonstrating that the C4 and CAM machineries co-exist within *P. oleracea* leaves under mild drought conditions, we open up a new window of opportunity for investigating the biochemical and regulatory mechanisms underpinning the co-occurrence of these two CCMs. Knowledge generated using C4/CAM facultative species such as *P. oleracea* may shed new light on alternative biochemical arrangements for future bioengineering initiatives aiming to combine the high productivity of C4 and the stress-resistance traits offered by CAM into target crop species.

**ACKNOWLEDGMENTS**

This work was supported in part by the São Paulo Research Foundation (FAPESP - grant no. # 2016/04755-4 awarded to R.C.F.), by a Newton Advanced Fellowship funded by the Royal Society, UK (grant no. #NA140007 awarded to L.F. and J.H.) and by the US National Science Foundation (grant no. IOS-1754662 awarded to E.J.E). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. We also thank Nirja Kadu, Richard Eccles and Steve Paterson for the support given during the work at the Institute of Integrative Biology (University of Liverpool, UK), the members of the Centre for Genomic Research at the University of Liverpool that carried out the RNA-seq library production and Illumina sequencing, Emerson Alves da Silva (Instituto de Botânica de São Paulo) for support during osmotic potential measurements, and Luiz Lehmann Coutinho for granting access to the ESALQ Genomics Center Computer Cluster.

**AUTHOR CONTRIBUTIONS:**

LF and JH conceived the project and supervised the experiments, RCF and PPB conducted most of the experiments; MAR, LF, FRRA, DD, SFB, LVD, and JH conducted part of the experiments; SCSA conducted most bioinformatics analysis; VDG assisted the bioinformatic and statistical analysis; JJMV and EJE performed the phylogenetic analyses; RCF, LF and JH wrote the article with contributions from other authors.

**REFERENCES**

**Adams WW, Osmond CB**. **1988**. Internal CO2 supply during photosynthesis of sun and shade grown CAM plants in relation to photoinhibition *Fv/Fm*. *Plant Physiology* **86**: 117–123.

**Alves FRR, Melo HC, Crispim-Filho AJ, Costa AC, Nascimento KJT, Carvalho RF. 2016.** Physiological and biochemical responses of photomorphogenic tomato mutants (cv. Micro-Tom) under water withholding. *Acta Physiologiae Plantarum* **38**: 155.

**Amaral LIV do, Gaspar M, Costa PMF, Aidar MPM, Buckeridge MS**. **2007**. Novo método enzimático rápido e sensível de extração e dosagem de amido em materiais vegetais. *Hoehnea* **34**: 425–431.

**Black**  **CC, Chen JQ, Doong RL, Angelov MN, Sung SJS. 1996.** Alternative carbohydrate reserves used in the daily cycle of crassulacean acid metabolism. *In*: Winter K, Smith J, eds*. Crassulacean Acid Metabolism*. Heidelberg, Germany: Springer–Verlag Berlin, 31–45.

**Bohnert HJ, Cushman JC**. **2000**. The ice plant cometh: lessons in abiotic stress tolerance. *Journal of Plant Growth Regulation* **19**: 334–346.

**Borland AM, Griffiths H, Hartwell J, Smith JAC**. **2009**. Exploiting the potential of plants with crassulacean acid metabolism for bioenergy production on marginal lands. *Journal of Experimental Botany* **60**: 2879–2896.

**Borland AM, Guo HB, Yang X, Cushman JC**. **2016**. Orchestration of carbohydrate processing for crassulacean acid metabolism. *Current Opinion in Plant Biology* **31**: 118–124.

**Boxall SF, Dever LV, Kneřová J, Gould PD, Hartwell J. 2017.** Phosphorylation of phospho*enol*pyruvate carboxylase is essential for maximal and sustained dark CO2 fixation and core circadian clock operation in the obligate crassulacean acid metabolism species *Kalanchoë fedtschenkoi*. *The Plant Cell* **29**: 2519–2536.

**Boxall SF, Kadu N, Dever LV, Kneřová J, Waller JL, Gould PJD, Hartwell J. 2019.** Silencing *PHOSPHOENOLPYRUVATE CARBOXYLASE1* in the obligate crassulacean acid metabolism species *Kalanchoë laxiflora* causes reversion to C3-like metabolism and amplifies rhythmicity in a subset of core circadian clock genes. *bioRxiv* 684050; doi: https://doi.org/10.1101/684050

**Bräutigam A, Schlüter U, Eisenhut M, Gowik U.** **2017**. On the Evolutionary Origin of CAM Photosynthesis. *Plant Physiology* **174**: 473–477.

**Brilhaus D, Bräutigam A, Mettler–Altmann T, Winter K, Weber APM**. **2016**. Reversible burst of transcriptional changes during induction of crassulacean acid metabolism in *Talinum triangulare*. *Plant Physiology* **170**: 102–122.

**Carter PJ, Nimmo HG, Fewson CA, Wilkins MB. 1991.** Circadian rhythms in the activity of a plant protein kinase. *EMBO Journal* **10**: 2063–2068.

**Chastain CJ, Chollet R**. **2003**. Regulation of pyruvate, orthophosphate dikinase by ADP–/Pi–dependent reversible phosphorylation in C3 and C4 plants. *Plant Physiology and Biochemistry* **41**: 523–532.

**Chastain CJ, Fries JP, Vogel JA, Randklev CL, Vossen AP, Dittmer SK, Watkins EE, Fiedler LJ, Wacker SA, Meinhover KC, *et al.*** **2002**. Pyruvate, orthophosphate dikinase in leaves and chloroplasts of C3 Plants undergoes light–/dark–induced reversible phosphorylation. *Plant Physiology* **128**: 1368–1378.

**Chen Z, Hills A, Bätz U, Amtmann A, Lew VL, Blatt MR.** 2012. Systems dynamic modeling of the stomatal guard cell predicts emergent behaviors in transport, signaling, and volume control. *Plant Physiology* **159**: 1235–1251.

**Christin PA, Arakaki M, Osborne CP, Bräutigam A, Sage RF, Hibberd JM, Kelly S, Covshoff S, Wong GKS, Hancock L, *et al.*** **2014**. Shared origins of a key enzyme during the evolution of C4 and CAM metabolism. *Journal of Experimental Botany* **65**: 3609–3621.

**Christin PA, Arakaki M, Osborne CP, Edwards EJ**. **2015**. Genetic enablers underlying the clustered evolutionary origins of C4 photosynthesis in angiosperms. *Molecular Biology and Evolution* **32**: 846–858.

**Covshoff S, Hibberd JM**. **2012**. Integrating C4 photosynthesis into C3 crops to increase yield potential. *Current Opinion in Biotechnology* **23**: 209–214.

**Cruz AB, Bianchetti RE, Alves FRR, Purgatto E, Peres LEP, Rossi M, Freschi L**. **2018**. Light, ethylene and auxin signaling interaction regulates carotenoid biosynthesis during tomato fruit ripening. *Frontiers in Plant Science* **9**: 1–16.

**Cushman JC, Tillett RL, Wood JA, Branco JM, Schlauch KA**. **2008**. Large–scale mRNA expression profiling in the common ice plant, *Mesembryanthemum crystallinum*, performing C3 photosynthesis and crassulacean acid metabolism (CAM). *Journal of Experimental Botany* **59**: 1875–1894.

**D’Andrea RM, Andreo CS, Lara MV**. **2014**. Deciphering the mechanisms involved in *Portulaca oleracea* (C4) response to drought: metabolic changes including crassulacean acid–like metabolism induction and reversal upon re–watering. *Physiologia Plantarum* **152**: 414–430.

**De Angeli A, Zhang J, Meyer S, Martinoia E**. **2013**. *AtALMT9* is a malate-activated vacuolar chloride channel required for stomatal opening in Arabidopsis. *Nature Communications* **4**: 1–10.

**Dever LV, Boxall SF, Kneřová J, Hartwell J**. **2015**. Transgenic perturbation of the decarboxylation phase of crassulacean acid metabolism alters physiology and metabolism but has only a small effect on growth. *Plant Physiology* **167**: 44–59.

**Edwards EL. 2019**. Evolutionary trajectories, accessibility and other metaphors: the case of C4 and CAM photosynthesis. *New Phytologist.* doi: 10.1111/nph.15851

**Edwards EJ, Ogburn RM**. **2012**. Angiosperm responses to a low–CO2 world: CAM and C4 photosynthesis as parallel evolutionary trajectories. *International Journal of Plant Sciences* **173**: 724–733.

**Ferrari RC, Freschi L. 2019.** C4/CAM facultative photosynthesis as a means to improve plant sustainable productivity under abiotic–stressed conditions: regulatory mechanisms and biotechnological implications. *In*: Khan MIR, Reddy PS, Ferrante A, Khan NA, eds. *Plant Signaling Molecules*, Chennai, India: Woodhead Publishing, 517–532.

**Freschi L, Rodrigues MA, Tiné MAS, Mercier H**. **2010**. Correlation between citric acid and nitrate metabolisms during CAM cycle in the atmospheric bromeliad *Tillandsia pohliana*. *Journal of Plant Physiology* **167**: 1577–1583.

**Fukao Y, Hayashi M, Nishimura M. 2002**. Proteomic analysis of leaf peroxisomal proteins in greening cotyledons of *Arabidopsis thaliana*. *Plant Cell Physiol*ogy **43:** 689–696.

**Furbank RT**. **2011**. Evolution of the C4 photosynthetic mechanism: are there really three C4 acid decarboxylation types? *Journal of Experimental Botany* **62**: 3103–3108.

**Furumoto T, Yamaguchi T, Ohshima–Ichie Y, Nakamura M, Tsuchida–Iwata Y, Shimamura M, Ohnishi J, Hata S, Gowik U, Westhoff P, *et al.*** **2011**. A plastidial sodium–dependent pyruvate transporter. *Nature* **476**: 472–476.

**Gonnella M, Charfeddine M, Conversa G, Santamaria P**. **2010**. Purslane: a review of its potential for health and agricultural aspects. *The European Journal of Plant Science and Biotechnology* **4**: 131–136.

**Griffiths H. 1989.** Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. *In:* Lüttge, U, ed. *Vascular Plants as Epiphytes: Evolution and Ecophysiology*. Berlin: Springer-Verlag, 42-86.

**Guralnick LJ, Jackson MD**. **2001**. The occurrence and phylogenetics of crassulacean acid metabolism in the Portulacaceae. *International Journal of Plant Sciences* **162**: 257–262.

**Hafke JB, Hafke Y, Smith JAC, Lüttge UE, Thiel G. 2003**. Vacuolar malate uptake is mediated by an anion-selective inward rectifier. *The Plant Journal* 35, 116–128.

**Haider MS, Barnes JD, Cushman JC, Borland AM**. **2012**. A CAM– and starch–deficient mutant of the facultative CAM species *Mesembryanthemum crystallinum* reconciles sink demands by repartitioning carbon during acclimation to salinity. *Journal of Experimental Botany* **63**: 1985–1996.

**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. *Plant Journal* **10**: 1071–1078.

**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. *Plant Journal* **20**: 333–342.

**Hartwell J. 2006**. The circadian clock in CAM plants. *In*: Hall AJ, McWatters H, eds. *Annual Plant Reviews Volume 21: Endogenous Plant Rhythms*. Kundli, India: Blackwell Publishing, 211–236.

**Hartwell J, Dever L V, Boxall SF**. **2016**. Emerging model systems for functional genomics analysis of crassulacean acid metabolism. *Current Opinion in Plant Biology* **31**: 100–108.

**Hatch MD**. **1987**. C4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. *Bioehimica et Biophysica Acta* **895**: 81–106.

**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.

**Hibberd JM, Covshoff S**. **2010**. The Regulation of gene expression required for C4 photosynthesis. *Annual Review of Plant Biology* **61**: 181–207.

**Holtum JAM, Hancock LP, Edwards EJ, Winter K**. **2017**. Optional use of CAM photosynthesis in two C4 species, *Portulaca cyclophylla* and *Portulaca digyna*. *Journal of Plant Physiology* **214**: 91–96

**Kajala K, Covshoff S, Karki S, Woodfield H, Tolley BJ, Dionora MJA, Mogul RT, Mabilangan AE, Danila FR, Hibberd JM, *et al.*** **2011**. Strategies for engineering a two–celled C4 photosynthetic pathway into rice. *Journal of Experimental Botany* **62**: 3001–3010.

**Kanai R, Edwards GE**. **1999**. The biochemistry of photosynthesis. *In*: Sage R, Monson R, eds. *C4 Plant Biology*. San Diego: Academic Press, 49–87.

**Keeley JE, Rundel PW**. **2013**. Evolution of CAM and C4 carbon ‐ concentrating mechanisms. *International Journal of Plant Sciences* **164**: 54–77.

**Koch KE, Kennedy RA**. **1980**. Characteristics of crassulacean acid metabolism in the succulent C4 dicot, *Portulaca oleracea* L. *Plant Physiology* **65**: 193–197.

**Koch KE, Kennedy RA**. **1982**. Crassulacean acid metabolism in the succulent C4 dicot, *Portulaca oleracea* L under natural environmental conditions. *Plant Physiology* **69**: 757–761.

**Kovermann P, Meyer S, Hörtensteiner S, Picco C, Scholz–Starke J, Ravera S, Lee Y, Martinoia E**. **2007**. The Arabidopsis vacuolar malate channel is a member of the ALMT family. *Plant Journal* **52**: 1169–1180.

**Ku SB, Shieh YJ, Reger BJ, Black CC**. **1981**. Photosynthetic characteristics of *Portulaca grandiflora*, a succulent C4 dicot. *Plant physiology* **68**: 1073–1080.

**Laetsch WM. 1968**. Chloroplast specialization in dicotyledons possessing the C4-dicarboxylic acid pathway of photosynthetic CO2 fixation. *American Journal of Botany* **55**: 875-883.

**Lara M, Disante KB, Podestá FP, Andreo CS, Drincovich MF**. **2003**. Induction of a crassulacean acid like metabolism in the C4 succulent plant, *Portulaca oleracea* L.: physiological and morphological changes are accompanied by specific modifications in phosphoenolpyruvate carboxylase. *Photosynthesis Research* **77**: 241–254.

**Lara M V, Drincovich MF, Andreo CS**. **2004**. Induction of a crassulacean acid–like metabolism in the C4 succulent plant, *Portulaca oleracea* L.: study of enzymes involved in carbon fixation and carbohydrate metabolism. *Plant and Cell Physiology* **45**: 618–626.

**Matsuoka M**. **1995**. The gene for pyruvate,orthophosphate dikinase in C4 plants: structure, regulation and evolution. *Plant and Cell Physiology* **36**: 937–943.

**Mazen AMA**. **1996**. Changes in levels of phosphoenolpyruvate carboxylase with induction of crassulacean acid metabolism (CAM)–like behavior in the C4 plant *Portulaca oleracea*. *Physiologia Plantarum* **98**: 111–116.

**Mazen AMA**. **2000**. Changes in properties of phosphoenolpyruvate carboxylase with induction of crassulacean acid metabolism (CAM) in the C4 plant *Portulaca oleracea*. *Photosynthetica* **38**: 385–391.

**Meyer S, Mumm P, Imes D, Endler A, Weder B, Al-Rasheid KAS, Geiger D, Marten I, Martinoia E, Hedrich R**. **2010**. *AtALMT12* represents an R-type anion channel required for stomatal movement in Arabidopsis guard cells. *Plant Journal* **63**: 1054–1062.

**Miyanishi K, Cavers PB**. **1980**. The biology of canadian weeds. *Canadian Journal of Plant Science* **60**: 953–963.

**Monn****é M, Daddabbo L, Gagneul D, Obata T, Hielscher B, Palmieri L, Miniero DV, Fernie AR, Weber APM, Palmieri F**. **2018**. Uncoupling proteins 1 and 2 (*UCP1* and *UCP2*) from *Arabidopsis thaliana* are mitochondrial transporters of aspartate, glutamate, and dicarboxylates. *Journal of Biological Chemistry* **293**: 4213–4227.

**Monroe JD, Storm AR**. **2018**. Review: the Arabidopsis β–amylase (BAM) gene family: diversity of form and function. *Plant Science* **276**: 163–170.

**Moreno–Villena JJ, Dunning LT, Osborne CP, Christin PA**. **2017**. Highly expressed genes are preferentially co–opted for C4 photosynthesis. *Molecular Biology and Evolution* **35**: 94–106.

**Nelson EA, Sage TL, Sage RF**. **2005**. Functional leaf anatomy of plants with crassulacean acid metabolism. *Functional Plant Biology* **32**: 409–419.

**Neuhaus HE, Schulte N. 1996**. Starch degradation in chloroplasts isolated from C3 or CAM (crassulacean acid metabolism)–induced *Mesembryanthemum crystallinum* L. *Biochemical Journal* **318**: 945–953.

**Nimmo HG, Fontaine V, Hartwell J, Jenkins GI, Nimmo GA, Wilkins MB. 2001**. PEP carboxylase kinase is a novel protein kinase controlled at the level of expression. *New Phytologist.* **151**: 91–97.

**Osmond CB.** **1978**. Crassulacean acid metabolism: a curiosity in context. *Annual Review of Plant Physiology* **29**: 379–414.

**Palmieri L, Picault N, Arrigoni R, Besin E, Palmieri F, Hodges M**. **2008**. Molecular identification of three *Arabidopsis thaliana* mitochondrial dicarboxylate carrier isoforms: organ distribution, bacterial expression, reconstitution into liposomes and functional characterization. *Biochemical Journal* **410**: 621–629.

**Sage RF**. **2002**. Are crassulacean acid metabolism and C4 photosynthesis incompatible? *Functional Plant Biology* **29**: 775–785.

**Sage RF**. **2017**. A portrait of the C4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and hall of fame. *Journal of Experimental Botany* **68**: e11–e28.

**Smith JAC, Ingram J, Tsiantis MS, Barkla BI, Bartholomew DM, Bettey M. 1996.** Transport across the vacuolar membrane in CAM plants. *In*: Winter K, Smith J, eds*. Crassulacean Acid Metabolism*. Heidelberg, Germany: Springer–Verlag Berlin, 53–71.

**Schluter U, Denton AK, Bräutigam A**. **2016**. Understanding metabolite transport and metabolism in C4 plants through RNA–seq. *Current Opinion in Plant Biology* **31:** 83–90.

**Schultz CJ, Coruzzi GM**. **1995**. The aspartate aminotransferase gene family of Arabidopsis encodes isoenzymes localized to three distinct subcellular compartments. *The Plant Journal*: 61–75.

**Silvera K, Neubig KM, Whitten WM, Williams NH, Winter K, Cushman JC**. **2010**. Evolution along the crassulacean acid metabolism continuum. *Functional Plant Biology* **37**: 995–1010.

**Taylor SH, Ripley BS, Martin T, De–Wet LA, Woodward FI, Osborne CP**. **2014**. Physiological advantages of C4 grasses in the field: a comparative experiment demonstrating the importance of drought. *Global Change Biology* **20**: 1992–2003.

**Thompson AG, Brailsford MA, Beechey RB**. **1987**. Identification of the phosphate translocator from maize mesophyll chloroplasts. *Biochemical and Biophysical Research Communications* **143**: 164–169.

**Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE**. **2001**. Kranz anatomy is not essential for terrestrial C4 plant photosynthesis. *Nature* **414**: 543–546.

**Voznesenskaya EV, Franceschi VR, Kiirats O, Artyusheva EG, Freitag H, Edwards GE**. **2002**. Proof of C4 photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae). *Plant Journal* **31**: 649–662.

**Voznesenskaya EV, Koteyeva NK, Edwards GE, Ocampo G**. **2010**. Revealing diversity in structural and biochemical forms of C4 photosynthesis and a C3–C4 intermediate in genus *Portulaca* L. (Portulacaceae). *Journal of Experimental Botany* **61**: 3647–3662.

**Vozza A, Parisi G, De Leonardis F, Lasorsa FM, Castegna A, Amorese D, Marmo R, Calcagnile VM, Palmieri L, Ricquier D, *et al.*** **2014**. *UCP2* transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation. *Proceedings of the National Academy of Sciences* **111**: 960–965.

**Wai CM, Weise SE, Ozersky P, Mockler TC, MichaelI TP, VanBuren R.** 2019. Time of day and network reprogramming during drought induced CAM photosynthesis in *Sedum album*. *PLOS Genetics*. doi: 10.1371/journal.pgen.1008209

**Weise SE, Van Wijk KJ, Sharkey TD**. **2011**. The role of transitory starch in C3, CAM, and C4 metabolism and opportunities for engineering leaf starch accumulation. *Journal of Experimental Botany* **62**: 3109–3118.

**Wilkie SE, Warren MJ**. **1998**. Recombinant expression, purification, and characterization of three isoenzymes of aspartate aminotransferase from Arabidopsis thaliana. *Protein Expression and Purification* **12**: 381–389.

**Winter K. 2019**. Ecophysiology of constitutive and facultative CAM photosynthesis. *Journal of Experimental Botany*. doi: 10.1093/jxb/erz002.

**Winter K, Smith J. 1996.** Crassulacean acid metabolism: current status and perspectives. *In*: Winter K, Smith J, eds*. Crassulacean Acid Metabolism*. Heidelberg, Germany: Springer–Verlag Berlin, 1–13.

**Winter K, Holtum JAM**. **2014**. Facultative crassulacean acid metabolism (CAM) plants: powerful tools for unravelling the functional elements of CAM photosynthesis. *Journal of Experimental Botany* **65**: 3425–3441.

**Winter K, Holtum JAM**. **2017**. Facultative crassulacean acid metabolism (CAM) in four small C3 and C4 leaf–succulents. *Australian Journal of Botany* **65**: 3425–3441.

**Winter K, Sage RF, Edwards EJ, Virgo A, Holtum JAM.** **2019.** Facultative crassulacean acid metabolism in a C3–C4 intermediate. *Journal of Experimental Botany.* doi:10.1093/jxb/erz085

**Wheeler MCG, Tronconi MA, Drincovich MF, Andreo CS, Flügge U, Maurino VG. 2005.**

A comprehensive analysis of the NADP-malic enzyme gene family of Arabidopsis. *Plant Physiology* **139**: 39–51.

**Yang X, Cushman JC, Borland AM, Edwards EJ, Wullschleger SD, Tuskan GA, Owen NA, Griffiths H, Smith JAC, De Paoli HC, *et al.*** **2015**. A roadmap for research on crassulacean acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world. *New Phytologist* **207**: 491–504.

**Zhang J, Baetz U, Krugel U, Martinoia E, De Angeli A**. **2013**. Identification of a probable pore-forming domain in the multimeric vacuolar anion channel *AtALMT9*. *Plant Physiology* **163**: 830–843.

**Zimmerman CA**. **1976**. Growth characteristics of weediness in *Portulaca Oleracea* L. *Ecology* **57**: 964–974.

**SUPPLEMENTARY INFORMATION**

**Fig. S1** Diel leaf acid fluctuation in well-watered and droughted plants.

**Fig. S2** Surface view and fluorescence microscopy of *P. oleracea* leaves and stems.

**Fig. S3** Differentially expressed genes across all pairwise comparisons.

**Fig. S4** Validation of RNA-seq data via qPCR.

**Fig. S5** Mean TMM values for contigs of CCM core reactions.

**Fig. S6** Mean TMM values for contigs of CCM-related transporters reactions.

**Fig. S7** Phylogenetic trees for genes encoding core CCM enzymes (Separate PDF file).

**Fig. S8** Phylogenetic trees for genes encoding CCM-related transporters and pumps (Separate PDF file).

**Fig. S9** Phylogenetic trees for genes encoding core photorespiration-related enzymes (Separate PDF file).

**Fig. S10** Relative abundance of genes encoding photorespiration-related enzymes.

**Fig. S11** Transcript abundance of C4 and CAM marker genes upon drought and rewatering.

**Fig. S12** Chlorophyll *a* fluorescence in response to drought and rewatering.

**Fig. S13** Mean TMM values for contigs of carbohydrate metabolism genes.

**Fig. S14** Mean TMM values for contigs of genes encoding carbohydrate-related transporters.

**Table S1** Primer sequences used for qPCR (Separate Excel file).

**Table S2** Overall sequencing statistics.

**Table S3** Annotation and abundance of all reads mapped to the P. oleracea transcriptome (Separate Excel file).

**Table S4** Gene ontology (GO) enrichment analysis (Separate Excel file).

**Table S5** Contigs annotated as part of key C4/CAM modules (Separate Excel file).

**Table S6** C4/CAM-related DE contigs in response to drought (Separate Excel file).

**Table S7** Photorespiration-related DE contigs in response to drought (Separate Excel file).

**Table S8** Carbohydrate-related DE contigs in response to drought (Separate Excel file).

**Methods S1** Extended Materials and Methods.

**FIGURE CAPTIONS**

**Figure 1** Drought promotes CAM induction in C4 leaves and intensifies CAM expression in stems of *Portulaca oleracea*. (a) Leaf (left) and stem (right) cross-sections of well-watered plants. Black arrows and arrowheads indicate bundle sheath cells and vascular bundles, respectively. Bars = 250µm. (b) Diel fluctuation of malate content in leaf and stem from plants exposed to 34 days of well-watered or drought treatments. Malate was determined via enzymatic assays. In (b), data are means (±SE) of at least three biological replicates and asterisks indicate significant difference (P < 0.05). (c) Diel net CO2 assimilation (*A*) and transpiration (*E*) in leaves of plants exposed to well-watered or drought treatments. In (c), data are normalized against the leaf area. In (b-c), the shaded areas indicate the dark period.

**Figure 2** Drought-induced changes in transcriptional profile reveal key components of the C4/CAM machineries in *Portulaca oleracea*. Leaf and stem samples were harvested from plants maintained for 34 days under well-watered or droughted conditions. (a) Heatmaps indicate log2-fold change of droughted samples compared to well-watered samples. (b) Heatmaps indicate log2-fold change of stem samples compared to leaf samples. Black arrows indicate core reactions in both C4 and CAM biosynthetic pathways whereas gray lines terminated by a closed circle indicate regulatory interactions. Intermediate reactions are omitted. Biosynthetic enzymes and metabolites are represented with gray ovals and blue letters, respectively. TMM and log2-fold change values for genes shown in the heatmaps are presented in Table S5 and Fig. S5, and their phylogenetic relationship is presented in Fig. S7. Statistically significant differences in comparison with well-watered (a) or leaf (b) samples are represented as colored squares (adjusted p-value<0.05). Data are means (±SE) of at least three replicates. Metabolites: ALA, alanine; ASP, aspartate; OAA, oxaloacetate; MAL, malate; PEP, phospho*enol*pyruvate; Ppa, inorganic pyrophosphatase; PYR, pyruvate. Enzymes: AK, adenylate kinase; ALAAT, ALA aminotransferase; ASPAT, ASP aminotransferase; βCA, beta-carbonic anhydrase; NAD(P)-ME, NAD(P)-malic enzyme; NADMDH, NAD-malate dehydrogenase; PCK, PEP carboxykinase; PPC, PEP carboxylase; PPCK, PPC kinase; PPDK, PYR orthophosphate dikinase; PPDKRP, PPDK regulatory protein.

**Figure 3** Drought induces changes in diel transcript abundance of key C4/CAM genes in *Portulaca oleracea*. (a) qPCR data of genes exhibiting C4-like expression patterns. (b) qPCR data of genes exhibiting CAM-like expression patterns. (c) qPCR data of other CCM genes. Mean relative expression in leaf and stem samples was normalized against the first time point (8h) of well-watered leaf samples. The shaded areas indicate the dark period, and asterisks indicate significant difference (P<0.05). Data are means (±SE) of at least three replicates. AK, adenylate kinase protein; ALAAT, alanine aminotransferase; ASPAT, aspartate aminotransferase; βCA, beta-carbonic anhydrase; NAD(P)-ME, NAD(P)-malic enzyme; NADMDH, NAD-malate dehydrogenase; PCK, phospho*enol*pyruvate carboxykinase; Ppa, inorganic pyrophosphatase; PPC, phospho*enol*pyruvate carboxylase; PPCK, PPC kinase; PPDK, pyruvate orthophosphate dikinase; PPDKRP, PPDK regulatory protein.

**Figure 4** Drought modulates the transcriptional profile of genes encoding CCM-related transporters and pumps in *Portulaca oleracea*. Leaf and stem samples were harvested from plants maintained for 34 days under well-watered or droughted conditions. Schematic representation illustrating the putative intracellular localization of C4/CAM-related transporters and pumps, which are represented with gray ovals. Heatmaps indicate log2-fold change of droughted samples compared to well-watered samples. TMM and log2-fold change values for genes shown in the heatmaps are presented in Fig. S6 and Tables S5 and S6, and their phylogenetic relationship are presented in Fig. S8. Statistically significant differences in comparison with well-watered samples are represented as colored squares (adjusted p-value<0.05). Data are means (±SE) of at least three replicates. Metabolites: 2OG, 2-oxoglutarate; ASP, aspartate; MAL, malate; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PPi, inorganic phosphate. Transporters and pumps: ALMT, aluminum-activated malate transporter; BASS, sodium bile acid symporter family; DIC, dicarboxylate carrier; DIT, dicarboxylate transporter; PPT, PEP/phosphate translocator; PYR, pyruvate; tDT, tonoplast MAL/fumarate transporter; UCP-2, mitochondrial uncoupling protein 2; V-ATPase/VHA, V-type proton ATPase; V-PPase/AVP, pyrophosphate-energized membrane proton pump.

**Figure 5** Diel transcript fluctuation of genes encoding CCM-related transporters and pumps is altered by drought in *Portulaca oleracea*. (a) qPCR data of genes exhibiting C4-like expression patterns. (b) qPCR data of genes exhibiting CAM-like expression patterns. Mean relative expression in leaf and stem samples was normalized against the first time point (8h) of well-watered leaf samples. The shaded areas indicate the dark period and asterisks indicate significant difference (P<0.05). Data are means (±SE) of at least three replicates. ALMT, aluminum-activated malate transporter; BASS, sodium bile acid symporter family; DIT, dicarboxylate transporter; PPT, phosphoenolpyruvatephosphate translocator; tDT, tonoplast malate/fumarate transporter; UCP-2, mitochondrial uncoupling protein 2; VHA, V-type proton ATPase; AVP, pyrophosphate-energized membrane proton pump.

**Figure 6** Changes in water availability promotes completely reversible photosynthetic transitions in *Portulaca oleracea*. Plants were sampled after 0, 10, 22 and 34 days of drought treatment (D0, D1, D2 and D3, respectively) and during rewatering events initiated after 22 and 34 days of drought (R1 and R2, respectively). (a) Overall morphological aspects of well-watered (left) and droughted (right) plants. Bars: 7 cm. (b) Soil volumetric water content (SVWC) during the treatments. (c) Leaf and stem osmotic potential (*Ψs*). (d) Net CO2 uptake (*A*), stomatal conductance (*gs*) and transpiration (*E*) in leaves. (e) Titratable acidity in leaf and stem tissues. ΔH+ indicates dawn-dusk differences. In (e), standard error of the dawn-dusk difference = √((standard errordawn)2 + (standard errordusk)2). (f) Heatmaps indicate log2-fold change of well-watered compared to drought/rewatered leaves for C4-signature genes (qPCR data presented in Fig. S11). (g) Transcript abundance of phospho*enol*pyruvate carboxylase (PPC)-encoding genes normalized against D0 leaf samples. (h) Heatmaps indicate log2-fold change of well-watered compared to drought/rewatered leaves for CAM-signature genes (qPCR data presented in Fig. S11). In (c-e, g), asterisks indicate significant difference (P < 0.05). In (f,h), statistically significant differences are represented as colored squares (adjusted p-value<0.05). In (b-d), gray areas indicate rewatering events. Data are means (±SE) of at least three replicates. AK, adenylate kinase; ALAAT, alanine aminotransferase; ALMT, aluminum-activated malate transporter; ASPAT, aspartate aminotransferase; βCA, beta-carbonic anhydrase; DIC, dicarboxylate carrier; NAD-ME, NAD-malic enzyme; PPC, phosphoenolpyruvate carboxylase; PPCK, PPC kinase; PPDK, pyruvate orthophosphate dikinase.

**Figure 7** Coordinated changes in sugar metabolism accompany the drought-induced photosynthetic transitions of leaves and stems of *Portulaca oleracea*.Leaf and stem samples were harvested from plants maintained for 34 days under well-watered or drought conditions. (a) Diel fluctuations in starch, sucrose, glucose and fructose content. The shaded areas indicate the dark period and asterisks indicate significant difference (P<0.05). (b) Schematic representation of sugar metabolic reactions, with heatmaps indicating log2-fold change of droughted samples compared to well-watered samples for leaves and stems. Biosynthetic enzymes and transporters are represented with gray ovals and intermediate reactions are omitted. Statistically significant differences are represented as colored squares (adjusted p-value<0.05). Gene names were assigned based on Arabidopsis closest homolog according to sequence similarity (see Supplemental Methods S1). TMM and log2-fold change values for genes shown in heatmaps are presented in Table S8. Data are means (±SE) of at least three replicates. Metabolites: 1,3-BPG, 1,3-Bisphosphoglycerate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G1P, Glucose-1-phosphate; G6P, Glucose-6-phosphate; GAP, glyceraldehyde 3-phosphate; GLU, Glucose; MAL, maltose; PEP, phospho*enol*pyruvate; SUC, sucrose. Enzymes: AGPase, G1P adenylyltransferase; ADG, AGPase small subunit; APL, AGPase large subunit; AMY, amylase; BAM, Beta-amylase; BE, Starch branching enzyme; DPE, disproportionating enzyme; ENO, enolase; ERDL, vacuolar ERD6-like 6 glucose transporter; FBA, fructose-bisphosphate aldolase; GAPDH, GAP dehydrogenase; GBSS, granule-bound starch synthase; GLT, plastidic glucose transporter; GPT, G6P/phosphate translocator; GWD, alpha-glucan water dikinase; HXK, hexokinase; ISA, isoamylase; LDA, limit dextrinase; MEX, maltose exporter; PFK, 6-phosphofructokinase; PGI/SIS, G6P isomerase; PGK, phosphoglycerate kinase-2; PGlyM/IPGAM, phosphoglycerate mutase; PGM, phosphoglucomutase; PHS, starch phosphorylase; PWD, Phosphoglucan water dikinase; SEX, phosphoglucan phosphatase; SUC4, sucrose transport protein 4; SWEET, bidirectional sugar transporter; TMT, monosaccharide sensing protein; TPI, triosephosphate isomerase.