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RESEARCH PAPER

Facultative crassulacean acid metabolism in a C_3–C_4 intermediate

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Abstract

The Portulacaceae enable the study of the evolutionary relationship between C_4 and crassulacean acid metabolism (CAM) photosynthesis. Shoots of well-watered plants of the C_3–C_4 intermediate species *Portulaca cryptopetala* Speg. exhibit net uptake of CO_2 solely during the light. CO_2 fixation is primarily via the C_3 pathway as indicated by a strong stimulation of CO_2 uptake when shoots were provided with air containing 2% O_2. When plants were subjected to water stress, daytime CO_2 uptake was reduced and CAM-type net CO_2 uptake in the dark occurred. This was accompanied by nocturnal accumulation of acid in both leaves and stems, also a defining characteristic of CAM. Following rewatering, net CO_2 uptake in the dark ceased in shoots, as did nocturnal acidification of the leaves and stems. With this unequivocal demonstration of stress-related reversible, i.e. facultative, induction of CAM, *P. cryptopetala* becomes the first C_3–C_4 intermediate species reported to exhibit CAM. *Portulaca molokiniensis* Hobdy, a C_4 species, also exhibited CAM only when subjected to water stress. Facultative CAM has now been demonstrated in all investigated species of *Portulaca*, which are well sampled from across the phylogeny. This strongly suggests that in *Portulaca*, a lineage in which species engage predominately in C_4 photosynthesis, facultative CAM is ancestral to C_4. In a broader context, it has now been demonstrated that CAM can co-exist in leaves that exhibit any of the other types of photosynthesis known in terrestrial plants: C_3, C_4 and C_3–C_4 intermediate.

Keywords: C_4 photosynthesis, crassulacean acid metabolism, *Portulaca cryptopetala*, *Portulaca molokiniensis*, Portulacaceae.

Introduction

An estimated 10% of terrestrial vascular plants express either crassulacean acid metabolism (CAM) or C_4 photosynthesis (Smith and Winter, 1996; Winter *et al.*, 2015; Sage, 2016). Both photosynthetic pathways have evolved independently over 60 times. CAM is documented in more than 30 angiosperm families, and in one family in each of the cycads, gnetophytes, ferns, and lycophytes (Smith and Winter, 1996), while C_4 is known in 19 families of angiosperms (Sage and Sultmanis, 2016).

The CAM and C_4 pathways are comparable in many respects (Osmond, 1978; Hatch, 1987). Using a similar complement of enzymes, each pathway concentrates CO_2 in the vicinity of Rubisco thereby reducing the competitive inhibition by molecular oxygen of CO_2 uptake. In both pathways, atmospheric CO_2 initially fixed as HCO_3^- using oxygen-insensitive phospho*enol*pyruvate carboxylase (PEPc) is incorporated into a four-carbon intermediate from which CO_2 is ultimately

Abbreviations: BS, bundle sheath; FM, fresh mass; PEPc, phospho*enol*pyruvate carboxylase; PFD, photosynthetic photon flux density.

liberated in the vicinity of Rubisco. At the site of Rubisco, CO_2 attains concentrations that ensure that the enzyme functions overwhelmingly as a carboxylase.

Despite similarities, CAM and C_4 differ in important aspects. In C_4 plants, all of the processes associated with photosynthetic CO_2 assimilation occur during the light. PEPc and Rubisco are simultaneously active but are separated spatially, usually in two distinct types of cells (for exceptions, see e.g. Edwards and Vozsenskaya, 2011). The primary carboxylation by PEPc typically occurs in thin-walled mesophyll cells that surround thicker walled bundle-sheath (BS) cells. The four-carbon intermediate is transferred via plasmodesmata to BS cells where CO_2 is liberated and the refixation of the CO_2 by Rubisco takes place. In contrast to C_4 photosynthesis, CAM is essentially a single-cell phenomenon, during which the PEPc- and Rubisco-catalysed carboxylations operate at different times of the day–night cycle, i.e. their activity is separated temporally. During the night, CO_2 is fixed by PEPc and the four-carbon intermediate, malic acid, is stored in large vacuoles. During the following light period, the stomata close, PEPc is inactivated, and CO_2 released from the decarboxylation of malic acid is refixed by Rubisco.

Across the phylogenetic tree of angiosperms (Ogburn and Edwards, 2010, 2012), CAM and C_4 origins cluster in numerous distinct clades suggesting that certain plant lineages are prone to evolve both pathways (Edwards and Ogburn, 2012). It has been proposed that the distinct anatomical requirements of the CAM and C_4 pathways, coupled with differences in timing and regulation of their respective biochemical pathways, reduce the likelihood that both co-occur in the same organ. The possibility that parts of the C_4 and CAM cycles take place in the same cell has been suggested to be even less likely (Sage, 2002). In accordance, the reported instances of co-expression of CAM and C_4 within plants with Kranz anatomy are rare, with the only known cases being observed in *Portulaca* (Koch and Kennedy, 1980; Guralnick *et al.*, 2002). *Portulaca* is the only genus of the Portulacaceae, a family assigned to the order Caryophyllales in which CAM and C_4 have evolved multiple times (Christin *et al.*, 2014). Phylogenetic relationships of families within the Caryophyllales and the currently known distribution of CAM and C_4 photosynthesis among them have been recently featured in Holtum *et al.* (2018) (see their Fig. 3).

Originally, CAM and C_4 were reported for two species of *Portulaca* (*P. oleracea* and *P. grandiflora*), and in each, it appeared that CAM and C_4 were confined to different cell or tissue regions. More recent studies indicate that the co-existence of CAM and C_4 in the same photosynthetic organ is common in *Portulaca* (Winter and Holtum, 2014, 2017; Holtum *et al.*, 2017a; Winter, 2019). CAM has been demonstrated by CO_2 gas exchange and quantification of nocturnal acidification in seven species from four of the six major phylogenetic clades of *Portulaca*. In each case of CAM and C_4 co-expression, the expression of CAM was facultative (Guralnick *et al.*, 2002; D'Andrea *et al.*, 2014; Winter and Holtum, 2014, 2017; Holtum *et al.*, 2017a). CAM-type gas-exchange patterns and nocturnal acidification were not detected in well-watered plants, but were induced when the plants were subjected to water stress. When stressed plants were rewatered, their physiology returned to the original well-watered pattern. The observation of widespread CAM in *Portulaca* is supported by the evolutionary history of PEPc genes in *Portulaca* (Christin *et al.*, 2014). The putative gene encoding CAM-specific PEPc was apparently present before the divergence of *Portulaca*, and is similarly used for CAM in relatives of *Portulaca*, whereas PEPcs optimized for C_4 metabolism in *Portulaca* originated from a duplication event of a different paralog, which occurred at the base of *Portulaca*.

The coexistence of C_4 and CAM in leaves of C_4 *Portulaca* species raises interesting questions about the location of both pathways, i.e. whether they occur in different regions of the leaf or whether there is cell sharing. This issue is not yet fully resolved. In *Portulaca oleracea*, CAM-type nocturnal CO_2 fixation presumably takes place in centripetally located large parenchyma cells, yet critical daytime reactions of the CAM cycle may occur in the C_4 bundle-sheath cells (Lara *et al.*, 2003, 2004). By contrast, for *P. grandiflora* separate operation of the C_4 and CAM pathways in different regions of the leaf has been postulated, with C_4 in mesophyll cells associated with the bundle sheath cells and the complete CAM cycle taking place in the centripetal parenchyma cells (Guralnick and Jackson, 2001; Guralnick *et al.*, 2002; Holtum *et al.*, 2017a).

Species in five of the six phylogenetic clades of *Portulaca* are thought to use C_4 as the principal pathway of carbon acquisition (Ocampo *et al.*, 2013; Voznesenskaya *et al.*, 2017). All species examined exhibit C_4-type $\delta^{13}C$ values, Kranz anatomies, enzyme complements, and gas-exchange characteristics. *Portulaca* is not known to contain C_3 species *senso strictu*, but three species in the *Cryptopetala* clade, *P. cryptopetala*, *P. hirsutissima* and *P. mucronata*, have been characterized as C_3–C_4 intermediates on the basis of C_3-type $\delta^{13}C$ values, anatomy, location of glycine decarboxylase, and CO_2 compensation points (Voznesenskaya *et al.*, 2010, 2017; Ocampo *et al.*, 2013). It was inferred that the C_3–C_4 *Cryptopetala* clade evolved from C_4 progenitors and that it represents a reversion from a C_4 state (Ocampo and Columbus, 2012; Ocampo *et al.*, 2013). The reversion hypothesis was questioned by Christin *et al.* (2014) who argued, on the basis of the composition of PEPc genes, the distinct leaf anatomy in each major clade, and the diversity of the de-carboxylating enzymes used by the different clades, that C_4 evolved multiple times in parallel. The *Cryptopetala* clade may therefore be a lineage of *Portulaca* with a photosynthetic complement that reflects a pre-C_4 stage.

CAM in the *Cryptopetala* clade would strengthen the argument that CAM represents an ancestral state in *Portulaca*, being present prior to the evolution of C_4 photosynthesis. If so, the relationship between C_3–C_4 metabolism and CAM remains unclear. In C_3–C_4 intermediates, CO_2 is concentrated into BS-like compartments via the localization of the photorespiratory enzyme glycine decarboxylase (GDC) in the BS, and the shuttling of photorespiratory glycine into the BS for decarboxylation. This metabolism, termed C_2 photosynthesis, can raise CO_2 concentrations in the BS two to three times above the atmospheric value, but does not greatly alter $\delta^{13}C$ values from what are present in C_3 species (Keerberg *et al.*, 2014; Sage *et al.*, 2014). As proposed for C_4 plants, dual expression of C_2 metabolism and CAM could interfere with the optimal function of each, and hence it could be hypothesized that the two metabolic types are segregated either to different tissues or to different phases of development. Here we use gas exchange and measurements of titratable acidity to explore whether CAM is present in the annual/biennial *P. cryptopetala*, and in the perennial *P. molokiniensis* (Hobdy, 1987). *Portulaca cryptopetala* is a C_3–C_4 intermediate and a member of one of the three clades of *Portulaca* in which CAM has not yet been reported. *Portulaca molokiniensis* is a C_4 species that belongs to the C_4 *Oleracea* clade that is sister to the *Cryptopetala* clade.

Materials and methods

Seeds of *P. cryptopetala* and *P. molokiniensis* were obtained from the laboratory stock of one of us (RFS). Plants were grown from seed in either 0.5 litre terracotta pots with an upper diameter of 10 cm, or in 1 litre terracotta pots with an upper diameter of 13 cm. Pots contained potting mix (Miracle-Gro Lawn Products, Marysville, OH, USA). Plants were 1–3 months old when studied.

Two laboratory gas-exchange systems were used to measure 24 h patterns of CO_2 gas exchange of plants. Whole shoots were enclosed in either an 11×11×10 cm or an 11×11×16 cm Perspex cuvette. Roots plus pot remained outside the cuvette. The gas-exchange cuvettes were located inside controlled-environment chambers operating under 12 h light (28 °C):12 h dark (22 °C) cycles. Light was provided by LED grow lights (model LL4L-GP300, GrowPro300). Photon flux density at the level of the cuvettes is specified in the corresponding figure legends. Cuvettes were supplied with air containing 400 ppm CO_2 at flow rates of either 1.26 or 2.5 l min^{-1}. Net CO_2 exchange was measured in flow-through gas-exchange systems consisting of Walz components (gas mixing units, air pumps, cold traps, dew point mirrors; Walz GmbH, Effeltrich, Germany), LI-6252 CO_2 analyzers (Li-Cor, NE, USA) and CR-1000 data loggers (Campbell Scientific, UT, USA) (Holtum and Winter, 2003). For measurements at 2% O_2, N_2 flowing at 4.75 l min^{-1} was added to ambient air flowing at 0.5 l min^{-1}. CO_2 was removed by passing the mixture through soda-lime and then re-added via a mass-flow controller to obtain 400 ppm CO_2 before the gas mixture entered the cuvette. Exposures to air containing 2% O_2 lasted 30–60 min.

Well-watered plants were watered daily to field capacity. Drought treatments were imposed by withholding irrigation until net CO_2 uptake in the light was reduced to close to, at most, 10% of the value for well-watered plants, after which the plants were rewatered daily.

In a separate set of experiments, nine plants of each species were grown in the laboratory under 12 h light–12 h dark cycles. Photosynthetically active photon flux density (PFD) was 600 μmol m^{-2} s^{-1} supplied by a LED grow light (300 W Diamond series, Advanced LED Lights, Hiwasse, AR, USA). Temperature was 26 °C during light periods and 24 °C during dark periods. Plants watered daily to field capacity were deprived of water for several days and then rewatered as specified in the corresponding figure legends. Mature leaves were excised at the end of the light and dark periods from each well-watered, drought-stressed and rewatered plants, and then the fresh mass (FM) obtained, and leaf area measured using a LI-3100 area meter (Li-Cor). Samples were then frozen in liquid nitrogen and freeze-dried. After determination of dry mass, samples were boiled in 80 ml of 50% ethanol until the volume had about halved. Water was then added to bring the volume back to 80 ml and the extract was boiled until the volume again decreased by about half. The extracts were brought to the original volume with water, cooled to room temperature, and titrated with 5 mM KOH to pH 6.5.

Results

Well-watered plants of *P. cryptopetala* exhibited net CO_2 uptake during the day and net CO_2 loss at night (Fig. 1; see also Supplementary Fig. S1 at *JXB* online). The net rates of CO_2 exchange during the day and night increased as the plants grew. In the experiment of Fig. 1, 3 d after watering ceased (day 5 of the experiment), net CO_2 exchange began to decline in the light and the dark. The shape of the CO_2 exchange curve in the dark was noticeably more curved, and nocturnal CO_2 exchange approached the CO_2 compensation point. On day 6, net CO_2 uptake was present for the first time at night. Nocturnal uptake peaked during the night of day 7 and remained approximately constant until the night of day 9, the day prior to rewatering. Within 6 h of rewatering the plant on day 10, CO_2 uptake during the light had almost recovered to the rates observed before the imposition of water stress. No nocturnal net CO_2 uptake was present during the following dark periods.

On day 4, when the *P. cryptopetala* plant shown in Fig. 1 was still exhibiting the well-watered pattern of CO_2 uptake in the light and CO_2 loss at night, the transfer of shoots during the light from an air-stream containing 21% O_2 to an air-stream containing 2% O_2 was accompanied by an increase in the rate of net CO_2 uptake of up to 46%. When air containing 21% O_2 was resupplied, the rate of CO_2 uptake reattained the control pre-2% O_2 rate. A total of nine 2% O_2 treatments were performed on three plants and resulted in an increase of CO_2 uptake by 39±7% (mean ±SD, n=3). The range was 31–46%.

When a plant of *P. cryptopetala* was exposed to sequential watering, droughting, and rewatering cycles, the stress-related induction of net CO_2 uptake in the dark was observed during each period of water stress (Fig. 2). The shoot inside the gas-exchange cuvette continued to grow during the experiment as evidenced by the progressive increase in net CO_2 uptake during the light.

Leaves of well-watered *P. cryptopetala* either did not exhibit nocturnal acidification or, if it was present, the end of night/end of day differences in acidity were very low (Fig. 3). Following the imposition of water stress, strong nocturnal

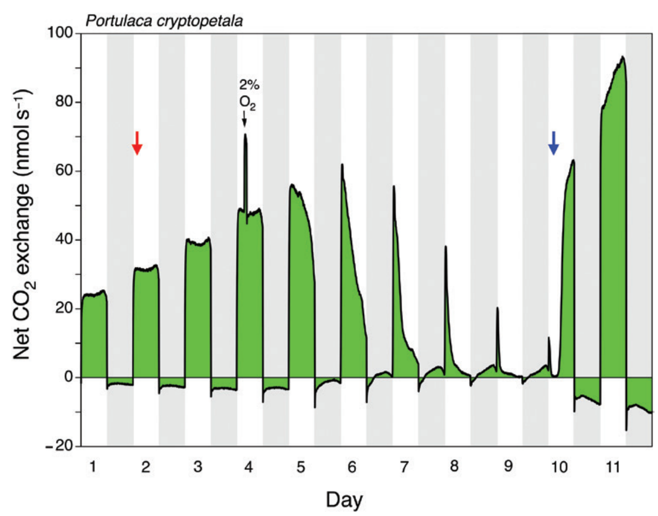

Fig. 1. Eleven days of net CO_2 exchange by the shoot (leaves plus stems) of a *Portulaca cryptopetala* plant growing in a pot. Watering was withheld on day 2 (red arrow) and recommenced on day 10 (blue arrow). During the light period of day 4, the shoot was exposed to air containing 2% O_2 (black arrow) for approx. 1 h. Shaded areas represent the 12 h dark periods. PFD incident to the top of the gas-exchange cuvette was 1000 μmol m^{-2} s^{-1}. At the end of the experiment, total leaf area was 71.1 cm^2, and leaf and stem dry masses were 0.26 and 0.095 g, respectively.

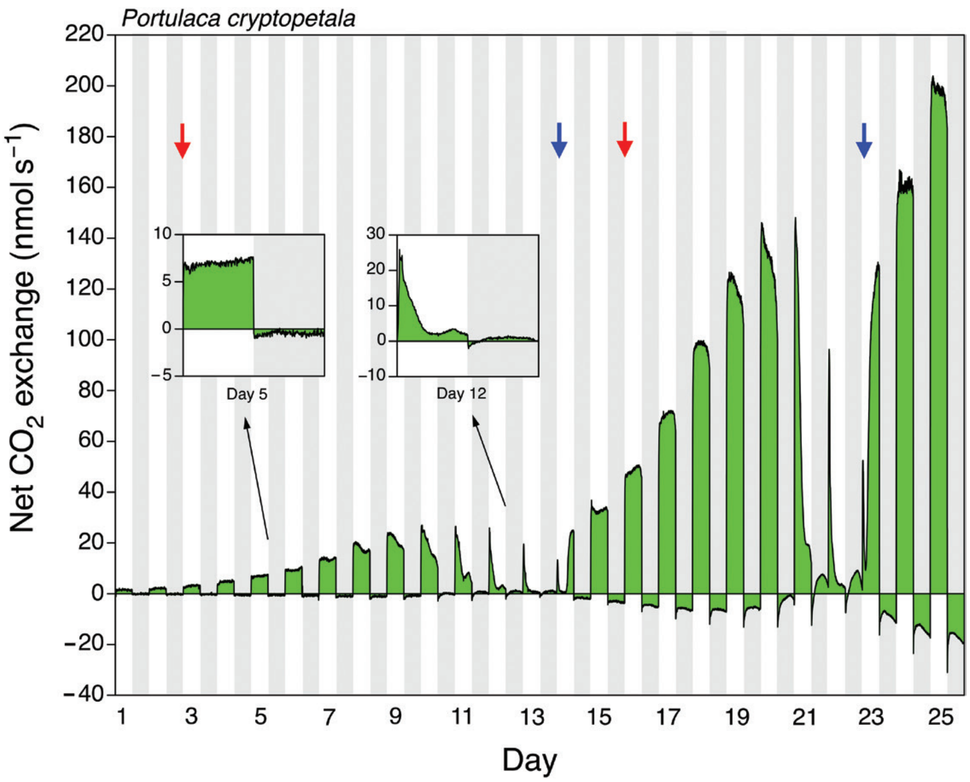

Fig. 2. Twenty-five days of net CO_2 exchange by the shoot (leaves and stems) of a potted *Portulaca cryptopetala* that was exposed to two wetting and drying cycles. Watering was withheld on days 3 and 16 (red arrows) and recommenced on days 14 and 23 (blue arrows). Shaded areas represent the 12 h dark periods. PFD incident to the top of the gas-exchange cuvette was 1350 μmol m^{-2} s^{-1}. At the end of the experiment, total leaf area was 152 cm^2 and leaf and stem dry masses were 0.578 and 0.372 g, respectively.

acidification was induced, reaching about 110 μmol H^+ g^{-1} FM. At the end of the night, the absolute leaf H^+ content was about 25-fold greater than in unstressed plants. Following rewatering, nocturnal leaf acidification was reduced markedly such that the end of the night–end of the day differences in H^+ levels were close to zero. The expression in Fig. 3 of acid levels on fresh mass, dry mass, and leaf area bases enables the calculation of acid concentrations in leaves, permits estimation of the effects of changes in leaf-water content that occur during the droughting process, and facilitates comparison with gas-exchange measurements of CO_2 exchange.

In stems of *P. cryptopetala*, in a manner similar to leaves, marked nocturnal acidification was induced when the plants were subjected to water stress (Fig. 4), with the end of the night acid pool increasing by about 10-fold in comparison to unstressed plants. In contrast to leaves, the stems of rewatered plants continued to exhibit nocturnal acidification, although the levels on a fresh mass basis were only about 14% of those observed in stems of droughted plants.

Well-watered shoots of *P. molokiniensis* exhibited net CO_2 uptake during the light and net CO_2 loss in the dark (Fig. 5). Following the imposition of water stress, a marked decrease in CO_2 uptake was accompanied by the induction of net CO_2 uptake in the dark. Rewatering was followed by a recovery of net CO_2 uptake during the light and a loss of nocturnal net CO_2 uptake. As was observed for *P. cryptopetala*, the exposure of shoots of *P. molokiniensis* to sequential watering, droughting, and rewatering cycles was accompanied by the stress-related induction of net CO_2 uptake at night during each period of water stress. The continued increase in net CO_2 uptake during the light demonstrated that the shoots of *P. molokiniensis* continued to grow during the experiment. The stress-induced, reversible induction of net dark CO_2 fixation shown in Fig. 5 was fully confirmed in three additional gas-exchange experiments with three different *P. molokiniensis* plants (see Supplementary Figs S2–S4).

In well-watered *P. molokiniensis*, the transfer of shoots during the light from an air-stream containing 21% O_2 to an air-stream containing 2% O_2 was accompanied by an increase in the rate of net CO_2 uptake by 8±3% (mean ±SD, n=3 different plants; total of 10 measurements) (e.g. Fig. 5; Supplementary Fig. S2). The range was 5–14%. As with *P. cryptopetala*, when air containing 21% O_2 was resupplied, the rate of CO_2 uptake reattained the control pre-2% O_2 rate.

In a manner similar to *P. cryptopetala*, nocturnal acidification was either not present or barely detectable in leaves of well-watered *P. molokiniensis* (Fig. 6). Leaf acidity increased at the end of the light and the dark periods when plants were stressed. The increase in acidity at the end of the dark was much greater than at the end of the light period, resulting in substantial net acidification during the night. Nocturnal acidification of similar magnitude in droughted *P. molokiniensis* has been observed previously (L. Guralnik, unpublished data, personal communication). The nocturnal acidification was completely lost following rewatering, although the background [H^+] remained somewhat greater than acidity levels at the beginning of the experiment.

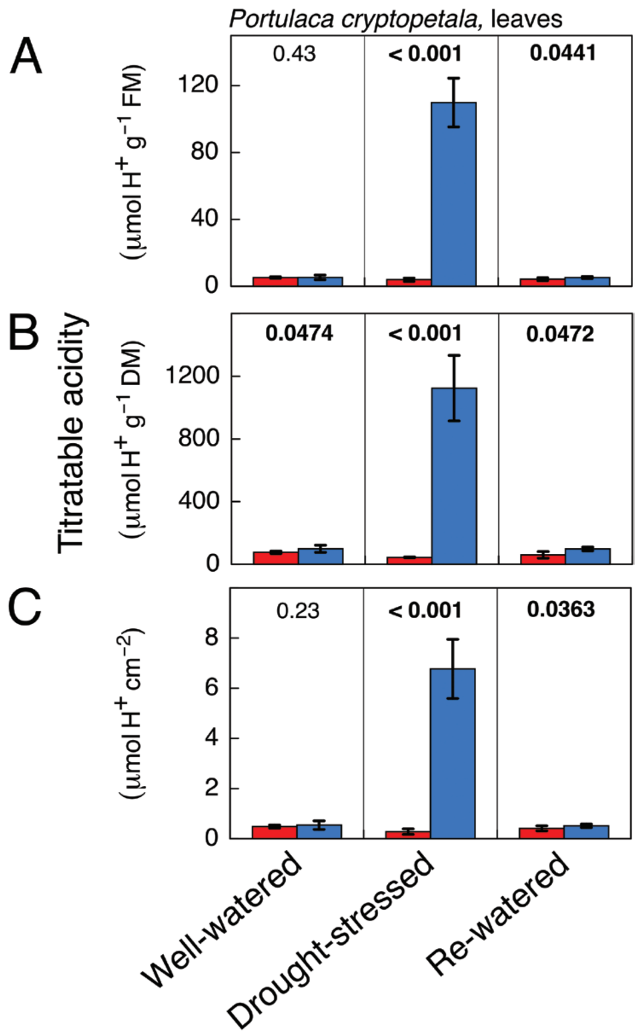

Fig. 3. Titratable acidity in recently fully expanded leaves of *Portulaca cryptopetala* at the end of the 12 h light period (red) and the end of the 12 h dark period (blue) in plants that were well-watered (left-hand column), droughted (middle column; 9 d without irrigation) and droughted and rewatered (right-hand column; 3 d with irrigation). The data are expressed on a fresh mass basis (A), a dry mass basis (B) and a leaf area basis (C). Mean ±SD (*n*=5 leaves; at a given time point each leaf was harvested from a different plant). The numerical values shown above the bars are *P* values (one-tailed *t*-test). Bold letters indicate that the values at the end of the dark period were significantly greater than those at the end of the day at *P*≤0.05.

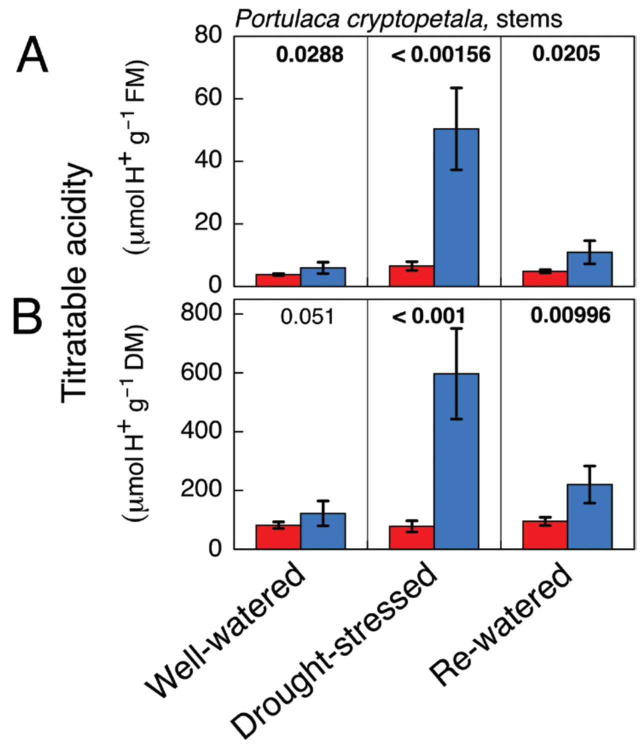

Fig. 4. Titratable acidity in stems of *Portulaca cryptopetala* at the end of the 12 h light period (red) and the end of the 12 h dark period (blue) in plants that were well-watered (left-hand column), droughted (middle column; 10 d without irrigation), and droughted and rewatered (right-hand column; 2 d with irrigation). The data are expressed on a fresh mass basis (A) and a dry mass basis (B). Mean ±SD (*n*=5 stems; at a given time point each stem was harvested from a different plant). The numeric values shown above the bars are *P* values (one-tailed *t*-test). Bold letters indicate that the values at the end of the dark period were significantly greater than those at the end of the day at *P*≤0.05.

Discussion

The demonstration of CAM in *P. cryptopetala* and in *P. molokiniensis* adds a new facet to our understanding of the diversity in origins, functioning, expression, and interrelationships of C_3, C_4, and CAM photosynthesis. CAM, long known to be co-expressed alongside C_3 photosynthesis in plants with succulent tissues, is now documented in eight C_4 species, all within *Portulaca* (Koch and Kennedy, 1980; Guralnick *et al.*, 2002; Christin *et al.*, 2014; Winter and Holtum, 2014, 2017; Holtum *et al.*, 2017a). With the evidence presented here for CAM in *P. cryptopetala*, we can now conclude that CAM can also co-occur in leaves with C_3–C_4 photosynthesis. In *Portulaca*, the C_4 and C_3–C_4 intermediate species that express CAM are dispersed across five of the six clades of *Portulaca* (Fig. 7). CAM has been detected in species with all of the forms of anatomy described for *Portulaca* (Atriplicoid, Pilosoid, Portulacelloid and C_3–C_4) and in both NAD-ME C_4 species (*P. oleracea* and *P. molokiniensis*) and in NADP-ME C_4 species (*P. pilosa*, *P. grandiflora* and *P. umbraticola*) (Voznesenskaya *et al.*, 2010, 2017; Ocampo *et al.*, 2013).

In *P. cryptopetala* and *P. molokiniensis*, as in other *Portulaca* with CAM, the expression of CAM is unmistakably facultative. Compared with rates of C_3 and C_4 photosynthesis in unstressed plants, the magnitudes of CAM-type dark CO_2 uptake and nocturnal acidification are relatively low, but both characters are clearly present in water-stressed plants and are absent, or close to absent, in well-watered plants (Figs 1–6). The observation that CAM can be repeatedly induced or lost following cycles of water supply and water stress in *P. cryptopetala* and *P. molokiniensis* (Figs 2, 5) reveals a tight relationship between the environmental trigger, in this case water stress, and the physiological reaction of the plants, independent of ontogeny (Winter and Holtum, 2007).

At present, in the absence of field studies, we can only speculate as to how a combination of C_4 and CAM traits in a single plant might potentially be of adaptive significance. The most obvious conclusion is that C_4 and C_2 provide a capacity for enhanced productivity and that CAM increases the ability to cope with

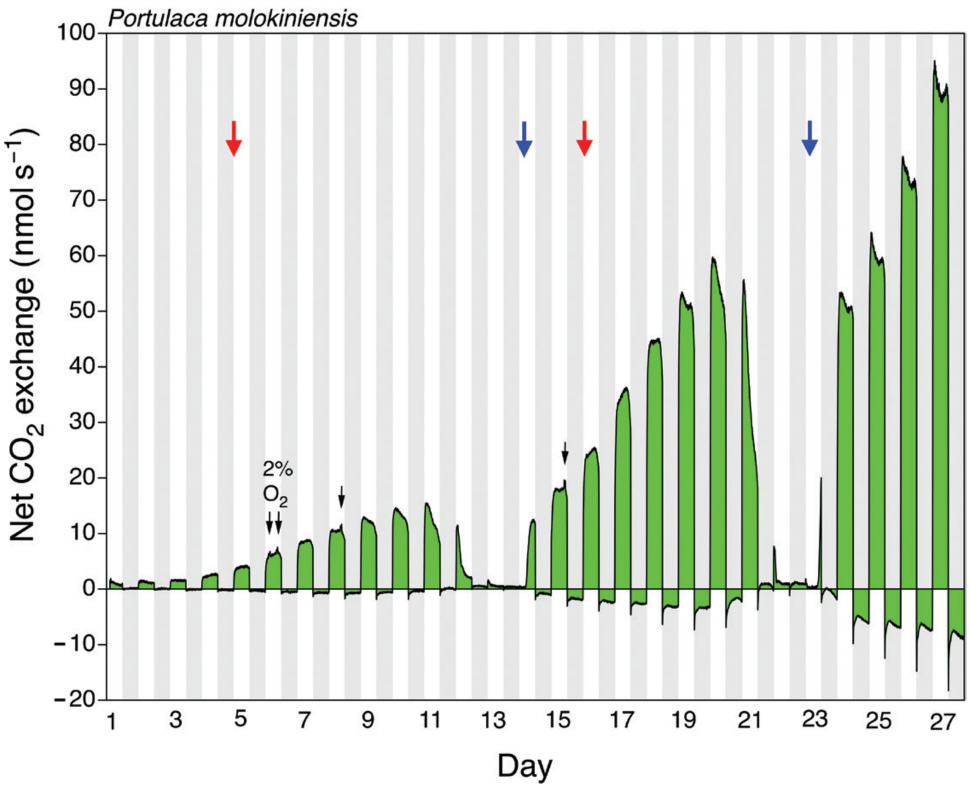

Fig. 5. Twenty-seven days of net CO_2 exchange by shoots of a potted *Portulaca molokiniensis* that was exposed to two wetting and drying cycles. Watering was withheld on days 5 and 16 (red arrows) and recommenced on days 14 and 23 (blue arrows). During the light periods of days 6, 8, and 15 the shoots were exposed to air containing 2% O_2 (black arrows). Shaded areas represent the 12 h dark periods. PFD incident to the top of the gas-exchange cuvette was 1000 µmol m^{-2} s^{-1}. At the end of the experiment, total leaf area was 97.5 cm^2, and leaf and stem dry masses were 0.405 and 0.060 g, respectively.

water stress (Winter and Ziegler, 1992). Particularly in warmer climates, the C_4 component could enable rapid growth and high nitrogen-use efficiency, and CAM could contribute to survival via its ability to reduce carbon and water loss when the supply of water is constrained. The rapid switching from CAM back to C_4 would be expected to enable a prompt response to rainfall events, an ability of relevance to species that are fast-growing, generally annual, weedy ecological opportunists of disturbed sites, e.g. *P. cryptopetala*, *P. grandiflora*, *P. oleracea*, and *P. pilosa*.

While an intermediate CO_2 compensation point and other characteristics (Voznesenskaya *et al.*, 2010, 2017; Ocampo *et al.*, 2013) support the notion that *P. cryptopetala* is not a C_4 species but rather a C_3–C_4 species, the stimulation of photosynthesis by up to 46% when *P. cryptopetala* was exposed to air containing 2% O_2 (Fig. 1; Supplementary Fig. S1), together with C_3-type $\delta^{13}C$ values, suggests that it is an intermediate in which, at current ambient CO_2 concentrations, uptake of atmospheric CO_2 in the light is catalysed largely by Rubisco. Presumably, this Rubisco signal is contributed to by Rubisco in C_3–C_4 tissue and CAM tissue.

In contrast to *P. cryptopetala*, the exposure of *P. molokiniensis* to 2% O_2 resulted in up to a 14% stimulation of photosynthesis (Fig. 5; Supplementary Fig. S2), a response more similar to that of C_4 plants. In C_4 plants, photosynthesis is typically unaffected by a transfer from 21 to 2% O_2 but, at current ambient [CO_2], it is not uncommon for plants to exhibit a small stimulation in photosynthesis as [O_2] is lowered from 21 to 5–10% followed by a small inhibition as [O_2] is further reduced to 2% or lower (Maroco *et al.*, 1997, 1998). The inhibition is thought to be related to a greater requirement for O_2-dependent ATP generation by C_4 photosynthesis compared with C_3 photosynthesis. The ATP is required to regenerate PEP, the primary substrate of the C_4 cycle. The [O_2] at which the stimulation-to-inhibition transition occurs is apparently species-specific and may be anywhere between 10 and 2%. The small stimulation in CO_2 uptake in well-watered plants of *P. molokiniensis* following exposure to 2% O_2 is probably not an effect of O_2 on C_4 metabolism; rather it reflects the effect of [O_2] on reducing photosynthesis in the large-celled chloroplast-containing parenchyma (Kim and Fisher, 1990) in which C_3 photosynthesis presumably occurs in well-watered plants, and in which CAM is induced when the plants are drought-stressed.

Christin *et al.* (2014) suggest that the occurrence of CAM and C_4 in *Portulaca* is the product of a partially shared evolutionary trajectory in which *Portulaca* was ancestrally a C_3–CAM plant. C_4 photosynthesis subsequently evolved multiple times while a functional CAM cycle was maintained. For enzymes other than PEPc, *Portulaca* co-opted the ancestral CAM genes for C_4 photosynthesis, but the C_4 PEPc genes appear to have arisen via *Portulaca*-specific gene duplication, and were independently optimized in each *Portulaca* clade. It is possible that the *Cryptopetala* clade may represent an ancestral C_3–CAM state common to all extant *Portulaca* (but see Hancock and Edwards (2014) for challenges to this type of inference).

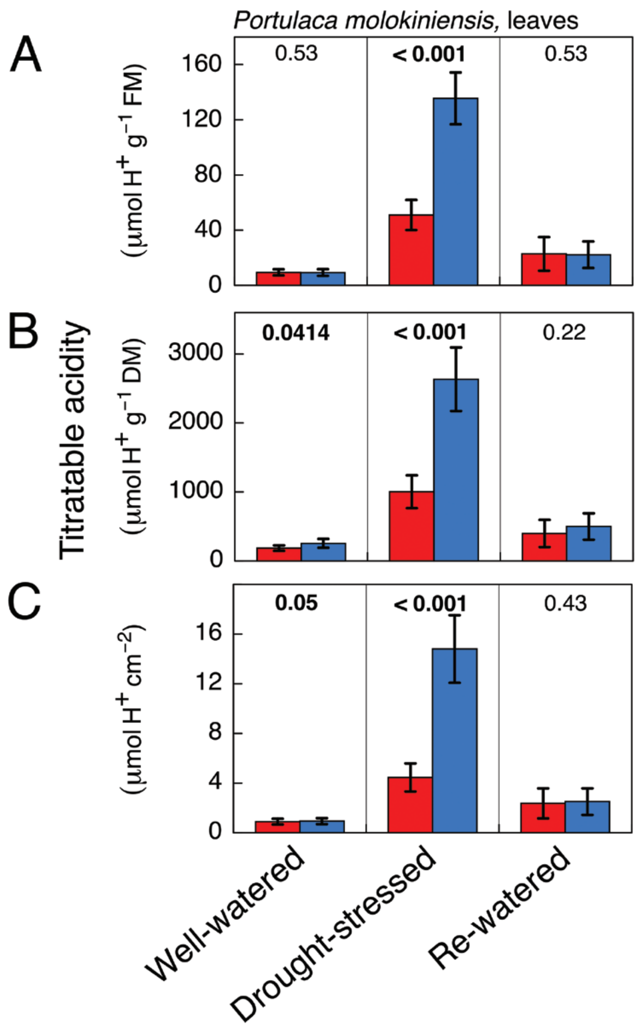

Fig. 6. Titratable acidity in recently fully expanded leaves of *Portulaca molokiniensis* at the end of the 12 h light period (red) and at the end of the 12 h dark period (blue) in plants that were well-watered (left-hand column), droughted (middle column; 12 d without irrigation), and droughted and rewatered (right-hand column; 6 d with irrigation). The data are expressed on a fresh mass basis (A), a dry mass basis (B), and a leaf area basis (C). Mean ±SD (n=5 leaves; at a given time point each leaf was harvested from a different plant). The numeric values shown above the bars are P values (one-tailed t-test). Bold letters indicate that the values at the end of the dark period were significantly greater than those at the end of the day at $P \leq 0.05$.

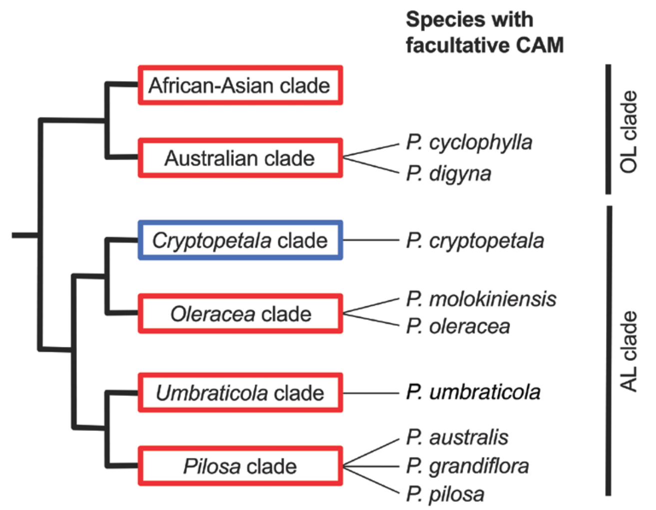

Fig. 7. Phylogenetic relationships of species within the genus *Portulaca*, based upon the analyses of Ocampo *et al.* (2013) and Moore *et al.* (2018), showing the currently known distribution of C_4 (red) and C_3–C_4 (blue) photosynthesis among them. The C_4 distribution is from Ocampo *et al.* (2013), and the CAM distribution is from Koch and Kennedy (1980), Guralnick *et al.* (2002), Winter and Holtum (2017), Holtum *et al.* (2017a), Winter (2019), and the current study.

Nevertheless, the presence of CAM but not full C_4 in *P. cryptopetala* is consistent with the notion that in *Portulaca* CAM is an ancestral state that has persisted despite the subsequent repeated evolution of C_4 photosynthesis.

As is the case for leaves in the C_3–C_4 *P. cryptopetala*, the stems of *Portulaca* species in general lack Kranz anatomy and the C_4 pathway (Voznesenskaya *et al.*, 2010). Observations of nocturnal acidification in stems as well as leaves of the C_3–C_4 *P. cryptopetala* (Figs 3, 4) and the C_4 species *P. oleracea* (Koch and Kennedy, 1980) and *P. grandiflora* (Guralnick *et al.*, 2002) may also be taken as evidence in support of the concept of a pre-C_4 presence of CAM in *Portulaca*.

There is little sign that the evolution of C_4 in organs with CAM has systematically enhanced or retarded the CAM phenotype in *Portulaca*. Nocturnal acidification in leaves of the C_3–C_4 *P. cryptopetala* does not overly differ in magnitude from acidification in the C_4–CAM species in the other three clades known with CAM. Nocturnal acid accumulation in leaves of *P. cryptopetala* of ca. 100 µmole H^+ g^{-1} FM (Fig. 3A) is comparable to values reported for *P. oleracea* (Koch and Kennedy, 1980) and *P. grandiflora* (Guralnick *et al.*, 2002), but greater than levels of ca. 75 µmole H^+ g^{-1} FM reported for *P. australis*, *P. digyna*, *P. molokiniensis*, and *P. pilosa*, and far in excess of the 8 µmole H^+ g^{-1} FM reported for *P. cyclophylla* (Holtum *et al.*, 2017b; Winter and Holtum, 2017). Although water-stressed *P. molokiniensis* (Fig. 6) accumulated less acid at night than did water-stressed *P. cryptopetala*, in terms of the absolute acidity stored in tissues, the acid levels in *P. molokiniensis* were greater. The reason for the difference was that following the imposition of stress, the background levels of acid increased in *P. molokiniensis* but not in *P. cryptopetala*. To further address the question of possible differences in the capacity for nocturnal acid accumulation between different species of *Portulaca*, a rigorous comparison of acid levels from a wide range of species growing under identical conditions is warranted.

Similarities exist between the expression of CAM in *Portulaca* (Portulacaceae) and in the Australian *Calandrinia* (Montiaceae) (Winter and Holtum, 2011; Holtum *et al.*, 2017b; Hancock *et al.*, 2018). Both are located in the sub-order Portulacineae (Carophyllales) where they nest among lineages in which CAM and succulence are common (Moore *et al.*, 2018; Ogburn and Edwards, 2013), and both are mainly composed of small, short-lived, succulent-leaved herbs of open arid to semi-arid sites (Eggli 2004; Nyffeler *et al.*, 2008; Kapitany, 2007). Indeed, in Australia it is not uncommon to see species of *Portulaca* and *Calandrinia* growing alongside each other. Facultative CAM appears widespread in both groups but, although full C_4 is present in *Portulaca*, there is currently no evidence of strong constitutive CAM in either lineage, despite both having diverged from their respective progenitors around 30 Ma ago (Arakaki *et al*., 2011; Hancock *et al*., 2018). In each of the lineages, it is unclear why full CAM has not evolved but facultative CAM has. The answer undoubtedly lies in historical contingencies that are the products of interactions between genetic composition and ecological opportunity over space and time (Edwards and Donoghue, 2013; Christin *et al*., 2014, 2015).

In the case of the C_4 pathway, detailed analyses of phylogeny, anatomy, genes, and physiological phenotypes in the ~40 C_3–C_4 intermediates known from ca. 20 monocot and eudicot genera has markedly assisted conceptualization of the importance of parallel and convergent evolution to the multiple emergence of the C_4 pathway, and of the processes that constrain and enable it (Sage *et al*., 2011, 2014; Christin *et al.* 2015). If plants with low-level CAM or facultative CAM are the CAM equivalent of C_3–C_4 intermediates, then many more C_3–CAM intermediates are known than are C_3–C_4 intermediates (Winter *et al*., 2015). Presumably, as has been demonstrated for the C_4 pathway intermediates, the C_3–CAM intermediates contain a subset of the anatomical and biochemical components of the CAM CO_2 pump that improve physiological performance over the C_3 system in the places where the plants are found (Heckmann, 2016). Addressing the core questions of CAM origins and expression will benefit from rigorous comparisons across lineages of genes and traits that have been acquired repeatedly during evolution of CAM.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Fourteen days of net CO_2 exchange of *Portulaca cryptopetala* during a wet–dry–wet cycle.

Fig. S2. Eleven days of net CO_2 exchange of *Portulaca molokiniensis* during a wet–dry–wet cycle.

Fig. S3. Sixteen days of net CO_2 exchange of *Portulaca molokiniensis* during a wet–dry–wet cycle.

Fig. S4. Twelve days of net CO_2 exchange of *Portulaca molokiniensis* during a wet–dry–wet cycle.

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