Facultative crassulacean acid metabolism in a C₃–C₄ intermediate

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Abstract

The Portulacaceae enable the study of the evolutionary relationship between C₄ and crassulacean acid metabolism (CAM) photosynthesis. Shoots of well-watered plants of the C₃–C₄ intermediate species Portulaca cryptopetala Speg. exhibit net uptake of CO₂ solely during the light. CO₂ fixation is primarily via the C₃ pathway as indicated by a strong stimulation of CO₂ uptake when shoots were provided with air containing 2% O₂. When plants were subjected to water stress, daytime CO₂ uptake was reduced and CAM-type net CO₂ 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 CO₂ 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 C₃–C₄ intermediate species reported to exhibit CAM. Portulaca molokiniensis Hobdy, a C₄ 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 C₄ photosynthesis, facultative CAM is ancestral to C₄. 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: C₃, C₄ and C₃–C₄ intermediate.

Keywords: C₄ photosynthesis, crassulacean acid metabolism, Portulaca cryptopetala, Portulaca molokiniensis, Portulacaceae.

Introduction

An estimated 10% of terrestrial vascular plants express either crassulacean acid metabolism (CAM) or C₄ 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 C₄ is known in 19 families of angiosperms (Sage and Sultmanis, 2016). The CAM and C₄ pathways are comparable in many respects (Osmond, 1978; Hatch, 1987). Using a similar complement of enzymes, each pathway concentrates CO₂ in the vicinity of Rubisco thereby reducing the competitive inhibition by molecular oxygen of CO₂ uptake. In both pathways, atmospheric CO₂ initially fixed as HCO₃⁻ using oxygen-insensitive phosphoenolpyruvate carboxylase (PEPc) is incorporated into a four-carbon intermediate from which CO₂ is ultimately
librated in the vicinity of Rubisco. At the site of Rubisco, CO₂ attains concentrations that ensure that the enzyme functions overwhelmingly as a carboxylase.

Despite similarities, CAM and C₄ differ in important aspects. In C₃ plants, all of the processes associated with photosynthetic CO₂ 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 Vozensenskaya, 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 CO₂ is liberated and the refixation of the CO₂ by Rubisco takes place. In contrast to C₄ photosynthesis, CAM is essentially a single-cell phenomenon, during which the PEP-catalysed carboxylations operate at different times of the day-night cycle, i.e. their activity is separated temporally. During the night, CO₂ is fixed by PEPs and the four-carbon intermediate, malic acid, is stored in large vacuoles. During the following light period, the stomata close, PEPs is inactivated, and CO₂ released from the decarboxylation of malic acid is refixed by Rubisco.

Across the phylogenetic tree of angiosperms (Ogburn and Edwards, 2010, 2012), CAM and C₄ 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 C₄ 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 C₄ 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 C₄ 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 C₄ have evolved multiple times (Christin et al., 2014). Phylogenetic relationships of families within the Caryophyllales and the currently known distribution of CAM and C₄ photosynthesis among them have been recently featured in Holtum et al. (2018) (see their Fig. 3).

Originally, CAM and C₄ were reported for two species of Portulaca (P. oleracea and P. grandiflora), and in each, it appeared that CAM and C₄ were confined to different cell or tissue regions. More recent studies indicate that the co-existence of CAM and C₄ 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 CO₂ 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 C₄ 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 C₄ metabolism in Portulaca originated from a duplication event of a different paralog, which occurred at the base of Portulaca.

The coexistence of C₄ and CAM in leaves of 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 CO₂ fixation presumably takes place in centripetally located large parenchyma cells, yet critical daytime reactions of the CAM cycle may occur in the C₄ bundle-sheath cells (Lara et al., 2003, 2004). By contrast, in P. grandiflora separate operation of the C₄ and CAM pathways in different regions of the leaf has been postulated, with C₄ 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 C₄ as the principal pathway of carbon acquisition (Ocampo et al., 2013; Vozensenskaya et al., 2017). All species examined exhibit C₄-type δ¹³C values, Kranz anatomy, enzyme complements, and gas-exchange characteristics. Portulaca is not known to contain C₃ species sensu strictu, but three species in the Cryptopetala clade, P. cryptopetala, P. huisuissima and P. mucronata, have been characterized as C₃–C₄ intermediates on the basis of C₃-type δ¹³C values, anatomy, location of glycine decarboxylase, and CO₂ compensation points (Vozensenskaya et al., 2010, 2017; Ocampo et al., 2013). It was inferred that the C₃–C₄ Cryptopetala clade evolved from C₄ progenitors and that it represents a reversion from a C₄ 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 C₄ evolved multiple times in parallel. The Cryptopetala clade may therefore be a lineage of Portulaca with a photosynthetic complement that reflects a pre-C₄ 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 C₄ photosynthesis. If so, the relationship between C₃–C₄ metabolism and CAM remains unclear. In C₃–C₄ intermediates, CO₂ is concentrated into BS-like compartments via the localization of the photorespiratory enzyme glycine decarboxylase (GDC) in the BS, and the shunting of photorespiratory glycine into the BS for decarboxylation. This metabolism, termed C₂ photosynthesis, can raise CO₂ concentrations in the BS two to three times above the atmospheric value, but does not greatly alter δ¹³C values from what are present in C₃ species (Keerberg et al., 2014; Sage et al., 2014). As proposed for C₃ plants, dual expression of C₂ 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 \textit{P. cryptopetala}, and in the perennial \textit{P. molokiniensis} (Hobdy, 1987). \textit{Portulaca cryptopetala} is a \textit{C}_3–\textit{C}_4 intermediate and a member of one of the three clades of \textit{Portulaca} in which CAM has not yet been reported. \textit{Portulaca molokiniensis} is a \textit{C}_4 species that belongs to the \textit{C}_4 \textit{Oleracea} clade that is sister to the \textit{Cryptopetala} clade.

**Materials and methods**

Seeds of \textit{P. cryptopetala} and \textit{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 \textit{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 \textit{CO}_2 at flow rates of either 1.26 or 2.5 l min\(^{-1}\). Net \textit{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 \textit{CO}_2 analyzers (Li-Cor, NE, USA) and CR-1000 data loggers (Campbell Scientific, UT, USA) (Holtum and Winter, 2003).

For measurements at 2% \textit{O}_2, \textit{N}_2 flowing at 4.75 l min\(^{-1}\) was added to ambient air flowing at 4.75 l min\(^{-1}\). \textit{CO}_2 was removed by passing the mixture through soda-lime and then re-added via a mass-flow controller to obtain 400 ppm \textit{CO}_2 before the gas mixture entered the cuvette. Exposures to \textit{O}_2 containing 2% \textit{O}_2 lasted 30–60 min.

Well-watered plants were watered daily to field capacity. Drought treatments were imposed by withholding irrigation until net \textit{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 (PPFD) was 600 μmol m\(^{-2}\) s\(^{-1}\) supplied by a LED grow light (300 W Diamond series, Advanced LED Lights, Hiwase, 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 \textit{P. cryptopetala} exhibited net \textit{CO}_2 uptake during the day and net \textit{CO}_2 loss at night (Fig. 1; see also Supplementary Fig. S1 at JXB online). The net rates of \textit{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 \textit{CO}_2 exchange began to decline in the light and the dark. The shape of the \textit{CO}_2 exchange curve in the dark was noticeably more curved, and nocturnal \textit{CO}_2 exchange approached the \textit{CO}_2 compensation point. On day 6, net \textit{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, \textit{CO}_2 uptake during the light had almost recovered to the rates observed before the imposition of water stress. No nocturnal net \textit{CO}_2 uptake was present during the following dark periods.

On day 4, when the \textit{P. cryptopetala} plant shown in Fig. 1 was still exhibiting the well-watered pattern of \textit{CO}_2 uptake in the light and \textit{CO}_2 loss at night, the transfer of shoots during the light from an air-stream containing 21% \textit{O}_2 to an air-stream containing 2% \textit{O}_2 was accompanied by an increase in the rate of net \textit{CO}_2 uptake of up to 46%. When air containing 21% \textit{O}_2 was resupplied, the rate of \textit{CO}_2 uptake reattained the control pre-2% \textit{O}_2 rate. A total of nine 2% \textit{O}_2 treatments were performed on three plants and resulted in an increase of \textit{CO}_2 uptake by 39±7% (mean ±SD, n=3). The range was 31–46%.

When a plant of \textit{P. cryptopetala} was exposed to sequential watering, droughting, and rewatering cycles, the stress-related induction of net \textit{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 \textit{CO}_2 uptake during the light.

Leaves of well-watered \textit{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
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 CO₂ 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 CO₂ uptake during the light and net CO₂ loss in the dark (Fig. 5). Following the imposition of water stress, a marked decrease in CO₂ uptake was accompanied by the induction of net CO₂ uptake in the dark. Rewatering was followed by a recovery of net CO₂ uptake during the light and a loss of nocturnal net CO₂ uptake. As was observed for P. cryptopetala, the exposure of shoots of P. molokiniensis to sequential watering, droughting, and rewetting cycles was accompanied by the stress-related induction of net CO₂ uptake at night during each period of water stress. The continued increase in net CO₂ 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 CO₂ 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% O₂ to an air-stream containing 2% O₂ was accompanied by an increase in the rate of net CO₂ 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% O₂ was resupplied, the rate of CO₂ uptake reattained the control pre–2% O₂ 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 rewetting, although the background [H⁺] remained somewhat greater than acidity levels at the beginning of the experiment.
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 C3, C4, and CAM photosynthesis. CAM, long known to be co-expressed alongside C3 photosynthesis in plants with succulent tissues, is now documented in eight C4 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 C3–C4 photosynthesis. In *Portulaca*, the C4 and C3–C4 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 C3–C4) and in both NAD-ME C4 species (*P. olereca* and *P. molokiniensis*) and in NADP-ME C4 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 C3 and C4 photosynthesis in unstressed plants, the magnitudes of CAM-type dark CO2 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 C4 and CAM traits in a single plant might potentially be of adaptive significance. The most obvious conclusion is that C4 and C2 provide a capacity for enhanced productivity and that CAM increases the ability to cope with...
water stress (Winter and Ziegler, 1992). Particularly in warmer climates, the C4 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 C4 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 CO2 compensation point and other characteristics (Voznesenskaya et al., 2010, 2017; Ocampo et al., 2013) support the notion that *P. cryptopetala* is not a C4 species but rather a C3–C4 species, the stimulation of photosynthesis by up to 46% when *P. cryptopetala* was exposed to air containing 2% O2 (Fig. 1; Supplementary Fig. S1), together with C3-type δ13C values, suggests that it is an intermediate in which, at current ambient CO2 concentrations, uptake of atmospheric CO2 in the light is catalysed largely by Rubisco. Presumably, this Rubisco signal is contributed to by Rubisco in C3–C4 tissue and CAM tissue.

In contrast to *P. cryptopetala*, the exposure of *P. molokiniensis* to 2% O2 resulted in up to a 14% stimulation of photosynthesis (Fig. 5; Supplementary Fig. S2), a response more similar to that of C4 plants. In C4 plants, photosynthesis is typically unaffected by a transfer from 21 to 2% O2 but, at current ambient [CO2], it is not uncommon for plants to exhibit a small stimulation in photosynthesis as [O2] is lowered from 21 to 5–10% followed by a small inhibition as [O2] is further reduced to 2% or lower (Maroco et al., 1997, 1998). The inhibition is thought to be related to a greater requirement for O2-dependent ATP generation by C4 photosynthesis compared with C3 photosynthesis. The ATP is required to regenerate PEP, the primary substrate of the C4 cycle. The [O2] at which the stimulation–inhibition transition occurs is apparently species-specific and may be anywhere between 10 and 2%. The small stimulation in CO2 uptake in well-watered plants of *P. molokiniensis* following exposure to 2% O2 is probably not an effect of O2 on C4 metabolism; rather it reflects the effect of [O2] on reducing photosynthesis in the large-celled chloroplast-containing parenchyma (Kim and Fisher, 1990) in which C3 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 C4 in *Portulaca* is the product of a partially shared evolutionary trajectory in which *Portulaca* was ancestrally a C3–CAM plant. C4 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 C4 photosynthesis, but the C4 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 C3–CAM state common to all extant *Portulaca* (but see Hancock and Edwards (2014) for challenges to this type of inference).
Nevertheless, the presence of CAM but not full C₄ 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 C₄ photosynthesis.

As is the case for leaves in the C₃–C₄ *P. cryptopetala*, the stems of *Portulaca* species in general lack Kranz anatomy and the C₄ pathway (Voznesenskaya et al., 2010). Observations of nocturnal acidification in stems as well as leaves of the C₃–C₄ *P. cryptopetala* (Figs 3, 4) and the C₄ species *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 C₄ 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., 2018).
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 composi-
tion and ecological opportunity over space and time (Edwards
and Donoghue, 2013; Christin et al., 2014, 2015).

In the case of the C₄ pathway, detailed analyses of phylog-
yeny, anatomy, genes, and physiological phenotypes in the
~40 C₃–C₄ intermediates known from ca. 20 monocot and
eudicot genera has markedly assisted conceptualization of the
importance of parallel and convergent evolution to the mul-
tiple emergence of the C₄ 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 C₃–C₄ intermediates, then many more
C₃–CAM intermediates are known than are C₃–C₄ intermedi-
ates (Winter et al., 2015). Presumably, as has been demonstrat-
ed for the C₄ pathway intermediates, the C₃–CAM intermediates
contain a subset of the anatomical and biochemical compo-
nents of the CAM CO₂ pump that improve physiological
performance over the C₃ system in the places where the plants
are found (Heckmann, 2016). Addressing the core questions of
CAM origins and expression will benefit from rigorous com-
parisons across lineages of genes and traits that have been ac-
quired repeatedly during evolution of CAM.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Fourteen days of net CO₂ exchange of Portulaca crypt-
topetala during a wet–dry–wet cycle.

Fig. S2. Eleven days of net CO₂ exchange of Portulaca molo-
kiniensis during a wet–dry–wet cycle.

Fig. S3. Sixteen days of net CO₂ exchange of Portulaca molo-
kiniensis during a wet–dry–wet cycle.

Fig. S4. Twelve days of net CO₂ exchange of Portulaca molo-
kiniensis during a wet–dry–wet cycle.

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