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Short communication

Optional use of CAM photosynthesis in two C_4 species, *Portulaca cyclophylla* and *Portulaca digyna*

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ABSTRACT

Low levels of crassulacean acid metabolism (CAM) are demonstrated in two species with C_4 photosynthesis, *Portulaca cyclophylla* and *P. digyna*. The expression of CAM in *P. cyclophylla* and *P. digyna* is facultative, i.e. optional. Well-watered plants did not accumulate acid at night and exhibited gas-exchange patterns consistent with C_4 photosynthesis. CAM-type nocturnal acidification was reversible in that it was induced following drought and lost when droughted plants were rewatered. In *P. cyclophylla*, droughting was accompanied by a small but discernible net uptake of CO_2 during the dark, whereas in *P. digyna*, net CO_2 exchange at night approached the CO_2 compensation point but did not transition beyond it. This report brings the number of known C_4 species with a capacity for expressing CAM to six. All are species of *Portulaca*. The observation of CAM in *P. cyclophylla* and *P. digyna* is the first for species in the opposite-leaved (OL) Portulacelloid-anatomy lineage of *Portulaca* and for the Australian clade therein. The other four species are within the alternate-leaved (AL) lineage, in the Atriploid-anatomy Oleracea and the Pilosoid-anatomy Pilosa clades. Studies of the evolutionary origins of C_4 and CAM in *Portulaca* will benefit from a more wide-range survey of CAM across its species, particularly in the C_3-C_4 intermediate-containing Cryptopetala clade.

1. Introduction

C_4 photosynthesis and crassulacean acid metabolism (CAM) have each evolved often, at least 65 times for C_4 (Sage, 2016) and perhaps a similar number of times for CAM (Edwards and Ogburn, 2012). The ca. 8100 known C_4 species are spread across ca. 418 genera in 19 angiosperm families (Sage and Sultmanis, 2016), whereas the roughly 16,000 CAM taxa are in 400+ genera from 36 families of angiosperms, gymnosperms, ferns and lycopsids (Smith and Winter, 1996; Winter and Smith, 1996; Yang et al., 2015). The number of CAM species is less certain than that of C_4 species because firstly, species with low levels of CAM cannot be identified using carbon isotopic analysis alone, and secondly, fewer than 10% of orchids have been surveyed. The Orchidaceae are one of the two most species-rich families of flowering plants and are believed to be the family with the largest number of species capable of CAM.

Only five families contain C_4 species as well as CAM species, *viz.* Aizoaceae, Asteraceae, Euphorbiaceae, Hydrocharitaceae and Portulacaceae (Sage, 2016). Even more uncommon is the simultaneous expression of both C_4 and CAM in one plant. Four such C_4-CAM species are known, all within the genus *Portulaca* (Koch and Kennedy, 1980; Kraybill and Martin, 1996; Guralnick and Jackson, 2001; Guralnick et al., 2002; Christin et al. 2014; Winter and Holtum, 2017). In the four species, C_4 and low-level CAM are present in leaves, whereas stems are presumably C_3 and low-level CAM (C_3-CAM in the terminology of Winter et al., 2015).

Present in all continents bar Antarctica, *Portulaca* is a mainly tropical and subtropical genus that probably originated in South America. Members of its clades have independently dispersed to other continents from South and North America (Eggli, 2004; Ocampo and Columbus, 2010; Hernández-Ledesma et al., 2015). An evolutionary analysis of 44 of the 110+ species of *Portulaca* identified an opposite-leaved (OL) lineage and an alternate-leaved

Abbreviations: AL, alternate leaved lineage of *Portulaca*; BS, bundle-sheath cells; C_4, C_4 pathway of photosynthesis; CAM, crassulacean acid metabolism; OL, opposite leaved lineage of *Portulaca*; PEPC, phospho*enol*pyruvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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(AL) lineage (Ocampo et al., 2013). The OL lineage contains two clades, an Australian clade and an Asian clade, both of which comprise only C_4 species. Within the AL lineage are four clades, the C_4-containing Oleracea, Pilosa and Umbraticola clades, and the Cryptopetala clade that contains known and putative C_3-C_4 intermediates (Voznesenskaya et al., 2010).

The four known *Portulaca* C_4 species with CAM are members of the AL lineage. *Portulaca oleracea* L. is in the NAD malic enzyme, Oleracea clade (Koch and Kennedy, 1980; Christin et al., 2014; D'Andrea et al., 2014; Winter and Holtum, 2014), whereas *P. australis* Endl. (Winter and Holtum, 2017), *P. grandiflora* Hook. (Guralnick et al., 2002) and *P. pilosa* L. (=*P. mundula*; Matthews et al., 1992; Kraybill and Martin, 1996; Guralnick and Jackson, 2001; Winter and Holtum, 2017) are within the NADP malic enzyme, Pilosa clade. There are no reports of CAM in the OL lineage, nor in the Umbraticola and Cryptopetala clades of the AL lineage.

In each of the known C_4-CAM *Portulaca* species, leaves of well-watered plants exhibit only C_4 photosynthesis. Leaves of droughted plants exhibit both C_4 and CAM, switching back to solely C_4 photosynthesis when the plants are rewatered (Winter and Holtum, 2014, 2017). The CAM component is thus fully reversible, i.e. optional or facultative.

Here we explore whether features of CAM are present in two members of the OL lineage and its Australian clade. In the case of C_4-CAM species, carbon isotope signatures are not informative as the signals for C_4 and CAM overlap. Our investigation thus involves measurements in individual plants of characters that are used to define CAM, nocturnal fluctuations of titratable acidity and patterns of day-night gas-exchange. We demonstrate that low-level facultative CAM is present in otherwise C_4 species in the OL lineage, specifically in two species of the Australian clade, *P. cyclophylla* F. Muell. and *P. digyna* F. Muell.

2. Material and methods

2.1. Study Species

Portulaca digyna is a prostrate annual herb with stems to 150 mm long. Inhabiting woodland or sandy/gravelly floodplains in savannas and hot grasslands of tropical northern Australia (Fig. 1.), *P. digyna* appears after summer rainfall (ALA, 2016). The opposite, succulent leaves, broadly ovate to narrowly elliptic, are 3–8 mm long and set on short petioles of 0.5–1 mm.

Portulaca cyclophylla is a spreading prostrate geophyte with branches to 100 mm that die back in dry conditions to a perennial taproot that re-shoots after rain (Fig. 2). The moderately fleshy leaves, green in well-watered plants, but generally grey or brown in the field, are 6–10 mm in diameter, opposite and almost circular (Kapitany, 2007). A species of arid gravel and sand plains, *P. cyclophylla* has been collected from the Gascoyne, Little Sandy Desert, Murchison, Pilbara and northern Kimberley regions of Western Australia (ALA, 2016).

2.2. Plant material and CO_2 exchange

Plants of *P. cyclophylla* were grown from seeds supplied by A. Kapitany (australiansucculents.com) and originally collected from near Meekatharra, Western Australia (26.594° S, 118.495° E). Seeds of *P. digyna* were harvested from plants collected near Mareeba, Queensland (16.933281° S, 145.32896° E).

Seeds were germinated in terracotta pots (10 cm upper diameter, 0.4 L volume) containing potting mix. For each experiment, the shoots of a plant were enclosed inside a Perspex cuvette (internal dimensions of 11 × 11 × 10 cm). The attached roots plus pot remained outside the cuvette.

A plant, with the gas-exchange cuvette sealed around the shoot, was located inside a controlled environment chamber (EGC, OH, USA) operating under 12 h light (28 °C):12 h dark (22 °C) cycles. Photon flux density was 1000 μmol m^{-2} s^{-1} at the top of the cuvette. Air containing 400 ppm CO_2 was delivered to the cuvette at 1.26 L min^{-1}. Net CO_2 exchange of the shoot inside the cuvette was measured continuously for up to 24 day-night cycles with data-points obtained every four minutes. The flow-through gas-exchange system consisted of Walz components (Walz, FRG) and a LI-6252 CO_2 analyzer (Li-Cor, NE, USA) (Holtum and Winter, 2003). Drought treatments were imposed by withholding irrigation which was daily in the well-watered treatment. For each species, gas-exchange analysis during watering, drought and rewatering treatments was performed on two separate plants. The responses of the replicates to the treatments were consistent with each other. Data are shown for one of the two replicates.

2.3. Determination of titratable acidity

In a separate experiment, *P. cyclophylla* and *P. digyna* were grown outdoors in pots as described above. Leaves from mature plants that were watered, droughted and rewatered, were excised at dusk and dawn and frozen in liquid nitrogen prior to sequential extraction in boiling 50% ethanol and in water. The extracts were titrated with 5 mM KOH to pH 6.5.

2.4. Anatomical investigation

Fluorescence from hand-sections of leaves either unstained, or stained with Nuc Blue Live Cell Stain (Thermo Fisher Scientific), was observed following excitation at 405 nm using an Olympus FV1000 confocal microscope.

3. Results

The distribution of *P. digyna* correlates with the extent in northern Australia of the Köppen vegetation classes 'tropical grasslands' and 'savannas' (Fig. 1), an area where seasonal rainfall mainly occurs in the Austral summer (December to February). In contrast, *P. cyclophylla* occupies the southern limits of the Western Australian summer rainfall zone (Fig. 2), a range not clearly defined by one or two Köppen vegetation classes. In landscapes occupied by the annual *P. digyna*, moderately to highly variable rainfall averages between 100 and 600 mm, whereas in the arid gravel- and sand-plain landscapes occupied by *P. cyclophylla* rainfall averages 50–350 mm and is highly to extremely variable (BOM, 2016).

Shoots of well-watered plants of *P. cyclophylla* and *P. digyna* growing inside a gas-exchange cuvette exhibited net CO_2 uptake solely during the light (Figs. 1 and 2), with daily CO_2 exchange increasing as the shoots grew. Following cessation of watering, plants continued to grow until the plant-available water in the pot became limiting. In both species, the resultant onset of drought was associated with a reduction in CO_2 uptake in the light. The reduction of CO_2 uptake during the day was accompanied by a progressive reduction in CO_2 loss at night culminating in low but detectable net nocturnal CO_2 uptake in *P. cyclophylla* (Fig. 2). For *P. digyna*, net CO_2 exchange at night approached the compensation point but did not transition beyond it (Fig. 1).

Following rewatering, both species rapidly reattained their original pattern of net CO_2 uptake restricted to the light. The rates of CO_2 uptake following rewatering were greater than at the onset of the experiment because the plants continued to grow inside the cuvette throughout the experiment.

In well-watered plants of each species the titratable acidity of extracts from leaves harvested at dusk and at dawn were similar (Table 1). Upon droughting, the titratable acidity at dawn exceeded

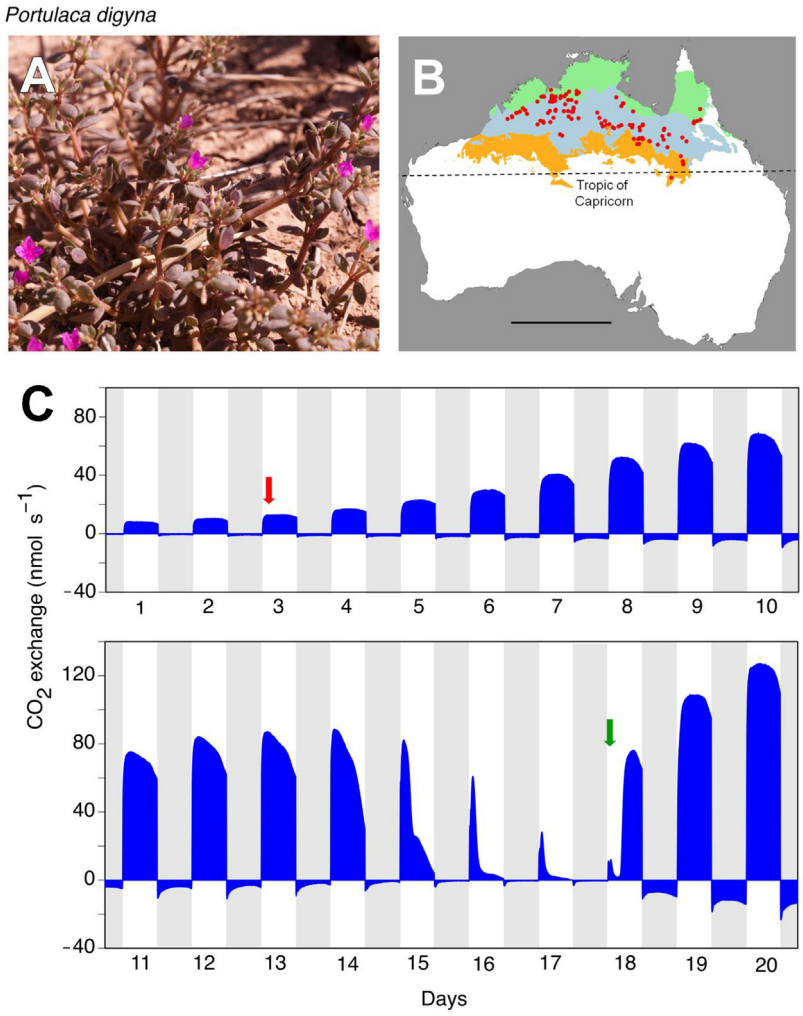

Fig. 1. (A) *Portulaca digyna* growing in situ alongside the Stuart Highway, 70 km south of Matranka, Northern Territory (Lat. −15.53726°S, Long. 133.20362°W); (B) sites of collection of specimens of *P. digyna* deposited in Australia's Virtual Herbarium (AVH, 2016) shown in relation to the location of the Köppen Climate Classification classes: tropical savanna (), hot grassland (winter drought) () and hot desert (winter drought) (). The bar represents 1000 km. (C) Twenty days of net CO_2 exchange by the above-ground shoot of a potted *P. digyna*. Measurements were performed at 400 ppm CO_2 in a controlled environment chamber maintained under 12 h light/12 h dark cycles. Watering was withheld on day 3 (red arrow) and recommenced on day 18 (green arrow). Shaded areas represent the dark periods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Titratable acidity of extracts of leaves from *Portulaca cyclophylla and Portulaca digyna* that were well-watered, droughted and rewatered. Values are means and standard deviations for three plants of each species. Differences between dawn and dusk values samples were compared using unpaired 1-tailed *t*-tests; ns = not significantly different ($\rho > 0.05$).

Species and treatment	Titratable acidity (μmol H^+ g^{-1} fresh mass)			
	End of day	End of night	Difference	Significance
Portulaca cyclophylla				
Well-watered	7.0 ± 0.5	7.4 ± 1.4	+0.4	ns
10 days without water	12.5 ± 3.1	21.0 ± 2.6	+8.5	≤0.05
5 days after rewatering	7.8 ± 2.5	8.4 ± 0.7	+0.6	ns
Portulaca digyna				
Well-watered	5.9 ± 1.6	6.4 ± 1.4	+0.5	ns
7 days without water	22.5 ± 1.2	76.4 ± 4.7	+53.9	≤0.05
9 days without water	12.0 ± 1.2	57.8 ± 7.3	+45.8	≤0.05
3 days after rewatering	12.0 ± 2.6	12.4 ± 1.2	+0.4	ns

that at dusk by up to 2-fold in *P. cyclophylla* and about 5-fold in *P. digyna*. After rewatering daily for 3–5 days, nocturnal acidification was lost, the dusk and dawn titratable acidities were again similar within each species.

For both species, the leaf anatomy is characterised by a horizontal row of vascular bundles below the adaxial surface of the leaf (Fig. 3). The bundles exhibit Kranz anatomy that is more pronounced on the adaxial (upper) sides. There are few abaxial bundle-sheath cells and mesophyll cell density is low. Parenchyma cells, with large vacuoles and few chloroplasts, are located abaxially to the vascular bundles. The vasculature conforms to the Portulacelloid type (Voznesenskaya et al., 2010; Ocampo et al., 2013).

4. Discussion

Portulaca digyna and *P. cyclophylla* are C_4 species that also express low-level CAM. In *P. cyclophylla*, CAM activity is evident as nocturnal accumulation of titratable acidity in leaves and accompanying net CO_2 uptake in the dark. *Portulaca digyna* also underwent nocturnal acidification with CO_2 exchange at night approaching but not exceeding the compensation point. The latter feature in association with nocturnal acid accumulation is frequently termed CAM-cycling, implying that respiratory CO_2 is the predominant CO_2 source for nocturnal acid synthesis (Ting, 1985). For both species, the expression of CAM was clearly facultative, i.e. optional, in that

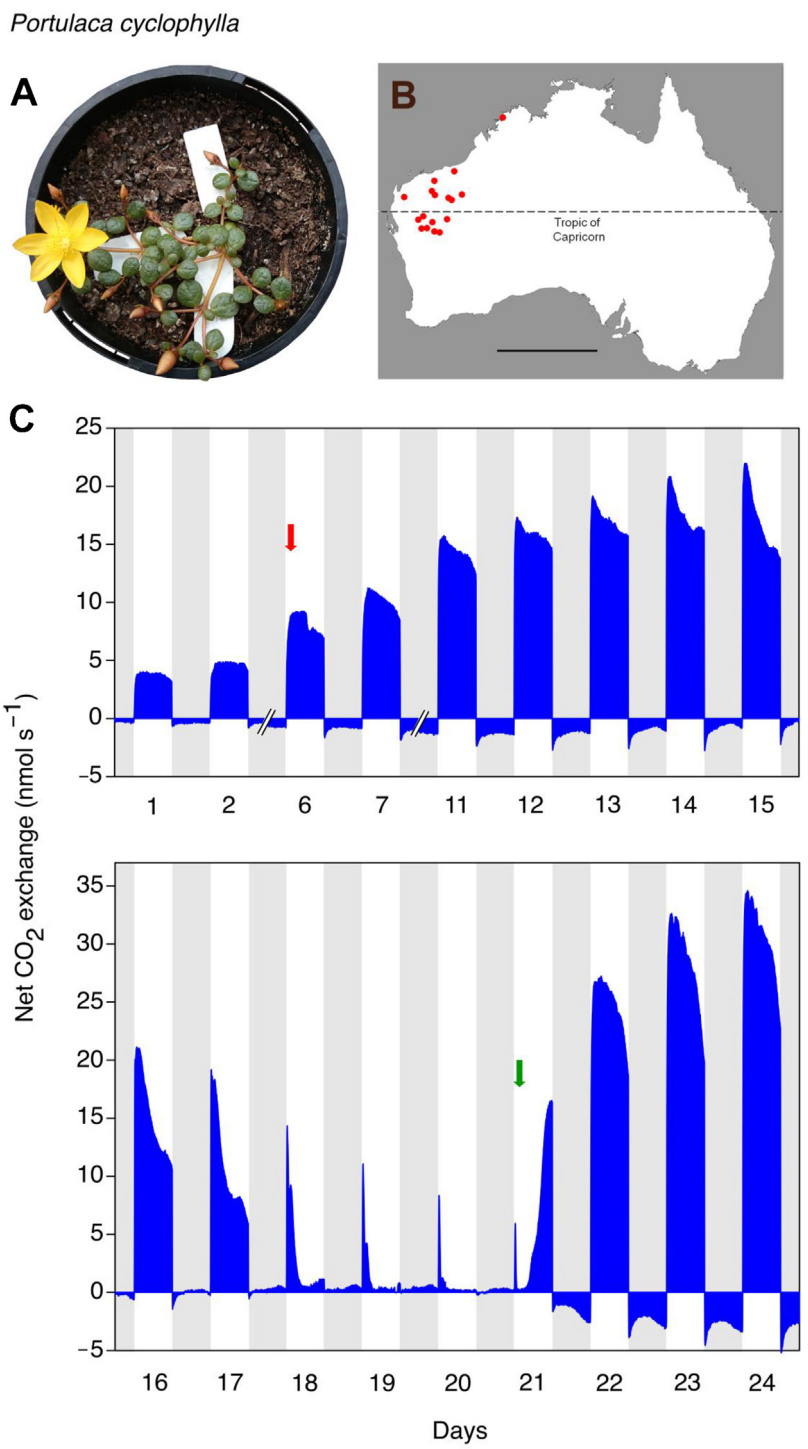

Fig. 2. (A) *Portulaca cyclophylla* growing in a 100 mm diameter pot; (B) sites of collection of specimens of *P. cyclophylla* deposited in Australia's Virtual Herbarium (AVH, 2016). The bar represents 1000 km. (C) Twenty-four days of net CO_2 exchange by the above-ground shoot of a potted *P. cyclophylla*. Measurements were performed at 400 ppm CO_2 in a controlled environment chamber maintained under 12 h light/12 h dark cycles. Watering was withheld on day 6 (red arrow) and recommenced on day 21 (green arrow). Shaded areas represent the dark periods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nocturnal acidification was absent in well-watered plants, was induced by drought, and was lost when water was resupplied.

During droughting, the time-course of nocturnal CO_2 exchange became slightly more curved. This curvature, typically not seen in C_3 tissues maintained under constant temperatures at night, is often observed in nocturnal gas-exchange by low-level CAM plants (see time-courses of C_3 *Calophyllum longifolium* and *Ochroma pyramidale* and various facultative and low-level CAM species in Winter and Holtum 2014, 2015), and probably mirrors the effects on net CO_2 exchange of variations in PEPC activity at night (Winter, 1982).

The demonstration of CAM activity in *P. cyclophylla*, a geophyte of arid landscapes, and in *P. digyna*, an annual of tropical grasslands and savanna, is the first evidence for CAM in the OL lineage of *Portulaca*. The lineage contains species from Africa, Asia and Australia, and includes the pan-tropical weed *P. quadrifida* L. CAM is now known from both the OL and AL *Portulaca* lineages and from three

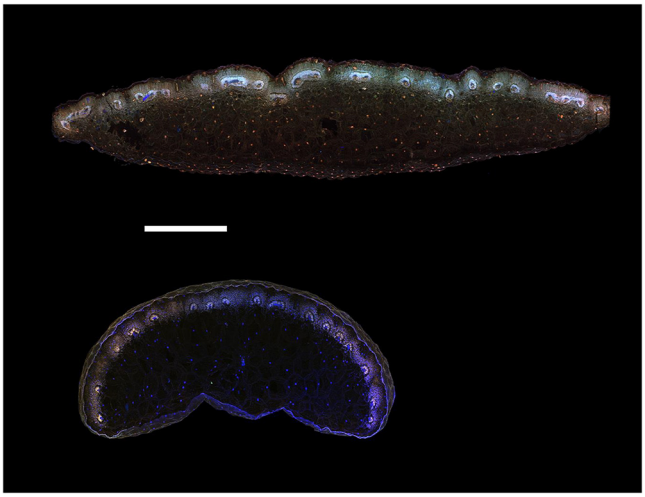

Fig. 3. Cross-sections of an unstained *Portulaca cyclophylla* leaf (upper photograph) and a leaf of *P. digyna* stained with Nuc Blue Live Cell Stain (lower photograph). Note the portulacelloid Kranz venation in the upper parts of the leaves and the subtending large parenchyma cells with small numbers of chloroplasts. The bar represents 1 mm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the six clades within them. *Portulaca digyna*, which exhibits Portulacelloid leaf anatomy, has been placed in the Australian clade (within the OL lineage; Ocampo et al., 2013) but *P. cyclophylla* has not been included in any recent molecular or anatomical treatments (Ocampo and Columbus, 2010; Nyffeler and Eggli, 2010; Voznesenskaya et al., 2010; Ocampo et al., 2013) and has not been assigned to any clade. On the basis of its opposite leaves, Kranz anatomy, Portulacelloid leaf structure and distribution in Australia, we postulate that *P. cyclophylla* is most likely within the Australian clade of *Portulaca*.

Globally, including the two species reported here, only six species of plants are known to simultaneously express both CAM and C_4. The six species reside within *Portulaca* and all exhibit facultative CAM. Although only perhaps 5% of *Portulaca* have been surveyed for CAM, the existing observations support the notion that low-level CAM, in its facultative form, may be widespread in *Portulaca* and, by extrapolation, could be present in other succulent-leaved C_4 species harboured in sections of the angiosperm phylogenetic tree where C_4 and CAM species cluster (Edwards and Ogburn, 2012). To date, it has been only demonstrated that C_4 co-exists with facultative CAM in *Portulaca* but there is no reason why C_4 could not co-exist with constitutive CAM.

The presence of CAM, albeit at a low level, in four of the five C_4 clades, including the three anatomical groups described for *Portulaca* (Atriploid, Pilosoid and Portulacelloid) and both biochemical groups (NAD- and NADP- malic enzyme), is consistent with the hypothesis that C_4 in *Portulaca* has evolved from an ancestral CAM type (Sage, 2002; Nyffeler et al., 2008). The notion that not only has C_4 photosynthesis arisen independently multiple times in *Portulaca* and that the presence of a C_3-C_4 clade is another instance of C_4 emerging, is consistent with the evolutionary history of C_4-specific PEPC genes, and anatomical and biochemical differences among the C_4 lineages (Christin et al., 2014; Hancock and Edwards, 2014). A less supported interpretation is that the nesting of the C_3-C_4 clade within otherwise C_4 *Portulaca* lineages represents a C_4 to C_3-C_4 reversion (Ocampo and Columbus, 2012; Ocampo et al., 2013 Ocampo et al., 2013). Resolving whether facultative CAM is the ancestral CAM state in *Portulaca* requires more comprehensive physiological sampling of gas-exchange and titratable acidity across all *Portulaca* species and other closely related lineages.

In an attempt to explain the paucity of species with C_4 and CAM, Sage (2002) suggested that the operation of C_4 and CAM might be incompatible in the same cell but struggled to explain why. The issue is complicated by the fact that C_4 generally requires two cell types that shuttle metabolites between them, whereas CAM is a single-cell phenomenon in which the principal reactions are separated temporally. Sage (2002) concluded that the operation in the same cell of PEPC-catalysed CAM-type CO_2 uptake in the dark and C_4-type CO_2 uptake in the light could result in futile cycles and loss of regulatory control. However, it is conceivable that C_4 and CAM could share only one of the C_4 cell types, the BS cells, as the biochemistry of CAM in the light is similar to reactions that occur in C_4 BS cells during the light. PEPC, which is nocturnally active in CAM but is diurnally active in C_4 mesophyll cells, may be encoded by closely related yet distinct genes in *Portulaca* but for other enzymes of the CAM and C_4 pathways that operate during the light, *Portulaca* appears to use the same genes (Christin et al., 2014).

There is evidence for C_4 and CAM cell-sharing in *Portulaca* (Lara et al., 2003, 2004). In *P. oleracea*, PEPC is located in the BS-encircling mesophyll cells and in centripetally-located large parenchyma cells but Rubisco was only detected in the BS. If Rubisco is genuinely restricted to the BS in *P. oleracea*, the operation of functional CAM in the large parenchyma cells would require CO_2 released during deacidification in the light to diffuse from the parenchyma cells to the BS where it would be refixed by Rubisco (Lara et al., 2004). It is also possible that malate could diffuse into the BS cells where it would be decarboxylated. Under both scenarios, not only would the C_4 and CAM pathways be operating together in the BS, but CAM would not be a single-cell pathway. Further experiments are required to confirm the participation of the BS cells in the CAM cycle.

In contrast to the unexpected observations for *P. oleracea*, in *P. grandiflora* the intracellular locations of PEPC and Rubisco are consistent with the separate operation of the C_4 and CAM pathways in different regions of the leaf, with C_4 in mesophyll cells associated with the BS and CAM in the centripetal parenchyma cells (Guralnick and Jackson, 2001; Guralnick et al., 2002).

The expression of facultative CAM in the C_4-CAM *Portulaca* documented to date presumably reflects environmental selection pressure for life-cycle extension at low water cost. The six C_4-CAM species that have been studied are all small prostrate or decumbent succulents. *Portulaca digyna*, *P. grandiflora* and *P. oleracea* are annuals whereas *P. australis*, *P. cyclophylla* and *P. pilosa* are geophytes that are functionally annual in that the shoots die back to a tuber at the end of a season. Germinating from seed or resprouting from tubers after unpredictable rain events, early rapid plant growth supported by C_4 photosynthesis in the well-watered plants presumably provides the carbon for bulking-up the plant and for forming the large parenchyma cells that can store water and have chloroplasts, thus potentially enabling CAM. As the environment dries, the reduction in day-time CO_2 exchange and the facultative development of CAM reduces water-loss considerably but maintains some net carbon gain, or at least in species in which nocturnal carbon gain is not attained, reduces nocturnal loss of carbon. Presumably the seed- or tuber-forming life of the plant is extended, as has been experimentally demonstrated for *Mesembryanthemum crystallinum* (Aizoaceae; Winter and Ziegler, 1992) and postulated for annual facultative herbs such as *Calandrinia polyandra* (Montiaceae; Winter and Holtum, 2011) and even for woody species such as *Jatropha curcas* (Euphorbiaceae; Winter and Holtum, 2015) which exhibits extremely low levels of CAM. Assessment of the ecological performance of these C_4-CAM species clearly requires season-long study in the field.

The accumulating evidence for facultative CAM in succulent species previously considered solely C_3, C_4 or even constitutively CAM (Winter and Holtum, 2011, 2015, 2017; Holtum et al., 2016)

reflects the increased number of species for which gas-exchange and acidity data are becoming available. The targeting of species for these physiological analyses is better informed by surveys in which phylogenetic and isotopic information are coupled. Key features associated with facultative CAM, plant succulence and habitats in which water is transiently available, are globally common (Ogburn and Edwards, 2010), suggesting that the abundance and ecological significance of facultative CAM may be currently under-appreciated. With respect to *Portulaca*, it will be of interest to determine whether facultative CAM is present in the as yet untested Asian and Umbraticola clades, and particularly in the C_3-C_4 intermediate-containing Crytopetala clade, as this would strengthen the case for CAM preceding C_4.

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