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Evolutionary physiology: the extent of C_4 and CAM photosynthesis in the genera *Anacampseros* and *Grahamia* of the Portulacaceae

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Abstract

The Portulacaceae is one of the few terrestrial plant families known to have both C_4 and Crassulacean acid metabolism (CAM) species. There may be multiple origins of the evolution of CAM within the Portulacaceae but the only clear evidence of C_4 photosynthesis is found in members of the genus *Portulaca.* In the *Portulaca*, CAM succulent tissue is overlaid with the C_4 tissue in a unique fashion where both pathways are operating simultaneously. Earlier reports have shown that the clade containing the genera *Anacampseros* and *Grahamia* may also contain C_4 photosynthetic species similar to the *Portulaca*, which would indicate multiple origins of C_4 photosynthesis within the family. The aim of the present study was to ascertain the true photosynthetic nature of these genera. An initial survey of the carbon isotope composition of the *Anacampseros* ranged from –12.6‰ to –24.0‰, indicating very little CAM activity in some species, with other values close to the C_4 range. *Anacampseros* (=*Grahamia*) *australiana* which had been previously identified as a C_4 species had a carbon isotope composition value of –24.0‰, which is more indicative of a C_3 species with a slight contribution of CAM activity. Other *Anacampseros* species with C_4-like values have been shown to be CAM plants. The initial isotope analysis of the *Grahamia* species gave values in the range of –27.1‰ to –23.6‰, placing the *Grahamia* species well towards the C_3 photosynthetic range. Further physiological studies indicated increased night-time CO_2 uptake with imposition of water stress, associated with a large diurnal acid fluctuation and a marked increased phospho*enol*pyruvate carboxylase activity. This showed that the *Grahamia* species are actually facultative CAM plants despite their C_3-like carbon isotope values. The results indicate that the *Grahamia* and *Anacampseros* species do not utilize the C_4 photosynthetic pathway. This is the first to identify that the *Grahamia* species are facultative CAM plants where CAM can be induced by water stress. This work supports earlier physiological work that indicates that this clade containing *Anacampseros* and *Grahamia* species comprises predominantly facultative CAM plants. This report suggests there may be only one clade which contains C_4 photosynthetic members with CAM-like characteristics.

Key words: *Anacampseros*, carbon isotope composition, C_4 photosynthesis, Crassulacean acid metabolism (CAM), evolution, *Grahamia*, PEP carboxylase, Portulacaceae.

Introduction

In terrestrial plants, two metabolic adaptations that concentrate CO_2 are known: the C_4 and the Crassulacean acid metabolism (CAM) pathway of photosynthesis. The C_4 pathway is found in 19 plant families and ~7000 species (Sage *et al*., 1999; Sage, 2001), while CAM has evolved in >30 families and occurs in at least 20 000 species (Winter and Smith, 1996; Sage and Monson, 1999). Both metabolic pathways evolved independently in well over two dozen distinct lineages (Winter and Smith, 1996; Sage, 2004), and both pathways have evolved in four higher plant families (Aizoaceae,

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Asteraceae, Euphorbiaceae, and Portulacaceae). In one family, the Portulacaceae, CAM and C_4 evolved in close relatives, and can even co-occur in the same species in the genus *Portulaca*. Evolution of CAM and C_4 appears to occur by different intermediate steps, which may be incompatible (Sage, 2002), so the presence of CAM and C_4 in a common evolutionary lineage represents an interesting question of significance to the understanding of complex trait evolution.

The C_4 pathway first evolved 24–35 million years ago in grasses and later in dicots in response to decreasing CO_2 concentration in the atmosphere (Sage, 2004; Christin *et al*., 2008). The steps involved in the evolution of the C_4 pathway include the formation of distinct mesophyll and bundle sheath (Kranz anatomy) compartments, followed by localization of the photorespiratory enzyme glycine decarboxylase to the bundle sheath (Hylton *et al*., 1988; Moore *et al*., 1988; Rawsthorne, 1988; Erhleringer and Monson, 1993; Sage, 2004). This forms a modest CO_2 concentration system where oxygenation products are shuttled to the bundle sheath for decarboxylation during photorespiration, with the result that CO_2 levels are elevated around bundle sheath chloroplasts. Subsequent to this, a C_4 cycle is engaged by up-regulating phospho*enol*pyruvate carboxylase (PEPCase) and the expression of the other enzymes in the C_4 pathway. The main evolutionary driver for C_4 evolution thus appears to be the scavenging of photorespiratory CO_2, and thus C_4 photosynthesis evolves in habitats that consistently experience conditions conducive to high ribulose bisphosphate (RuBP) oxygenase activity by Rubisco. Recent work has shown that the Kranz anatomy is not essential for terrestrial C_4 photosynthesis to occur, but can occur in a single cell with a spatial separation of the C_4 and C_3 pathway within a single chlorenchyma cell (Voznesenskaya *et al*., 2001, 2002; Edwards *et al*., 2004).

CAM photosynthesis occurs in primitive vascular plants, indicating a very ancient origin; however, most of the modern lineages appear to have arisen in the same time frame as the C_4 lineages (during the last 35 million years). CAM is more taxonomically diverse than C_4 photosynthesis, with many CAM lineages scattered across many monocot and dicot families (Winter and Smith, 1996; Sage, 2004). CAM photosynthesis appears to have originated as a means to scavenge respiratory CO_2 under conditions where the carbon balance is restricted in environments where water availability becomes restricted temporarily or seasonally, such as in deserts or rock outcrops, or as in epiphytes. As with C_4, CAM involves major changes to the leaf structure as well as metabolism. Succulence is the obvious structural innovation as it facilitates the capture of night-time CO_2 released by respiration (Gibson, 1982; Ting, 1985; Sage, 2002); however, tight packing of the mesophyll cells is another key structural feature that appears to enhance CAM performance by restricting CO_2 loss during phase III of CAM (Guralnick *et al*., 1986, 2001; Maxwell *et al*., 1997; Nelson and Sage, 2005). The structural adaptations required to effect CAM, and the changes in enzyme regulation required to create a CAM diurnal cycle, are two features which are hypothesized to produce major barriers to the origin of CAM and C_4 photosynthesis in a common evolutionary lineage. Consistent with this idea, only one of the dozens of evolutionary lineages with CAM or C_4 has both pathways present. This is the section of the Portulacaceae that includes *Portulaca*, *Grahamia*, and *Anacampseros* (Hershkovitz and Zimmer, 1997).

To distinguish C_3, C_4, and CAM plants within a given phylogeny, carbon isotope composition values can be used as an initial indicator to estimate the proportional contribution of RuBP carboxylase and PEPCase to the overall carbon gain of the plant (Winter and Holtum, 2002). C_3 plants will show values of around –29‰ when 100% of the CO_2 is captured by RuBP carboxylase, with a less negative upper limit of –23‰ to –20‰ due to chemical, diffusional, and environmental constraints (Winter and Holtum, 2002). C_4 plants which utilize PEPCase as the initial enzyme to capture CO_2 can typically have a range from –10‰ to –16‰ (Sage *et al*., 2007). CAM plants can range from both ends of the spectrum depending on the contribution of the CAM pathway to the overall carbon gain of the plant. Failure to account for this could lead to a misidentification of a CAM plant as being a C_3 or C_4, and hence it is usually necessary to complement carbon isotope analyses with additional physiological investigation to ensure proper identification of CAM in a candidate species (Winter and Holtum, 2002).

The Portulacaceae is a medium-sized family with ~30 genera and 450 species with a wide distribution, but is predominant in the Southern hemisphere (Eggli and Ford-Werntz, 2002). The Portulacaceae has species which are strict C_3 plants (typically those found in Western North America; Guralnick *et al*., 2001); others which are C_3 plants with some attributes of CAM; others which are C_4 plants which also display some CAM characteristics; and other species which are facultative CAM plants (switching between C_3 and CAM photosynthesis). Hence, with respect to photosynthesis, the Portulacaceae is one of the most diverse plant families in the plant kingdom. The only genus known to have C_4 photosynthetic members is the genus *Portulaca*, which forms a distinct clade in the Anacampseroid section (Hershkovitz and Zimmer, 1997). *Portulaca* most probably evolved the C_4 pathway from CAM ancestors (Guralnick and Jackson, 2001). Prior research has shown that *Portulaca grandiflora* has both the C_4 and CAM pathway operating simultaneously in the mesophyll leaf tissue (Guralnick *et al*., 2002); however, the two pathways are segregated into distinct tissue regions. CAM resides in succulent cells positioned

towards the interior of the leaf, while C_4 is localized to a few layers of mesophyll cells towards the exterior of the leaf and near the stomata (Guralnick and Jackson, 2001).

The Anacampseroid clade which contains both C_4 and CAM members is composed of two subclades; one containing the genus *Portulaca* and the other subclade containing the genera *Anacampseros*, *Grahamia*, *Talinopsis*, *Xenia*, and *Tallinaria* which contains many facultative CAM plants (Hershkovitz and Zimmer, 1997; Guralnick and Jackson, 2001). In addition, earlier reports in the literature have indicated that *Grahamia bracteata* and two *Anacampseros* species, *A. kurtzii* and *A. australiana* (which have since been placed in *Grahamia*; Nyffeler, 2007), may also be C_4 photosynthetic species (Kellogg, 1999). This raises the possibility of multiple origins of C_4 photosynthesis overlaying CAM tissue within the Anacampseroid clade. The placement of the genus *Grahamia* and *Anacampseros* as having C_4 members is not clearly resolved, as much of the early evidence of a C_4 pathway may be incorrect due to the possible operation of a CAM pathway (Watson and Dallwitz, 1992). In this study, the objective is to clarify and identify the photosynthetic pathway utilized within *Grahamia* and *Anacampseros* using carbon isotope assessment, gas exchange, titratable acidity analysis, and biochemical assays of PEPCase activity. The results will aid in clarifying the origins of C_4 and CAM photosynthesis within the Portulacaceae, thereby allowing for better understanding of how two seemingly incompatible traits might arise in a common evolutionary lineage.

Materials and methods

Plant material

Cuttings of *G. bracteata*, *G. coahuilensis*, *G. frutescens*, and *A. vulcanensis* were obtained from the Museum of Succulents (Zurich, Switzerland). Plants were then transplanted into pots in a glasshouse. *Anacampseros australiana* (also known in the literature as *Grahamia australiana*) plants were purchased from the Rare Plant Research Institute (Portland, OR, USA). All plants grown were irrigated with 1/2 strength Hoagland's solution and irrigated prior to sampling. The plants were grown under natural light conditions supplemented with artificial light to maintain a light level of 400–600 mmol m^{-2} s^{-1}. The day/night temperature in the glasshouse was 27/17 °C. Water was withheld for 5 d prior to sampling for the water stress conditions. The carbon isotope composition was assessed for all of the living material in the study, and a series of specimens of *Anacampseros* collected from herbarium sheets at the Royal Botanical Garden at Kew, UK (Table 1). The $\delta^{13}C$ of living specimens was collected from leaves, ground to a fine powder, and sent to Washington State University (College of Sciences Stable Isotope Core; http://www.isotopes.wsu.edu). Herbarium specimens were analysed at the Stable Isotope centre of the University of California, Davis (http://stable-isotopes.geology.ucdavis.edu). In addition, leaf material of plants of known photosynthetic pathways also growing under the same conditions was collected and sent along with the *Grahamia* species. Carbon isotope fractionation values were determined on leaf samples taken from plants using a standard procedure relative to PDB (Pee Dee Belemnite) limestone as the carbon isotope standard (Bender *et al*., 1973).

Table 1. *Carbon isotope ratios of* Anacampseros *and* Grahamia *species from herbarium specimens*

The samples were stored at either Kew Gardens, Richmond, UK, or the Missouri Botanical Gardens, St Louis, Missouri, or live material from Western Oregon University[a]). Isotope values are indicated with sample size if >1.

Species	$\delta^{13}C/^{12}C$ isotope value (‰)	Photosynthetic mode
Anacampseros		
arachnoids	–12.6	
albidiflora	–13.8, *n*=2	
albissima	–14.5	Facultative CAM
albissima	–19.0, *n*=2	
australiana	–23.8	
baesckii	–17.3	
comptonii	–18.20	
filamentosa	–13.00	Facultative CAM
filamentosa	–14.6	
lanigera (=*filamentosa*)	–13.10	
namaquensis	–13.5	
papyracea	–12.8	
quinaria	–16.3	
rhodesicae	–14.5, *n*=2	
ruschii	–17.5	
somaliensis	–23.3	
subnuda	–15.2	
telephiastrum	–14.8	Facultative CAM
telephiastrum	–19.6	
thodesica	–23.2	
urstulata	–19.8	
wischkonin	–21.1	
vulcanensis[a]	–23.7, *n*=2	
rufescens[a]	–17.8, *n*=2	Facultative CAM
australiana[a]	–24.0, *n*=2	
Grahamia		
bracteata[a]	–23.7, *n*=2	
frutescens[a]	–26.8, *n*=2	
coahuilensis[a]	–24.1, *n*=2	
Other Portulacaceae		
Montia sibirica[a]	–32.6, *n*=2	C_3
Lewisia cotyledon[a]	–25.2, *n*=2	Weak CAM
Portulaca oleracea[a]	–13.6, *n*=2	C_4

Titratable acidity

Three to six leaves were collected in the morning and evening, and were frozen (–20 °C) until assayed. Leaf samples were weighed, ground in glass-distilled water, and titrated with 0.01 N KOH to a pH 7 end-point.

PEPCase activity

Approximately 0.5 g of leaf tissue for PEPCase activity was collected in triplicate in the afternoon under well-watered and water-stress conditions. The samples were assayed spectrophotometrically by following the oxidation of NADH at 340 nm as previously described (Guralnick and Ting, 1987).

CO_2 uptake

Rates of photosynthesis were measured with an LCpro+ portable infrared CO_2 gas analyser from ADC BioScientific Ltd, Great Amwell, UK, using the conifer chamber. The plants were measured

over 24 h with a 16 h photoperiod. Light was increased/decreased stepwise at the beginning and end of the photoperiod, respectively, with a maximum light intensity of 500 PPFD (photosynthetic photon flux density). The source of light was an LCpro+ conifer chamber red and blue LED lamp attachment. Other conditions were set on ambient mode with diurnal temperatures approximating 25/21 °C, 370 ppm CO_2, and 50% relative humidity. The area of the leaf was determined using ImageJ 1.36b software from the National Institutes of Health, Bethesda, MD, USA.

Results

Carbon isotope composition

The carbon isotope analysis showed *G. bracteata* with a value of –23.7‰, *G. coahuilensis* –24.1‰, *A. australiana* –24.0‰, and *G. frutescens* –26.8‰ (Table 1). The *Anacampseros* species showed a wide range of isotope values from –12.6‰ to –24‰. The carbon isotope composition values were shifted towards the C_4 range, with 18 of the 24 species having values of less than –20‰. For comparison, the CAM cycling species, *Lewisa cotyledon*, had a value of –25.2‰ while the C_3 *Montia sibirica* had a value of –32.6‰. Two known facultative CAM species, *A. rufescens* and *A. vulcanensis*, had rather different carbon isotope values of –17.8‰ and –23.7‰, respectively. The C_4 species, *Portulaca oleracea*, had a carbon isotope composition of –13.5‰. Due to a lack of plant material of *A. australiana* (except where noted) and *G. frutescens*, the following results are for *G. bracteata* and *G. coahuilensis*.

Titratable acidity

Well-watered plants of *G. bracteata*, *A. australiana*, and *G. coahuilensis* showed very high titratable acidity levels of >200 μeq g^{-1} FW and little or no diurnal acid fluctuations (Table 2). During the imposition of water stress, both *G. bracteata* and *G. coahuilensis* showed a large diurnal acid fluctuation. *Grahamia bracteata* had a fluctuation of >200 μeq g^{-1} FW, while *G. coahuilensis* showed a diurnal acid fluctuation of 148 μeq g^{-1} FW. *Anacampseros australiana* showed a smaller diurnal acid fluctuation increase of 58 μeq g^{-1} FW. All three *Grahamia* species under water-stress conditions showed a significant difference from the am to pm acid levels.

PEPCase activity

The well-watered plants of *G. bracteata* and *G. coahuilensis* had PEPCase activity of 28–36 μmol mg^{-1} chl h^{-1} (Table 2) while *A. australiana* had a slightly higher activity of 125 μmol mg^{-1} chl h^{-1}. After imposing 5 d of water stress, *G. bracteata* and *G. coahuilensis* both showed a significant induction of enzyme activity, with a 16–26-fold increase in the PEPCase activity (P <0.05, Table 2). *Anacampseros australiana* did not show a large induction of PEPCase activity when compared with the other plants under water-stress conditions.

CO_2 gas exchange

Gas exchange activity of control plants of *G. bracteata* showed daytime CO_2 uptake between 10 μmol m^{-2} s^{-1} and 15 μmol m^{-2} s^{-1} over the course of the light period (Fig. 1A). At night there was a net loss of CO_2 due to respiration. Imposing water stress triggered a change in the CO_2 uptake pattern, with primarily night-time CO_2 uptake and daytime uptake at the beginning and end of the light period (Fig. 1B). During most of the light period there was little if any net CO_2 gas exchange observed.

Gas exchange activity of control plants of *G. coahuilensis* also showed daytime CO_2 uptake between 10 μmol m^{-2} s^{-1} and 15 mol m^{-2} s^{-1} over the course of the light period (Fig. 2A). After 3 d of water stress, in *G. coahuilensis* there was no observable shift to night-time CO_2 uptake (Fig. 2B). Water stress imposed for 9 d caused a dramatic decrease in daytime CO_2 uptake with very little increase in nocturnal CO_2 uptake (Fig. 2B). Early morning and late afternoon uptake remained quite positive after 9 d of water stress.

Table 2. *Diurnal acid fluctuation and PEPCase activity of* Grahamia bracteata, Grahamia coahuilensis, *and* Anacampseros australiana *under well-watered and 5 d water-stress conditions*

Species	Titratable acidity (μeq g^{-1} FW)			PEPCase activity (μmol NADH mg^{-1} chl h^{-1})
	am	pm	Δ (am–pm)	
A. australiana				
Well-watered	208 (77)	243(84)	–35	125 (14)
Stress	311 (12)	253 (6)	58*	304 (50)
G. bracteata				
Well-watered	226 (47)	214 (8)	12	28.7 (9)
Stress	338 (11)	137 (9)	201*	448 (164)*
G. coahuilensis				
Well-watered	176 (15)	164 (24)	12	36 (20)
Stress	423 (34)	283 (35)	140*	962 (175)*

* A significant difference between am and pm acid levels or well-watered and stressed PEPCase activity (P < 0.05, n=3–5, t-test).

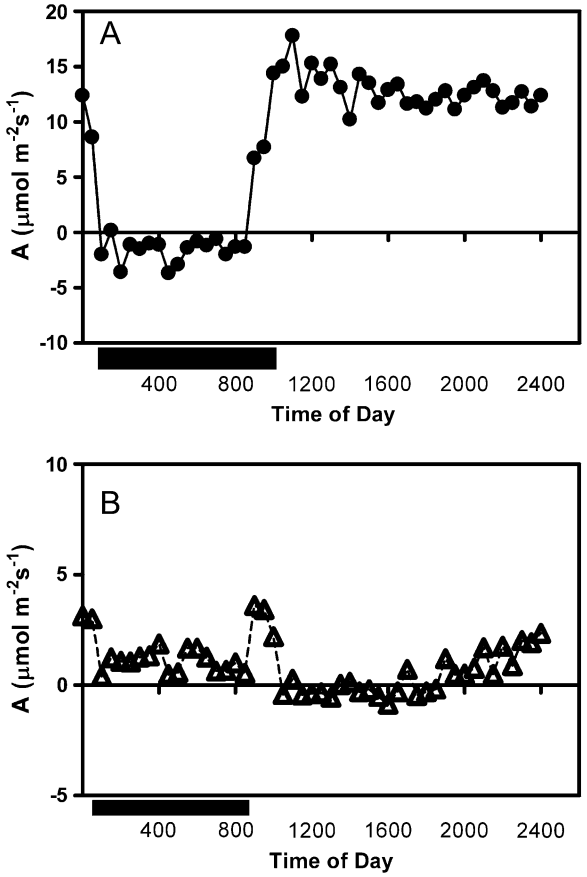

Fig. 1. Diurnal course of CO_2 gas exchange of *G. bracteata* (A) of control and (B) 5 d water-stressed plants. The results summarize the results of a 2 d sampling period. The dark bar indicates the night period.

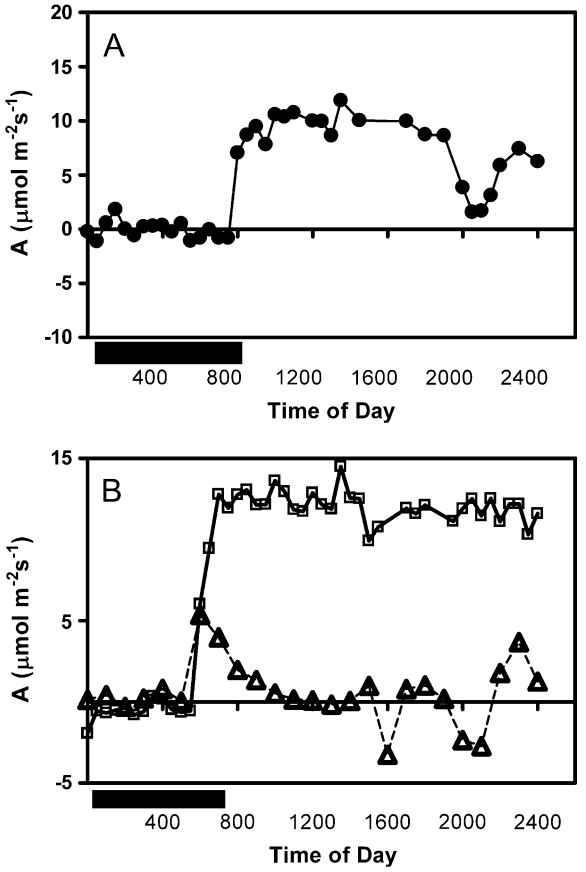

Fig. 2. Diurnal course of CO_2 gas exchange of *G. coahuilensis* (A) control and (B) 3 d (open squares) and 9 d water-stressed (open triangles) plants. The results summarize the results of a 2 d sampling period. The dark bar indicates the night period.

Discussion

A number of interesting questions arise from studies of the Portulacaceae; how did the C_4 and CAM pathway evolve within the Portulacaceae, are there multiple origins, and how may have C_4 and CAM evolved in the same species, particularly within a common leaf tissue. Many of the C_4 species within the Portulacaceae are also succulent, with increased titratable acidity levels compared with typical C_4 species (Kraybill and Martin, 1996; Guralnick *et al*., 2002). Since both pathways require the same suite of enzymes to function, understanding how these pathways evolved in this family and in the same leaf tissue could reveal important insights into how complex metabolic pathways modify common enzyme systems to serve distinct metabolic roles.

Prior work studying the Portulacaceae demonstrates that CAM evolved from their C_3 ancestors to weak CAM first, followed by the formation of facultative CAM in more derived lineages (Guralnick and Jackson, 2001). Facultative CAM metabolism may have multiple origins within the family. The C_4 pathway appears to have evolved after CAM, possibly by the modification of a distinct layer of Kranz tissue that is separate from the CAM tissue in leaves of *Portulaca* species (Guralnick and Jackson, 2001). Additional reports in the literature for the Portulacaceae noted that the genus *Trianthema* has C_4 species, but this genus is now properly placed in the Aizoacaceae (Watson and Dallwitz, 1992). Early reports also indicated that certain *Anacampseros* species have the C_4 pathway, but there is little supporting evidence for this conclusion (Watson and Dallwitz, 1992). A recent phylogenetic analysis demonstrates that *Anacampseros* is closely aligned to *Grahamia* and indicates that certain *Grahamia* species, including the putative C_4 species, should be reclassified as *Grahamia* (Fig. 3; Nyffeler, 2007). Based on this phylogenetic analysis and the close alignment of the C_4 photosynthetic *Anacampseros* with the other members of the genus *Grahamia*, a physiological investigation was initiated to explore the possibility of multiple origins of C_4 photosynthesis and to determine the true photosynthetic characteristics of the genus *Anacampseros* and *Grahamia* in the Portulacaceae.

The initial analysis of the carbon isotope composition of *Anacampseros* ranged from –12.6‰ to –24.0‰,

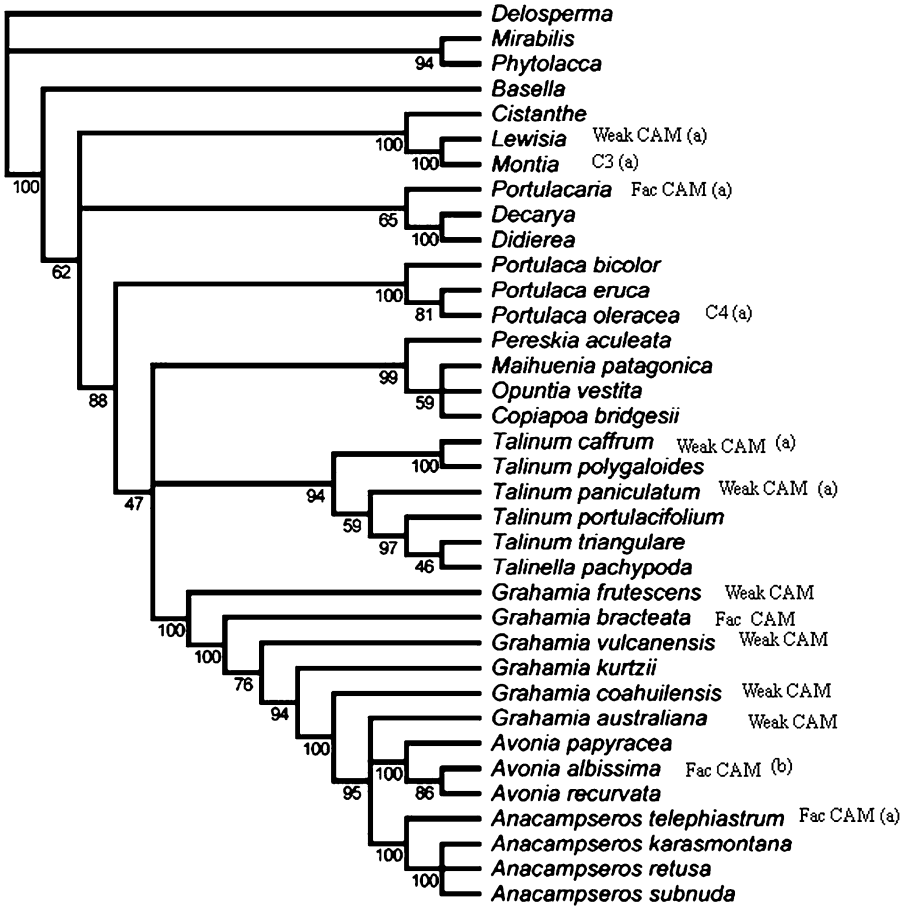

Fig. 3. Tree topology derived from phylogenetic analyses of the combined data (matK, ndhF, and nadI sequences) giving the strict consensus of two most parsimonious trees. Bootstrap values are given below the branches (redrawn from Nyffeler, 2007 with kind permission of The Botanical Society of America). Photosynthetic determinations of C_3, C_4, and CAM are based on this study, (a) Guralnick and Jackson (2001) and (b) Kluge and Ting (1978).

indicating that very little CAM activity is present in some species, while others have values close to the C_4 range, indicating either the presence of a C_4 pathway or strong CAM activity. While the presence of C_4 photosynthesis in species with C_4-like isotope values, such as *A. arachnoids* and *A. papyracea*, cannot definitively be ruled out, there was little additional evidence that these species are C_4. A superficial examination of *Anacampseros* herbarium material showed no sign of Kranz anatomy (R Sage, personal observation). Kranz anatomy in C_4 plants is often readily observed in leaves by examining them when backlit by a strong light, or by viewing the veins on end in cut sections using a strong hand lens or dissecting scope. Also, the presence of $\delta^{13}C$ values that are intermediate between C_3 and C_4 values in many of the *Anacampseros* species is commonly observed in CAM lineages, but not in C_3 and C_4. Notably, *Anacampseros* species which had carbon isotope composition values close to the C_4 range have been previously shown to be facultative CAM species. *Anacampseros filamentosa* showed –13.0‰ here, but previously showed a value of –15.4‰ in the field (Mooney *et al.*, 1977), and exhibits a large diurnal acid fluctuation when grown under glasshouse conditions (Guralnick and Jackson, 2001). *Anacampseros albissima* had a variable carbon isotope value from –14.5‰ to –19.0‰ and also has been shown to have a diurnal acid fluctuation typical of many CAM species (Kluge and Ting, 1978). Many other *Anacampseros* species (*A. tomentosa*, *A. dielsiana*, *A. marlothi*, *A. telephiastrum*, *A. retusa*, *A. minutum*, *A. lanceolata*, *A. crinitia*, and *A. rufescens*) have also shown diurnal acid fluctuations typical of facultative CAM species (Guralnick and Jackson, 2001). Together, these results are consistent with the conclusion that these *Anacampseros* species are mostly likely CAM species with varying contributions of the CAM pathway to the carbon isotope composition of the species.

Within the other *Anacampseros* clade (Hershkovitz and Zimmer, 1997) are found *A. kurtzii* and *A. australiana*, putative C_4 species (Watson and Dallwitx, 1992; Kellogg, 1999), *Grahamia bracteata*, and *Xenia* (=*Anacampseros*=*Grahamia*) *vulcanensis* which have not been identified

regarding which photosynthetic pathway they utilize. *Anacampseros* (=*Grahamia*) *australiana* has a carbon isotope composition value of –24.0‰, which is more indicative of a C_3 species with a slight contribution of CAM activity. The carbon isotope composition values of both *G. bracteata* and *X vulcanensis* were of –23.7‰, which is not indicative of C_4 photosynthesis. The other *Grahamia* species tested ranged from a value of –26.8‰ to –24.1‰. These data indicated that these *Grahamia* species are not C_4 plants, with the carbon isotope composition being too negative and not in the C_4 range. The results also indicated a minimal contribution of the CAM pathway during its growth, with the carbon isotope composition values being closer to those of C_3 plants (Winter and Holtum, 2002). The ranges of carbon isotope composition values obtained were similar to the CAM cycling species, *L. cotyledon* (Table 1; Guralnick *et al*., 2001). The isotope data are consistent with the possibility that the *Grahamia* species are facultative CAM plants because the values are similar to those of the facultative CAM species *Portulacaria afra* when grown under well-watered conditions (Guralnick and Ting, 1987); however, to be certain, direct physiological assessments are required. Therefore, a physiological investigation was undertaken to ascertain the true nature of the photosynthetic pathway utilized by the *Grahamia* species.

The well-watered *Grahamia* plants had very high titratable acidity levels and low levels of PEPCase activity (Guralnick *et al*., 1984; Guralnick and Ting, 1987). After 5 d of water stress, a large diurnal acid fluctuation was noted, as was a sizable increase of PEPCase activity in *A. australiana*, *G. bracteata*, and *G. coahuilensis*. The gas exchange pattern observed for water-stressed *G. bracteata* showed a typical CAM pattern with little or no midday CO_2 uptake. These results were similar to pre-drought and post-drought patterns of photosynthesis found in *P. afra* (Guralnick *et al*., 1984; Guralnick and Ting, 1987).

Grahamia coahuilensis was different from *G. bracteata* in its response to water stress. *Grahamia coahuilensis* did not have an increase in nocturnal CO_2 uptake as observed in *G. bracteata*. After 9 d of water stress, the gas exchange data showed only a slight positive increase of night-time CO_2 uptake; overall, however, night-time gas exchange remained close to zero while early morning and late afternoon CO_2 uptake remained high. The large diurnal acid fluctuation may be explained by substantial night-time refixation of respiratory CO_2 by PEPCase, with a relatively small contribution from fixation of atmospheric CO_2. This pattern has been observed in other Portulacaceae species such as *P. grandiflora* (Guralnick *et al*., 2002), and is commonly referred to as the CAM idling mode. CAM idling is often proposed as an initial phase in the evolution of the CAM pathway. The results support the conclusion that the *Grahamia* species are weak to strong facultative CAM plants where CAM can be induced by water stress. Based on the carbon isotope composition values, it can be concluded that the genus *Grahamia* does not utilize the C_4 photosynthetic pathway.

The data in this study support the hypothesis that C_4 photosynthesis within the Portulacaceae only originated in *Portulaca*. The clade containing the genera *Anacampseros*, *Talinopsis*, and *Tallinaria* which branches off from a common ancestor shared with *Portulaca* provides no evidence for C_4 species, and should be considered as being C_3, weak CAM, or facultative CAM species. These physiological results are similar to what has been observed in the closely aligned *Talinopsis* genera which had a carbon isotope value of –25.3‰ (LJ Guralnick, unpublished data) and a large diurnal acid fluctuation (Guralnick and Jackson, 2001).

There still may be multiple origins of C_4 photosynthesis within the Portulacaceae. Two apparent clades are present in *Portulaca* based on morphological and biochemical features. The *P. grandiflora* type has succulent, tubular leaves and an NADP-ME (malic enzyme) type of C_4 photosynthesis, while the *P. oleracea* type has succulent, flattened leaves with NAD-ME photosynthesis. NADP-ME and NAD-ME are often thought to represent distinct origins because the different biochemical and structural requirements of each subtype appear to preclude one type giving rise to another; however, this hypothesis does require validation (Muhaidat *et al*., 2007). Phylogenetic resolution is needed to confirm separate origins of the C_4 pathway in *Portulaca*, and possibly to indicate the immediate ancestors of the C_4 lineage(s). At present, there is no evidence for C_3–C_4 intermediacy in *Portulaca* or its relatives, nor for CAM–C_4 intermediacy, and it is not clear what the immediate ancestral genus is, since numerous genera such as *Portulacaria* and *Cistanthe* resolve as sister to *Portulaca* in recent phylogenies (Fig. 3). Because *Portulaca* represents the possible independent evolution of distinct biochemical and structural subtypes from a common CAM ancestor, the elucidation of phylogenetic relationships in this clade should be an important contribution to understanding photosynthetic pathway evolution in higher plants.

In conclusion, this report shows that *A. australiana* is not a C_4 species but does demonstrate attributes of CAM photosynthesis. It is also shown that *G. bracteata* is a facultative CAM species that does not utilize the C_4 photosynthetic pathway, and most *Anacampseros* species exhibit carbon isotope ratios that are consistent with this group also being facultative CAM and not C_4. It can therefore be recommended that follow-up work on C_4 evolution in the Portulacaceae should focus on *Portulaca* and its immediate ancestors. By identifying patterns of ancestry, a well-resolved phylogeny could allow researchers to focus their efforts on the branch-point species where important evolutionary developments occurred.

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