RESEARCH PAPER

The occurrence of C₂ photosynthesis in *Euphorbia* subgenus *Chamaesyce* (Euphorbiaceae)

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Abstract

This study investigated whether *Euphorbia* subgenus *Chamaesyce* subsection *Acutae* contains C₃–C₄ intermediate species utilizing C₂ photosynthesis, the process where photorespired CO₂ is concentrated into bundle sheath cells. *Euphorbia* species in subgenus *Chamaesyce* are generally C₄, but three species in subsection *Acutae* (*E. acuta*, *E. angusta*, and *E. johnstonii*) have C₃ isotopic ratios. Phylogenetically, subsection *Acutae* branches between basal C₃ clades within *Euphorbia* and the C₄ clade in subgenus *Chamaesyce*. *Euphorbia angusta* is C₃, as indicated by a photosynthetic CO₂ compensation point (G) of 69 μmol mol⁻¹ at 30 °C, a lack of Kranz anatomy, and the occurrence of glycine decarboxylase in mesophyll tissues. *Euphorbia acuta* utilizes C₂ photosynthesis, as indicated by a G of 33 μmol mol⁻¹ at 30 °C, Kranz-like anatomy with mitochondria restricted to the centripetal (inner) wall of the bundle sheath cells, and localization of glycine decarboxylase to bundle sheath mitochondria. Low activities of PEP carboxylase, NADP malic enzyme, and NAD malic enzyme demonstrated no C₄ cycle activity occurs in *E. acuta* thereby classifying it as a Type I C₃–C₄ intermediate. Kranz-like anatomy in *E. johnstonii* indicates it also utilizes C₂ photosynthesis. Given the phylogenetically intermediate position of *E. acuta* and *E. johnstonii*, these results support the hypothesis that C₂ photosynthesis is an evolutionary intermediate condition between C₃ and C₄ photosynthesis.

Key words: C₃–C₄ intermediate, C₄ photosynthesis, gas exchange, Kranz anatomy, photorespiration, photosynthetic evolution.

Introduction

C₄ photosynthesis has independently evolved over 60 times in divergent families of the angiosperms, making it one of the most convergent of evolutionary phenomena in the biosphere (Conway Morris, 2003; Sage *et al.*, 2011a). The process of C₄ evolution has been widely studied, largely in genera with species that exhibit traits intermediate between C₃ and C₄ plants. The best-studied genera with C₃–C₄ intermediate species are *Flaveria* (Asteraceae) and *Steinchisma* (=*Panicum sensu lato*, Poaceae) (Monson and Rawsthorne, 2000). C₃–C₄ intermediates have also been identified in *Heliotropium* (Boraginaceae), *Neurachne* (Poaceae), *Alternathera* (Amaranthaceae), *Cleome* (Cleomeaeceae), and *Mollugo* (Molluginaceae) (reviewed in Bauwe, 2011, and Sage *et al.*, 2011a). C₃–C₄ intermediate plants with no close relationship to C₄ lineages are also recognized in the Brassicaceae (*Moricandia*, *Diploctenium*) (Sage *et al.*, 1999). Based on extensive research in *Flaveria*, *Moricandia*, and *Panicum sensu lato*, elaborate models of C₄ evolution have been proposed (Monson and Moore, 1989; Monson, 1999; Monson and Rawsthorne, 2000; Sage, 2004). A central feature of these models is the appearance of photorespiratory CO₂ concentration, a process where photorespired CO₂ accumulates in the bundle sheath (BS) compartment, leading to enhanced efficiency of BS Rubisco (von Caemmerer, 1989; Monson and Rawsthorne, 2000). Photorespiratory CO₂ concentration occurs when glycine decarboxylase (GDC) is localized to the BS tissue, such that all of the glycine produced in mesophyll (M) cells by Rubisco oxygenation must move to the BS tissue for conversion to serine and CO₂ by GDC (Monson and Rawsthorne, 2000; Bauwe, 2011). The CO₂ released by GDC is trapped in the BS cell, where it can
accumulate and be refixed by BS Rubisco at much higher efficiency than is possible in M cells (von Caemmerer, 1989). GDC localization typically follows a mutation that prevents GDC expression in the M tissue (Hylton et al., 1988; Voznesenskaya et al., 2007; Bauwe, 2011). Associated with the GDC localization are a series of traits which are thought to enhance the capture and fixation of photorespired CO₂ by BS Rubisco. These include an enlargement of BS cells to form a Kranz-like anatomy, and the positioning of mitochondria and chloroplasts along the centripetal (inner) wall of the BS cell adjacent to the vascular tissue. These traits indicate that photorespiratory CO₂ concentration is a distinct carbon concentrating mechanism that specifically evolved to compensate for high rates of photorespiration. In recognition of this, Vogan et al. (2007) have proposed the term ‘C₂ photosynthesis’ to refer to photorespiratory CO₂ concentration. The use of ‘C₂ photosynthesis’ is logically and historically consistent with the prior use of the terms C₂ metabolism and C₂ photosynthesis. Tolbert (1997) used ‘C₂ metabolism’ to refer to the photorespiratory metabolic cycle, and hence it makes sense to label a carbon concentration mechanism based on photorespiratory metabolism as C₂ photosynthesis. Logical consistency is apparent in that ‘C₂’ refers to the number of carbons in the Rubisco product that shuttles CO₂ into the BS, just as ‘C₄’ refers to the number of carbons in the carboxylation product that shuttles CO₂ into the BS cells of C₄ plants.

While C₂ photosynthesis is widely thought to be the key intermediate stage in the evolution of C₄ photosynthesis (Monson and Rawsthorne, 2000; Sage, 2004), there is limited phylogenetic support for this hypothesis. Only in the genera Flaveria, Cleome, and Mollugo have detailed, molecular phylogenies demonstrated that C₂ photosynthesis occurs in species that branch at phylogenetic nodes between C₃ and C₄ clades (McKown et al., 2005; Feodorova et al., 2010; Christin et al., 2011). In the other clades where C₃–C₄ intermediate taxa occur, phylogenies with enough detail to resolve the relationships between C₃, C₄, and the C₃–C₄ species have yet to be published. Hence, to facilitate the study of C₄ evolution, it is useful to identify additional clades where C₂ occurs in species branching between C₃ and C₄ nodes within a phylogeny. In such studies, C₂ photosynthesis is indicated by reduced CO₂ compensation points of photosynthesis, non-linearity in the response of the CO₂ compensation point to O₂, localization of GDC to the BS tissue, high refixation of photorespired CO₂, and a line-up of mitochondria along the centripetal end of the BS cells adjacent to the vascular tissue (Bauwe et al., 1987; von Caemmerer, 1989; Monson, 1999; Monson and Rawsthorne, 2000).

One promising group to look for evolutionary intermediates is the subsection Acutae in the genus Euphorbia subgenus Chamaesyce (Euphorbiaceae). Based on morphological characters, Webster et al. (1975) hypothesized that the species in subsection Acutae make up the ancestral clade of subgenus Chamaesyce, which is a large group of C₄ species that derive from C₃ species in Euphorbia (Steinmann and Porter, 2002; Tropicos, 2010). Chamaesyce has been recognized as an independent genus (Webster, 1994), but this makes Euphorbia paraphyletic and thus its status as a distinct genus is not currently favoured (Tropicos, 2010). Webster et al. (1975) showed that two species in subsection Acutae—E. angusta and E. acuta—have C₃ isotopic values, while a third, E. lata, has a C₄ isotopic value. All other species in Euphorbia subgenus Chamaesyce are believed to be C₄ plants based on isotopic values, Kranz anatomy, gas exchange, or phylogenetic affinity to known C₄ species (Webster et al., 1975; Koutnik, 1987). Subsequently, Euphorbia johnstonii has been identified as a fourth species in subsection Acutae, and is thought, on morphological grounds, to be very close to E. acuta (Mayfield, 1991). Recent molecular phylogenies support the placement of subsection Acutae between C₃ species of Euphorbia and C₄ species of Euphorbia subgenus Chamaesyce (Steinmann and Porter, 2002; Yang and Berry, 2007), raising the possibility that the species in subsection Acutae may express evolutionarily intermediate traits between the C₃ and C₄ conditions.

All four species of Euphorbia subgenus Chamaesyce subsection Acutae grow in south-western Texas, USA and adjacent Mexico, and are abundant enough for easy collection and study in their field habit (Correll and Johnston, 1979). The purpose of this study was to collect live specimens of these species, describe their field ecology, and analyse their physiology and cell biology to determine if any of the species are C₃–C₄ intermediates conducting C₂ photosynthesis. The Caribbean species Euphorbia mesembryanthemifolia was included in the study as a representative of subgenus Chamaesyce (Herndon, 1996; Tropicos, 2010), which we assumed would have a well-developed C₄ pathway. The growth form of E. mesembryanthemifolia resembles that of E. acuta.

Materials and methods

Field work

Seeds of E. acuta Engelm. (synonymous with the Mexican species E. georgei Oudejans; Tropicos, 2010) and E. lata Engelm. were collected on 17 August 2007 from plants growing on a limestone outcrop along Highway 18, 8 km north of the Interstate 10 interchange at Fort Stockton, Texas. Seeds of E. angusta Engelm. were gathered on 7 April 2007 from plants in a limestone road cut along Highway 12, about 24 km north of Uvalde in Uvalde County, Texas. Euphorbia mesembryanthemifolia Jacq. seeds were collected near Progreso, Yucatan, Mexico on 26 June 2008. Sarah Taylor (University of Texas) collected E. johnstonii Mayfield plants on 11 October 2007 near Nuevo Leon, Mexico. These plants were couriered to Toronto. They lacked seeds and did not survive transplanting. It was possible to sample viable leaves of E. johnstonii for qualitative observations using light and transmission electron microscopy (TEM) as well as immunolocalization but living material could not be preserved for physiological/ biochemical analyses. Leaves of E. johnstonii were not used for quantitative analyses of leaf anatomy because they were not grown in the same conditions as the other four species.

To evaluate diurnal leaf temperatures of E. acuta in its natural habitat, Veriteq Spectrum data loggers (SP1700, Veriteq Corporation, Vancouver, British Columbia, Canada) were set up to log fine wire thermocouples in July 2010. Thirty-six gauge thermocouple junctions were placed against the bottom of attached leaves from
plants growing at the collection site 8 km north of Fort Stockton, Texas. Carbon isotope ratios were assayed on dried leaves from field-collected plants by the University of California, Davis stable isotope facility (http://stablesisotopefacility.ucdavis.edu).

Plant growth, whole-leaf gas exchange, and enzyme assays

Plants were grown from seed in a rooftop greenhouse located at the Earth Sciences Centre of the University of Toronto. Plants were grown in 4.0 l or 8.0 l pots in equal parts sand, Pro-Mix potting soil (Premier Horticulture, Inc., Quakertown, PA, USA), and sterilized topsoil. Plants were watered as necessary to avoid drought and fertilized weekly with equal parts of two commercial fertilizers (1:4 g l⁻¹, each of 24-8-16 Miracle-Grow All-Purpose plant food and 28-10-10 Miracle Grow Evergreen Tree and Shrub Food; Scotts-Canada, Mississauga, Ontario), with bimonthly supplements of approximately 300 ml of a 1 mM magnesium sulphate and 5 mM calcium nitrate solution. The approximate day/night temperature of the greenhouse was 30/25 °C and peak midday irradiance exceeded 1600 μmol photons m⁻² s⁻¹ on sunny days. Plants were periodically trimmed to a canopy area approximately equal to the pot width.

All measurements were conducted on recently expanded healthy leaves. Photosynthetic gas exchange was measured using a LI-6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA) during August and September 2008. Measurements were conducted at a leaf temperature of 30 °C, a photon flux density of 1300 μmol m⁻² s⁻¹, and leaf-to-air vapour pressure deficit (VPD) of 2.0 kPa. Because leaves were small (about 0.5 cm²), multiple leaves on a branch were placed in the chamber. CO₂ compensation points were calculated as the x-intercept of a linear regression through the five lowest intercellular CO₂ (Cᵢ) values on a graph of net CO₂ assimilation rate (A) versus Cᵢ. The initial point in each A/Cᵢ curve was determined at 370 μmol CO₂ mol⁻¹ air after which ambient CO₂ was lowered in four to five measurement steps to 30 μmol mol⁻¹, then raised to ambient again and allowed to stabilize at the original values of A and stomatal conductance. The CO₂ level was then raised to 600 and 800 μmol mol⁻¹ to assess the maximum rate of photosynthesis.

The activities of PEP carboxylase (PEPC), NADP-malic enzyme, NAD-malic enzyme, and PEP carboxykinase (PEPCK) were assayed using a coupled enzyme assay on freshly extracted leaf material. PEP carboxylase was assayed spectrophotometrically at 340 nm by coupling the production of OAA to NADH oxidation via malate dehydrogenase (modified from Ashton et al., 1990). The reaction mixture contained 50 mm Bicine (pH 8.0), 5 mM MgCl₂, 2 mM DTT, 2 mM NaHCO₃, 1 mm glucose 6-phosphate, 5 mM PEP, 0.25 mM NADH, and enzyme extract. The reaction was initiated by the addition of PEP. NAD-ME and NADP-ME were assayed by the formation of NADH and NADPH, respectively. The reaction mixture for NADP-ME contained 50 mM TRIS-HCl (pH 8.2), 1 mM EDTA, 20 mM MgCl₂, 2 mM DTT, 0.5 mM NADP⁺, 5 mM Na-malate, and enzyme extract (modified from Kanai and Edwards, 1973). The reaction was initiated by the addition of malate. The reaction mixture for the assay of NAD-ME contained 25 mM HEPES (pH 7.2), 5 mM DTT, 0.2 mM EDTA, 2.5 mM NAD⁺, 5 mM Na-malate, 8 mM (NH₄)₂SO₄, 75 μM coenzyme A, 2 mM MnCl₂, 25 μM NADH, and enzyme extract (modified from Hatch and Kagawa, 1974; Hatch et al., 1982). The reaction was initiated by the addition of MnCl₂. Activity of PEPC was assayed in the carboxylating direction by measuring the nucleotide-dependent production of OAA, coupled to NADH oxidation via malate dehydrogenase. The reaction mixture contained 80 mM MES (pH 6.7), 0.25 mM NADH, 5 mM DTT, 5 mM MnCl₂, 2 mM PEP, 2 mM ADP, 10 mM KHCO₃, and 5 units ml⁻¹ malate dehydrogenase (modified from Walker et al., 1995). Interfering PEPC activity was measured with PEP only, and the PEPC reaction was initiated with ADP. Activity of PEPCK was taken as the change in absorbance after the addition of ADP (Edwards et al., 1971).

All chemicals for enzyme assays were obtained from Sigma-Aldrich, St Louis, Missouri USA.

Leaf anatomy and ultrastructure

Leaf tissue was sampled for anatomical observations from the most recent, fully expanded, mature leaves. The internal anatomy of leaves was assessed on leaf sections harvested from the middle of the leaf (1 leaf plant⁻¹; three plants) and prepared for light and TEM as described by Sage and Williams (1995). Images of leaf cross-sections taken with a light microscope were used to measure leaf thickness, % M, % BS, and the ratio of M-to-BS tissue. Leaf thickness, % M, and % BS were determined from four separate sections per leaf. Leaf thickness was measured using Image J software (National Institutes of Health, Bethesda, MD, USA). The percentage of the leaf cross-section covered by M or BS tissue was determined by laying a grid of 100 random points over cross-sections of images and calculating the proportion of points falling on M or BS cells (Parkhurst, 1982; McKown and Dengler, 2007). TEM images were prepared from three BS and three M cells from each of three leaf sections from three separate plants. TEM images were used to quantify the area and number of chloroplasts and mitochondria per M and BS cell area using Image J software. Intervenial distances were determined from five images of leaves taken from three plants and cleared as described by McKown and Dengler (2007). Intervenial distance was determined by laying a grid of randomized points on an image and measuring the length of the shortest line that connected two adjacent veins through one of the randomized points. This was repeated ten times for each image. The 10 values determined for an image were averaged to produce an image value that was used in the statistical analysis of the data.

In situ immunolocalization

The preparation of tissue samples for immunolocalization of Rubisco, PEPC, and the P-subunit of the GDC complex follows Marshall et al. (2007). Polyclonal antisera used were anti-Nicotiana tabacum Rubisco (provided by NG Dengler, University of Toronto, Canada), anti-maize PEPC (obtained from J Berry, State University of New York, USA), and anti-pea GDC (P-subunit; received from S Rawsthorne, John Innes Centre, Norwich, UK). The cross-reactivity of each antibody was verified by running control labelling experiments on cross-sections of paraffin-embedded leaf tissues of Flexaria princeae (C₃), F. ramosissima (C₃-C₄), and F. trineria (C₄, NADP-ME) in which the enzymes were known to be expressed and accumulated in a tissue-specific manner (Edwards et al., 2001).

Data analysis

Results were analysed with SigmaPlot version 11.0 using a one-way analysis of variance and Tukey’s pairwise multiple comparison. Sample sizes varied between two and five for physiological and enzyme assays, and between 9 and 15 for anatomical and ultrastructural analysis. In the anatomical and ultrastructural study, an individual microscope section was considered as the sampling unit.

Results

Ecological habit

The four species listed for Euphorbia subgenus Chamaesyce subsection Acuta by Webster et al. (1975) and Mayfield
(1991), are *E. acuta*, *E. angusta*, *E. johnstonii*, and *E. lata*. *Euphorbia acuta* occurs on dry limestone uplands of the Edwards plateau and semi-arid scrublands of Brewster, Crane and Pecos Counties in Western Texas, USA (Fig. 1; Correll and Johnston, 1979). *Euphorbia angusta* grows on dry limestone outcrops in the Edwards plateau of west-central Texas, while *E. lata* occurs on dry calcareous soils and sandy plains of the western end of the Edwards plateau and adjacent trans-Pecos region of Texas (Fig. 1; Correll and Johnston, 1979). *Euphorbia johnstonii* is restricted to calcareous soils and caliche outcrops in northern regions of the Mexican states of Nuevo Leon and Tamaulipas (Mayfield, 1991). *Euphorbia mesembryanthemifolia* is widespread in warm, seasonally dry, coastal regions of the Caribbean basin and Gulf of Mexico, where it is found on sandy or rocky areas affected by salt spray (Herndon, 1996). All species are presumed to be photosynthetically active in the summer, based on the presence of a robust, fleshy leaf canopy observed for each species during the summer collection periods. Flowering for each species is greatest in mid-to-late summer, when monsoon rains provide periodic precipitation.

Midday leaf temperatures of *E. acuta* peaked near 39 °C between 12–14 July 2010 (Fig. 2). Leaf and air temperatures were usually similar, although on 13 July the mid-morning leaf temperatures were 3–4 °C above air temperature. Maximum summer temperatures from 1971–2010 in Fort Stockton, Texas, averaged 31.5 °C for June, 33.5 °C for July, and 32.2 °C for August (PRISM, 2010). Peak temperatures for July exceeded 36 °C in 15 of the 40 years in the 1971–2010 record (PRISM, 2010). Comparison of the results in Fig. 2 with the long-term climate records indicate that the leaf and air temperatures observed for *E. acuta* represent typical thermal profiles on average (13 July) to warmer than average (14 July) summer days. From this, it is concluded that leaf temperatures for *E. acuta* are above 30 °C for much of the day during summer, and will frequently peak above 35 °C. Similar temperature profiles probably exist for the other members of *Euphorbia* subsection *Acutae* in western Texas, given similarity in leaf size and shapes (Fig. 1).

**Gas exchange, enzyme activities, and carbon isotopes**

Gas exchange characteristics were determined for *E. angusta*, *E. acuta*, and *E. lata*. All three species have similar maximum rates of net CO₂ assimilation in the low-to-mid 30 μmol m⁻² s⁻¹ range (Table 1). *Euphorbia lata* had a typical C₄ type response of net photosynthesis rate to intercellular CO₂ concentration (the A/C_i response), with a steep initial slope, a sharp transition to CO₂ saturation, and a CO₂ compensation point of photosynthesis (Γ) of 6 μmol mol⁻¹ (Fig. 3; Table 1). By comparison, *E. angusta* and *E. acuta* had similarly shaped A/C_i responses, similar initial slopes, and a gradual transition to CO₂ saturation; however, while *E. angusta* exhibited a C₃-like Γ of 69 μmol mol⁻¹ at 30 °C, the Γ of *E. acuta* was half this value, being 33 μmol mol⁻¹ (Fig. 3; Table 1). Γ values of 20–35 μmol mol⁻¹ at 30 °C are characteristic of C₂ species (Monson and Rawsthorne, 2000; Vogan et al., 2007). Water use efficiency...
were less than 8% of the values for these enzymes in *E. lata* and *E. mesembryanthemifolia*. Activities of NAD-malic enzyme and PEPCK were similar and very low in all four species.

Carbon isotope ratios were C₄-like in *E. angusta* and *E. acuta* averaging between ~26.0‰ and ~28.5‰ (Table 1). In *E. johnstonii*, the δ¹³C value was ~27.9±0.5 (mean ±SE, *n*=4). *Euphorbia lata* had a typical C₄ δ¹³C value of ~15.0‰ (Table 1).

**Leaf anatomy and ultrastructure**

Leaves of *E. angusta*, *E. acuta*, and *E. johnstonii* are unifacial with single layers of adaxial and abaxial palisade parenchyma (Figs 4A, 5A). Leaves of *E. lata* and *E. mesembryanthemifolia* are bifacial with a single layer of adaxial palisade parenchyma and either two layers of abaxial spongy mesophyll (*E. lata*; Fig. 4C) or non-chlorenchymatous water-storage tissue and internal chlorenchymatous palisade parenchyma (*E. mesembryanthemifolia*; Fig. 4D). The distance between veins is lowest in *E. angusta*, intermediate in *E. acuta* and greatest in *E. lata* and *E. mesembryanthemifolia*, being about 50% greater in the latter two species than in *E. angusta* (Table 1). *Euphorbia angusta* has about 40% greater relative area of M tissue in cross-section than *E. acuta* and *E. lata*, while relative BS area of *E. angusta* is 40–50% lower than the BS area of *E. acuta* and *E. lata* (Table 1). The % BS area is similar between *E. angusta* and *E. mesembryanthemifolia*,
due to the presence of water storage tissue in \textit{E. mesembryanthemifolia} which reduced its relative BS area. Individual M cell size of \textit{E. angusta} is about twice that of \textit{E. acuta} and \textit{E. lata}, while \textit{E. angusta} has the smallest BS size of all four species (Table 1). These differences lead to a greater M-to-BS tissue ratio in \textit{E. angusta}. Notably, \textit{E. acuta} has a similar M-to-BS tissue ratio as \textit{E. lata} and \textit{E. mesembryanthemifolia}.

The BS cells of \textit{E. angusta} contain numerous peripherally situated chloroplasts, mitochondria, and peroxisomes, while all BS organelles of \textit{E. lata} and \textit{E. mesembryanthemifolia} are positioned along the centripetal cell wall adjacent to the vascular tissue (Figs 4, 6, 7). \textit{Euphorbia acuta} also has a peripheral distribution of chloroplasts in both M and BS cells (Figs 4B, 6B), but the BS mitochondria and peroxisomes are centripetally situated along the inner BS cell wall (Figs 6B, 7B). Chloroplasts and mitochondria in BS cells of \textit{E. acuta} are more numerous than in the BS cells of the other three species (Table 2). Mesophyll chloroplasts are fewer in \textit{E. lata} and \textit{E. mesembryanthemifolia} than the other two species and \textit{E. lata} has the fewest BS mitochondria of the four species (Table 2). The organelle distribution within M and BS cells in \textit{E. johnstonii} mirrors that of \textit{E. acuta} at both the light level (Fig. 5) and the TEM level (data not shown).

The size of the M chloroplasts does not differ greatly between the four species, while the BS chloroplasts of \textit{E. lata} and \textit{E. mesembryanthemifolia} are two to three times larger than those of \textit{E. angusta} and \textit{E. acuta} (Table 2). \textit{E. angusta} and \textit{E. acuta} have larger chloroplasts in the BS than the BS tissue, while the reverse was true for \textit{E. lata}. When the differences in chloroplast number are combined with the size of individual chloroplasts, the % of the cell area covered by chloroplasts is greater in the M than BS cells of \textit{E. angusta} and \textit{E. acuta} (Table 2). The percentage of cell area covered by chloroplasts tended to be greater in M cells of \textit{E. angusta} and \textit{E. acuta} than \textit{E. lata} and \textit{E. mesembryanthemifolia}, while in the BS cells, it was greater in \textit{E. lata} and \textit{E. mesembryanthemifolia} (Table 2). Bundle sheath chloroplasts in \textit{E. lata} and \textit{E. mesembryanthemifolia} had low granal stacking while granal stacking in the chloroplasts of \textit{E. angusta} and \textit{E. acuta} BS and M cells was common (Fig. 7).

Individual mitochondria in the BS cells of \textit{E. acuta} are two to three times larger than BS mitochondria of \textit{E. angusta}, \textit{E. lata}, and \textit{E. mesembryanthemifolia} (Figs 6, 7; Table 2). Mesophyll mitochondria are smallest in \textit{E. lata} and \textit{E. mesembryanthemifolia} (Table 2). Mitochondria of all species occupy a similar percentage of the M cell area, but the combination of more and larger mitochondria in the BS

**Fig. 3.** The response of net CO$_2$ assimilation rate to intercellular CO$_2$ concentration in \textit{E. angusta}, \textit{E. acuta}, and \textit{E. lata} at 30 °C. Curves shown are representative of two (\textit{E. lata}) or three individual curves (\textit{E. acuta} and \textit{E. angusta}).

**Fig. 4.** Light micrographs of cross-sections through leaves of (A) \textit{E. angusta} (Euan); (B) \textit{E. acuta} (Euac); (C) \textit{E. lata} (Eula); (D) \textit{E. mesembryanthemifolia} (Eume). Arrowheads denote centripetal localization of chloroplasts in Kranz bundle sheath. BS, Bundle sheath; M, palisade mesophyll; S, spongy mesophyll; W, water storage cell. Bars=20 µm.
Table 2. Organelle size and number for four *Euphorbia* subgenus *Chamaesyce* species  
Means ± SD. Letters indicate the statistical differences between species at *P* <0.05, *n*=9.

<table>
<thead>
<tr>
<th>Units</th>
<th>Species</th>
<th><em>E. angusta</em></th>
<th><em>E. acuta</em></th>
<th><em>E. lata</em></th>
<th><em>E. mesembryanthemifolia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per mesophyll cell</td>
<td>–</td>
<td>16±4.7 b</td>
<td>12.8±3.6 c</td>
<td>5.9±2.3 a</td>
<td>6.8±2.4 a</td>
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<td>Per bundle sheath cell</td>
<td>–</td>
<td>7.6±2.1 a</td>
<td>20.7±2.9 c</td>
<td>9.7±3.2 a</td>
<td>13.9±2.8 b</td>
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<td>Per mesophyll cell area</td>
<td>µm²×10⁻³</td>
<td>44.2±16.4 b</td>
<td>67.0±12.7 c</td>
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<td>25.3±2.6 a</td>
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<tr>
<td>Per bundle sheath cell area</td>
<td>µm²×10⁻³</td>
<td>32.5±8.3 a</td>
<td>54.5±15.5 b</td>
<td>23.1±10.1 a</td>
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<td>–</td>
<td>1.5±0.7 ab</td>
<td>1.4±0.5 ab</td>
<td>1.6±0.6 b</td>
<td>0.8±0.4 a</td>
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<tr>
<td>Chloroplast size</td>
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<td></td>
<td></td>
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<tr>
<td>Mesophyll µm²</td>
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<td>7.3±1.7 ab</td>
<td>5.5±0.6 a</td>
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<td>Bundle sheath µm²</td>
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<td>10.0±1.7 b</td>
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<td>mesophyll/bundle sheath size ratio</td>
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<td>1.5±0.5 b</td>
<td>1.6±0.2 b</td>
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<tr>
<td>Chloroplast area per mesophyll area %</td>
<td></td>
<td>31.2±8.4 b</td>
<td>37.4±5.8 b</td>
<td>21.6±10.1 ab</td>
<td>21.0±2.0 a</td>
</tr>
<tr>
<td>Chloroplast area per bundle sheath area %</td>
<td></td>
<td>17.1±6.8 a</td>
<td>18.7±4.9 a</td>
<td>29.3±6.5 b</td>
<td>34.7±10.9 b</td>
</tr>
<tr>
<td>Total chloroplast area ratio, M to BS</td>
<td>–</td>
<td>2.1±0.9 b</td>
<td>2.1±0.6 b</td>
<td>0.8±0.4 a</td>
<td>0.6±0.2 b</td>
</tr>
<tr>
<td>Mitochondria number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per mesophyll cell</td>
<td>–</td>
<td>10.1±8.5 b</td>
<td>4.6±3.6 ab</td>
<td>3.4±2.4 a</td>
<td>8.3±3.1 ab</td>
</tr>
<tr>
<td>Per bundle sheath cell</td>
<td>–</td>
<td>7.0±3.2 ab</td>
<td>22.4±7.5 c</td>
<td>3.8±2.2 a</td>
<td>13.3±5.3 b</td>
</tr>
<tr>
<td>Per mesophyll cell area</td>
<td>µm²×10⁻³</td>
<td>24.6±14.4 a</td>
<td>24.3±18.9 a</td>
<td>21.8±17.4 a</td>
<td>31.2±15.0 a</td>
</tr>
<tr>
<td>Per bundle sheath cell area</td>
<td>µm²×2×10⁻³</td>
<td>31.0±14.6 b</td>
<td>60.3±26.8 c</td>
<td>9.0±6.2 a</td>
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</tr>
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<td>Mesophyll to bundle sheath ratio</td>
<td>–</td>
<td>1.2±1.4 a</td>
<td>0.4±0.1 a</td>
<td>1.9±1.3 a</td>
<td>1.2±1.1 a</td>
</tr>
<tr>
<td>Mitochondria size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesophyll µm²</td>
<td></td>
<td>0.5±0.1 b</td>
<td>0.4±0.3 ab</td>
<td>0.2±0.2 a</td>
<td>0.2±0.1 a</td>
</tr>
<tr>
<td>Bundle sheath µm²</td>
<td></td>
<td>0.4±0.1 a</td>
<td>0.7±0.1 b</td>
<td>0.3±0.2 a</td>
<td>0.2±0.1 a</td>
</tr>
<tr>
<td>mesophyll/bundle sheath size ratio</td>
<td>–</td>
<td>1.5±0.7 b</td>
<td>0.6±0.3 a</td>
<td>0.9±0.6 ab</td>
<td>1.0±0.5 ab</td>
</tr>
<tr>
<td>Mitochondria area per mesophyll area %</td>
<td></td>
<td>1.2±0.7 a</td>
<td>1.0±1.1 a</td>
<td>0.7±0.6 a</td>
<td>0.6±0.3 a</td>
</tr>
<tr>
<td>Mitochondria area per bundle sheath cell %</td>
<td></td>
<td>1.0±0.5 a</td>
<td>4.4±2.0 b</td>
<td>0.3±0.2 a</td>
<td>0.7±0.3 a</td>
</tr>
<tr>
<td>Total mitochondria area ratio, M to BS</td>
<td>–</td>
<td>1.5±1.6 ab</td>
<td>0.2±0.2 a</td>
<td>2.4±1.9 b</td>
<td>1.0±0.5 ab</td>
</tr>
</tbody>
</table>

**Fig. 5.** Light micrographs of cross-sections through leaves of *E. johnstonii* (Eujo). Arrowheads mark centripetal location of chloroplasts in bundle sheath cells. B, Bundle sheath; M, mesophyll. Bars=20 µm.

cells of *E. acuta* result in a percentage of the BS cell area occupied by mitochondria that is 4–15 times greater than that of *E. angusta*, *E. lata*, and *E. mesembryanthemifolia* (Table 2).

The presence of brown stain demonstrated the occurrence of Rubisco, PEPC, and GDC in the immunolocalizations of the species studied here. Immunolocalization images showed Rubisco to be scattered around the periphery of the M tissue in *E. angusta* and *E. acuta*, and absent from the M tissue of *E. lata* and *E. mesembryanthemifolia* (Fig. 8). The stain for Rubsico is peripherally located in the BS of *E. angusta*, while in *E. acuta* it forms a pronounced layer along the inner walls of the BS cells. In *E. lata* and *E. mesembryanthemifolia*, Rubisco stain is present in the inner half of the BS cells. PEPC stain is very faint in all leaf cells of *E. angusta* (not shown) and *E. acuta* (Fig. 9A), and is pronounced in the M cells of *E. lata* (Fig. 9B) and *E. mesembryanthemifolia* (Fig. 9C).

The GDC stain is pronounced in M cells of *E. angusta*, where it forms dark spots that correspond to individual mitochondria (see arrows in Fig. 10A). By contrast, the GDC stain is restricted to the inner region of the BS cells in
E. lata and E. mesembryanthemifolia (Fig. 10C, D). Diffuse staining was apparent in the mesophyll of E. lata, but it is non-specific background staining and does not correspond to individual mitochondria. In E. acuta, the GDC stain is evident as a dark band along the inner BS wall. In this region, individual mitochondria stand out as dark spots of stained GDC (Fig. 10B). Immunolocalization of GDC for E. johnstonii was also attempted and a dark-staining area was identified only in the inner portion of the BS cells (data not shown).

Discussion

In this study, evidence is presented that Euphorbia subgenus Chamaesyce contains species that concentrate photorespired CO₂ into the BS cells using the C₂ metabolic cycle. Euphorbia acuta is a C₂ species based on its Γ value of 33.2 μmol mol⁻¹, Kranz-like arrangement of enlarged isodiametric BS cells, higher density of chloroplasts along the centripetal BS wall, and localization of GDC to BS cells. Bundle sheath cells in E. acuta also have increased numbers of mitochondria and larger mitochondria than the C₃ species E. angusta, and mitochondria are located centripetally within BS cells. Increased size and number of BS mitochondria are common in C₂ species, presumably reflecting a need for greater GDC capacity to process all of the photorespiratory metabolites produced by the leaf (Monson and Rawsthorne, 2000). Euphorbia johnstonii also exhibits a Kranz-like anatomy with GDC-positive staining and enhanced aggregation of chloroplasts and other organelles along the inner wall of the enlarged BS cells. Therefore, E. johnstonii is probably a C₂ species as well, although definitive confirmation of this will require measurements of photosynthetic characteristics. In contrast to E. acuta, E. angusta is clearly a C₃ species, based on its Γ value of 69 μmol mol⁻¹, a C₃ carbon isotope ratio, lack of well-defined and enlarged BS cells, low activity of C₄-cycle enzymes, and no evidence of chloroplast, mitochondria, and GDC localization to the inner wall of the BS cells. Euphorbia lata is a NADP-ME type of C₄ species based on a low Γ value, a carbon isotope ratio of ~15.0‰, high activities of PEPC and NADP-malic enzyme, and the obvious presence of Kranz anatomy. Euphorbia mesembryanthemifolia is also a NADP-ME C₄ species, as shown by the high PEPC and NADP-ME activities and Kranz anatomy. These results for E. lata and E. mesembryanthemifolia, in combination with prior work by Gutierrez et al. (1974), indicate all C₄ species
in *Euphorbia* subgenus *Chamaesyce* are NADP-ME species. Both *E. lata* and *E. mesembryanthemifolia* have low granal stacking in the BS chloroplasts relative to M chloroplasts. Chloroplast dimorphism with respect to thylakoid development has been reported for other C₄ *Euphorbia* species (Kim et al., 2000) and is typical of NADP-ME subtypes (Edwards and Walker, 1983; Dengler and Nelson, 1999). Mitochondrial number and size is reduced in the two C₄ species relative to *E. acuta*, presumably a result of the decreased metabolic role of the mitochondria in leaves of NADP-ME type C₄ species. Photospiration is low in NADP-ME subtypes due to high BS CO₂ and relatively low BS O₂ levels (Kanai and Edwards, 1999; Sage et al., 2011b).

Many C₂ species express a C₄ metabolic cycle of varying strength. Edwards and Ku (1987) have called C₂ species with a modest C₄ cycle Type II C₃–C₄ intermediates, while C₂ species lacking a C₄ cycle have been termed Type I C₃–C₄ intermediates. There is no evidence that *E. acuta* operates a C₄ metabolic cycle thereby classifying it as a Type I C₃–C₄ species. Photospiration is low in NADP-ME subtypes due to high BS CO₂ and relatively low BS O₂ levels (Kanai and Edwards, 1999; Sage et al., 2011b).

Phylogenetic data confirm that subsection *Acutae* is basal to all the C₄ species in subgenus *Chamaesyce* and thus support the hypothesis that photosynthetic CO₂ concentration (C₂ photosynthesis) is an obligatory step for the evolution of C₄ photosynthesis. *Euphorbia angusta* and *E. acuta* branch at a node between the more basal C₃ species in the genus *Euphorbia*, and more distal C₄ species in subgenus *Chamaesyce* (Steinmann and Porter, 2002; Yang and Berry, 2007). With the placement of *E. acuta* between C₃ and C₄ nodes in the *Euphorbia* phylogeny, the *Euphorbia* subsection *Acutae* represents a fourth confirmed case where C₂ photosynthesis appears in an evolutionary intermediate position between C₃ and C₄ clades. Other confirmed cases include *Flaveria*, *Cleome*, and *Mollugo* (McKown et al., 2005; Feodorova et al., 2010; Christin et al., 2011). These multiple observations provide strong evidence that C₂ photosynthesis is an obligatory step for C₄ evolution. However, the existence of numerous C₂ species that are unrelated to C₄ clades demonstrates that C₂ photosynthesis

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**Fig. 7.** Transmission electron micrographs of chloroplasts and mitochondria in the centripetal regions of bundle sheath cells of *Euphorbia* species. (A) *E. angusta*; (B) *E. acuta*; (C) *E. lata*; (D) *E. mesembryanthemifolia*. B, bundle sheath; C, chloroplast; M, mitochondrion; P, peroxisome; VT, vascular tissue. Bars = 0.5 μm.
does not automatically lead to the C₄ condition (Hylton et al., 1988, for Moricandia; Christin et al., 2011, for Mollugo; Sage et al., 2011a). This, along with the ecological success of C₂ species such as E. acuta, Mollugo verticillata, and M. nudicaulis (Christin et al., 2011), demonstrates the value of recognizing C₂ photosynthesis as a distinct photosynthetic adaptation in its own right. In light of this, it is proposed that the term ‘C₃–C₄ intermediate’ commonly used for C₂ species be restricted in use to species with intermediate traits that can be phylogenetically placed between C₃ and C₄ clades. Such a convention could include all species that are evolutionary intermediates, regardless of whether they express C₂ photosynthesis.

The evolutionary transition from C₃ to C₄ photosynthesis is marked by modifications in the characteristics of the leaf tissues and cell structure (Dengler and Taylor, 2000). Relative to C₃ species, the majority of species exhibiting C₄ photosynthesis have closer vein spacing, reduced M-to-BS tissue ratio, and an asymmetric positioning of organelles in the BS cells (Dengler and Taylor, 2000; Muhaidat et al., 2007; Voznesenskaya et al., 2007). Using a phylogenetic analysis, McKown and Dengler (2007) proposed a stepwise acquisition of anatomical traits during the evolution of C₄ photosynthesis from C₃ ancestors in Flaveria. Initial developmental innovations leading to C₃–C₄ intermediacy include increases in BS chloroplast numbers, decreases in the M-to-BS tissue ratio and increases in BS cell size. These features possibly enabled subsequent C₄ evolution by facilitating the acquisition of the C₄ metabolic steps (Monson, 1999; Sage, 2004). Results from this study demonstrate that a number of the critical traits associated with the C₄ pathway in E. lata and E. mesembryanthemifolia (for example, enlarged BS size, reduced M cell size, and asymmetric positioning of organelles) are also present in E. acuta and E. johnstonii. Chloroplast and mitochondria number as well as mitochondrial size are also enhanced in the BS of E. acuta. As noted for Flaveria (McKown and Dengler, 2007), the C₄-like traits in E. acuta occur in tandem with changes in GDC localization but prior to expression of PEPC in M tissue. The presence of these traits in E. acuta are...
consistent with those reported in C_2 species in Cleome, Flaveria, and Mollugo (Marshall et al., 2007; McKown and Dengler, 2007; Voznesenskaya et al., 2007; Christin et al., 2011). Together, these examples provide strong support that reduced M-to-BS tissue ratio, increased organelle number and size in enlarged BS cells, and the polar distribution of BS organelles are common intermediate steps in the evolution of C_4 photosynthesis in the eudicots.

Modifications in vein patterning that led to increases in vein density in C_4 species precede changes in BS organelle content and cell size in Flaveria; it has thus been hypothesized that changes in vein patterning may be a precondition for the evolution of C_3-C_4 intermediacy (McKown and Dengler, 2007). Euphorbia angusta has a number of traits which may facilitate the shift from the C_3 to C_2 and then the C_4 pathway. Interveinal distance in E. angusta is less than in E. acuta and either of the C_4 species, and the BS tissue is enlarged relative to what is considered typical in C_3 plants. In a survey of 21 C_3 to C_4 lineages, Muhaidat et al. (2007) observed that the C_3 species had a mean % area of BS-like tissue of 8.1%, which is less than the 14.9% observed here in E. angusta. Notably, chloroplast numbers on a BS area basis in E. angusta are statistically similar to the values in E. acuta and both C_4 species, indicating significant involvement of the E. angusta BS cells in leaf photosynthesis. The combination of close vein spacing, enlarged BS size, and enough chloroplasts for significant photosynthetic activity may provide the BS tissue of E. angusta with the potential to carry the photorespiratory load of the leaf following a mutation that knocks out GDC expression in the M tissue. The knockout of M GDC expression is proposed to be the key mutation that establishes photosynthetic CO_2 concentration and hence C_2 photosynthesis in the leaf (Monson et al., 1984; Bauwe, 2011). To evaluate whether E. angusta has increased potential to facilitate C_2 evolution, it would be useful to examine its relatives in the more basal C_3 clades of Euphorbia.

In addition to supporting physiological, molecular, and cell biology studies of C_4 origins, Euphorbia subgenus Chamaesyce is well positioned to support studies addressing the ecological factors facilitating C_4 evolution. The leading explanations for the origin of C_4 photosynthesis postulate that the C_4 pathway arose from increasingly sophisticated modifications that served to compensate for high levels of photorespiration (Monson et al., 1984; Ehleringer et al., 1991; Bauwe, 2011). The initial innovations facilitated the refixation of photorespired CO_2 in the BS, leading to an optimized C_2 pathway following loss of mesophyll GDC expression (Monson and Rawsthorne, 2000). As such, it is hypothesized that the C_4 pathway evolved in habitats where photorespiration represents a large drag on C_3 photosynthesis. The ecological habitat of the species studied here supports this hypothesis. The semi-arid landscapes of western Texas and north-eastern Mexico are most likely to be the location where C_4 photosynthesis evolved in Euphorbia, given the restriction of species from subsection Acuta to this region. As shown in Fig. 2, daily summer temperatures can climb well above 30 °C, soil water can be episodically scarce, and the vapour pressure difference between leaf and air is high. High VPD would restrict stomatal conductance, thus aggravating photorespiratory inhibitions. Elevated temperatures would therefore favour high rates of photorespiration, particularly at the lower CO_2 levels of recent geological time (Sage, 2004).
Photorespiration was unlikely to be the only factor promoting C₄ evolution, as indicated by the presence of C₄-like traits in the C₃ plant *E. angusta*, notably close vein spacing. Close vein spacing may have arisen in C₃ plants in response to very high evaporative demands, which occur in hot, semi-arid to arid climates (Sage, 2004). High VPD may favour close vein spacing in order to sustain water flux to the M tissue under high evaporative demand. The presence of a summer monsoon in the habitat of *E. angusta* also appears to be critical, as it would provide enough soil moisture to allow for summertime photosynthesis, even in hot conditions promoting high photorespiration and transpiration. Consistently, other lineages where C₂ and C₄ photosynthesis evolved also occur in hot, summer monsoon climates with some level of water or salinity stress (Sage et al., 2011a). C₂ and C₃ species of *Flaveria* and *Heliotropium* are active in summer monsoon habitats of west Texas and subtropical Mexico (Frohlich, 1978; Powell, 1978; Vogan et al., 2007); *Mollugo* is generally a tropical genus from monsoon-affected regions with hot summers (Christin et al., 2011), and the close relatives of the C₄ clades in *Cleome* occur on hot, rocky or sandy soils of the subtropics and tropics (Feodorova et al., 2010). Together, these patterns present in *Euphorbia* subgenus *Chamaesyce* and the other C₂ groups indicate that C₄ evolution is favoured by environments where photorespiration in C₃ plants is high; however, selection pressures that compensate for high evaporative demand establish the preliminary conditions such as close vein spacing that subsequently facilitate the origin of the C₂ pathway.

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