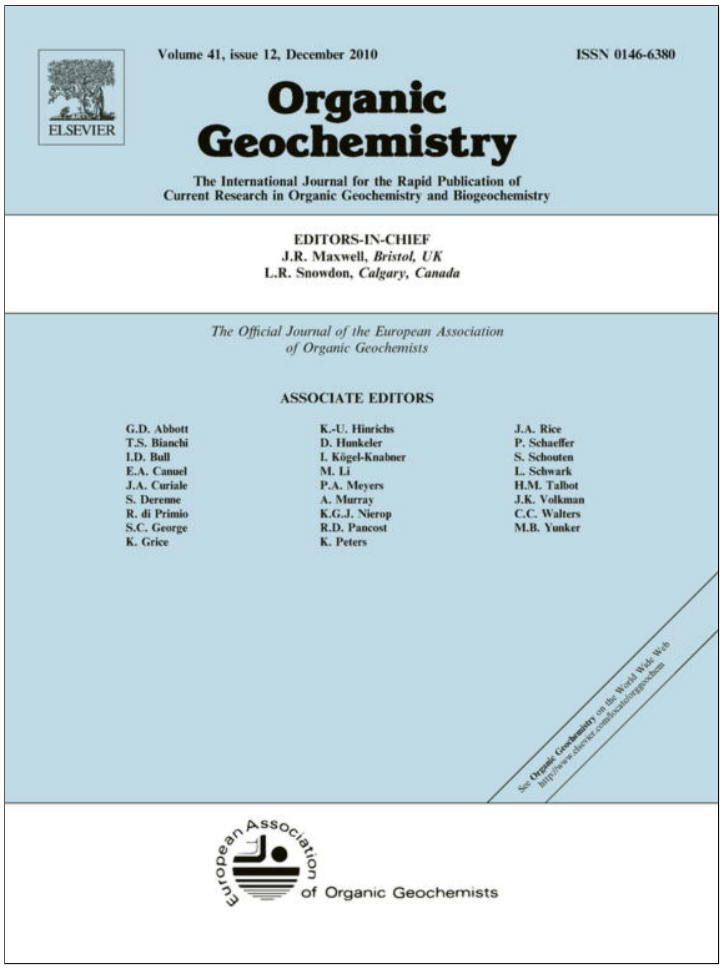

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Crassulacean acid metabolism influences D/H ratio of leaf wax in succulent plants

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ABSTRACT

This study sought to characterize hydrogen isotopic fractionation during biosynthesis of leaf wax *n*-alkanes in succulent plants capable of crassulacean acid metabolism (CAM). The metabolic and physiological features of CAM represent crucial strategies for survival in hot and dry climates and have been hypothesized to impact hydrogen isotope fractionation. We measured the stable carbon and hydrogen isotopic compositions ($\delta^{13}C$ and δD, respectively) of individual *n*-alkanes in 20 species of succulent plants from a global collection of the Huntington Botanical Gardens, San Marino, California. Greenhouse conditions and irrigation with water of constant δD value enabled determination of interspecies differences in net D/H fractionation between source water and leaf wax products. Carbon isotope ratios provide constraints on the extent of CAM vs. C_3 photosynthesis and indicate a wide range of CAM use, with $\delta^{13}C$ values ranging from −33.01‰ to −18.54‰ (C_{27}–C_{33} *n*-alkanes) and −26.66‰ to −17.64‰ (bulk tissue). Despite the controlled growth environment, we observed ca. 90‰ interspecies range in δD values from −193‰ to −107‰. A positive correlation between $\delta^{13}C_{bulk}$ and δD_{C31} values with $R^2 = 0.60$ ($\delta^{13}C_{C31}$ and δD_{C31} values with $R^2 = 0.41$) implicates a metabolic isotope effect as the dominant cause of interspecies variation in the hydrogen isotopic composition of leaf wax *n*-alkanes in CAM-intermediate plants.

1. Introduction

Succulent plants using crassulacean acid metabolism (CAM) exhibit substantial flexibility in metabolic pathways, particularly for carbon fixation. Carbon isotopes in plant bulk tissue and leaf waxes can be used to evaluate the use of differing carbon fixation pathways. C_3 plants, using the Calvin–Benson cycle, produce leaf wax *n*-alkanes with $\delta^{13}C$ values of −35 ± 5% and C_4 plants, using the Hatch-Slack cycle, generate values of −20 ± 5% (Collister et al., 1994; O'Leary, 1981). Succulent plants capable of CAM have extremely variable $\delta^{13}C$ values for bulk tissue, typically intermediate between those of C_3 and C_4 plants, although the ranges partially overlap (Osmond et al., 1973). This carbon isotope variability derives from the ability of CAM-enabled plants to use CAM or C_3-like pathways to varying degrees (Osmond et al., 1989). Thus, CAM plants provide an opportunity to investigate hydrogen isotopic fractionation associated with changing metabolic pathways.

CAM metabolism is an adaptation to drought and is distinguished by the night time fixation of CO_2 into malic acid, allowing stomata to open only at night when the relative humidity is higher (Osmond et al., 1989). Decarboxylation and net CO_2 fixation then proceed in the light while stomata are closed. CAM effectively minimizes water loss, but also slows photosynthesis and growth. Succulent plant species may therefore employ variable degrees of CAM, including cycling between C_3 and CAM pathways, and the degree of CAM use can be monitored by way of the carbon isotopic discrimination recorded in plant tissue (Osmond et al., 1973; Sternberg et al., 1984b,c). Several studies have reported effects of CAM on the composition of hydrogen isotopes in cellulose (Sternberg et al., 1984c) and individual *n*-alkyl lipids (Chikaraishi and Naraoka, 2007). An early study of bulk lipids did not reveal differentiation of δD values between C_3 and CAM plants (Sternberg et al., 1984a), and this might be attributable to the large isotopic offsets between acetogenic and isoprenoid lipids (Chikaraishi et al., 2004; Sessions et al., 1999; Zhang and Sachs, 2007) that are necessarily conflated in bulk analysis.

In contrast, there is limited evidence for a difference in δD values of individual *n*-alkanes between C_3 plants and CAM plants. Data are available for three species: *Ananas comosus* (pineapple, δD_{C31}, −194‰, $\delta^{13}C_{bulk}$, −13.6‰, $\delta^{13}C_{C31}$, −20.5‰) from Thailand, *Lycoris radiata* (red spider lily, δD_{C31}, −186‰, $\delta^{13}C_{bulk}$, −21.9‰, $\delta^{13}C_{C31}$, −27.8‰) and *Colocasia esculenta* (coco yam, δD_{C31}, −179‰, $\delta^{13}C_{bulk}$, −27.1‰, $\delta^{13}C_{C31}$, −34.0‰) from Japan (Chikaraishi and Naraoka, 2003). We note that $\delta^{13}C_{bulk}$ values indicate that these are C_3-CAM intermediates displaying a wide range of CAM use; *A. comosus*, displays the least negative and most CAM-like $\delta^{13}C$ value. Comparison of the $\delta^{13}C_{bulk}$ and $\delta^{13}C_{C31}$ values yields a positive correlation with $R^2 = 0.99$, indicating that $\delta^{13}C_{C31}$ represents a valid proxy for CAM activity. The δD values reported for these CAM plants are more negative than for C_3 and C_4 plants sampled in the same study, although unknown source waters make comparisons of net fractionation

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between water and lipids uncertain. Another study in Guangzhou, China by Bi et al. (2005) reported data for three species: *Euphorbia trigona* (African milk tree, δD_{C31}, −182‰, $\delta^{13}C_{C31}$, −29.4‰), *Opuntia dillenii* (prickly pear, δD_{C31}, −152‰, $\delta^{13}C_{C31}$, −25.1‰) and *Hylocereus undatus* (night blooming cactus, δD_{C31}, −171‰, $\delta^{13}C_{C31}$, −21.8‰). We have recently reported data for a single CAM plant from the Mojave Desert, *Opuntia basilaris*, with $\delta^{13}C_{bulk}$, −13.5‰, indicating full CAM expression under drought stress, and affording a δD value more positive than for C_3 plants sampled in the same study. The δD value of the C_{33} *n*-alkane was −124‰ and the value for the environmental water was −79‰ (Feakins and Sessions, 2010). The calculated net fractionation of −47‰ is smaller than the −91 ± 32‰ net fractionation for C_3 plants ($C_{27,29,31}$ *n*-alkanes). Comparison is tenuous given the limited data, so we initiated a study to test whether increasing CAM use alters net hydrogen isotopic fractionation.

Hydrogen isotope studies of non-CAM plants have identified large (up to 100‰) interspecies variability in net isotopic fractionation (e.g. Chikaraishi and Naraoka, 2003; Hou et al., 2007b; Krull et al., 2006; Liu and Huang, 2005). In some cases the variability has been linked to variation in life form (i.e. tree, shrub and grass), with the largest offset observed for grass (Hou et al., 2007b; Liu et al., 2006). Other studies have linked variation in δD values to differences between C_3 and C_4 pathways (Chikaraishi, 2003; Smith and Freeman, 2006). Given the large interspecies variability reported for non-CAM species and the known range of variability in $\delta^{13}C$ values for CAM plants, we anticipated variable net hydrogen isotopic fractionation in CAM plants.

Most CAM plants are succulents and form a significant component of many arid sub-tropical environments. However, the nature of arid climates – with intermittent rain, large inter-annual variability in precipitation and large inter-storm variability in isotopic composition – is a major disadvantage for field-based sampling. Our study therefore sought to characterize the carbon and hydrogen isotopic compositions of leaf waxes in succulent plant species derived from a global collection, grown in one location and in a controlled environment. We report paired measurements of δD and $\delta^{13}C$ values of *n*-alkanes for 20 species of succulent plants that have a propensity to use CAM metabolism. To our knowledge, this is the first study to systematically evaluate the sensitivity of hydrogen isotopic fractionation recorded in individual leaf wax *n*-alkanes to varying degrees of CAM metabolism.

2. Sample selection

Leaf samples were collected from a variety of specimens from the global collection of succulents cultivated in the 'desert greenhouse' at the Huntington Botanical Gardens, San Marino, California (hereafter 'the Huntington'; Table 1). The collection includes specimens from several continents representing species that are rare, endangered or of special historical or economic importance. Most are succulents and all are <1 m tall. We chose plants in a greenhouse environment where all water for growth is supplied by irrigation from an on-site well (δD −45.09 ± 0.24‰). This minimizes variation in water δD value that might otherwise influence the plant leaf wax lipids. In addition, the greenhouse environment greatly reduces the environmental variability experienced by plants, including factors such as rooting depth, canopy position and microclimate, as well as isotopic disequilibrium between soil water and water vapor. All the plants experience the same semi-controlled environmental conditions in a covered greenhouse. Temperature ranges between 3 and 42 °C annually, with an average diurnal temperature range of 25 °C. Relative humidity averaged lows of 26% during June and July 2005. No long term monitoring of relative humidity was available, but all species experienced equivalent environmental conditions. Leaf samples were collected to assess interspecies variation in chain length and carbon and hydrogen isotopic composition of leaf wax *n*-alkanes.

3. Methods

3.1. Leaf sampling and extraction

Samples were collected on October 17, 2006 by cutting at the junction of the leaf to the stalk. Entire leaves were collected in order to ensure integration of the signal from the whole leaf, given the possibility of isotopic gradients along its length (Helliker and Ehleringer, 2000; Sessions, 2006). For most samples, 1–5 leaves from a single plant were collected to obtain sufficient material,

Table 1
Plant specimens used.

ID[a]	Origin[b]	Class[c]	Order	Family	Species	CAM[d]
42493	Madagascar	Liliopsida	Asparagales	Anthericaceae	*Anthericum suffulicosum*	
28401	Madagascar	Liliopsida	Asparagales	Asphodelaceae	*Aloe deltoideodonta*	*
41364	Kenya	Liliopsida	Asparagales	Ruscaceae	*Dracaena ellenbechiana*	
54466	Kenya	Magnoliopsida	Asterales	Asteraceae	*Senecio nyikensis*	*
22933	Yemen	Magnoliopsida	Asterales	Asteraceae	*Senecio sempervivus*	*
38724	S. Africa	Magnoliopsida	Caryophyllales	Aizoaceae	*Conophytum quaesitum*	*
42491	S. Africa	Magnoliopsida	Caryophyllales	Aizoaceae	*Fenestraria aivantiaca*	*
42291	S. Africa	Magnoliopsida	Caryophyllales	Aizoaceae	*Lithops marmorata*	*
2802	Bolivia	Magnoliopsida	Caryophyllales	Cactaceae	*Pereskia cacharica*	*
n/a	S.E. Africa	Magnoliopsida	Cucurbitales	Cucurbitaceae	*Gerrardanthus macrorhizus*	
21096	Madagascar	Magnoliopsida	Cucurbitales	Cucurbitaceae	*Xerosicyos danguyi*	*
93751	Australia	Magnoliopsida	Gentianales	Apocynaceae	*Hoya australis sp. rupicola*	*
49170	W. Africa	Magnoliopsida	Malpighiales	Euphorbiaceae	*Euphorbia poissonii*	*
20200	S. Africa	Magnoliopsida	Malpighiales	Passifloraceae	*Adenia glauca*	
60908	S. America	Magnoliopsida	Piperales	Piperaceae	*Peperomia scandens*	*
73108	S. Africa	Magnoliopsida	Saxifragales	Crassulaceae	*Crassula rupestris*	*
75904	Venezuela	Magnoliopsida	Saxifragales	Crassulaceae	*Echeveria compressacolis*	*
20690	Mexico	Magnoliopsida	Saxifragales	Crassulaceae	*Echeveria runyeonii*	*
78254	S. Africa	Magnoliopsida	Saxifragales	Crassulaceae	*Kalanchoe luciae*	*
35755	Angola	Magnoliopsida	Vitales	Vitaceae	*Cyphostemma currorii*	

[a] Huntington herbarium voucher number corresponding to an archived specimen.
[b] Country of source; sampled plants were grown in the Huntington greenhouse.
[c] All species from the division Magnoliophyta.
[d] While CAM assessments have not been reported for all species, genera reported to display some form of CAM are marked with * (Bastide et al., 1993; Fioretto and Alfani, 1988; Hanscom and Ting, 1978; Holthe et al., 1987; Kluge et al., 1979; Rayder and Ting, 1983; Robinson et al., 1993; Winter et al., 2005).

depending on the size of the leaf. Only mature leaves were collected from the base of the plant. Samples were cut into ca. 1 cm^2 pieces with solvent-cleaned scissors and freeze dried. Then, 1–2 g of each sample was used for extraction. The *n*-alkanes were extracted (3×) in 5 ml each of hexane using a pumping action with a Pasteur pipette. The extract was transferred to a silica gel column (5 cm × 4 mm Pasteur pipette; 5% H_2O-deactivated silica gel, 100–200 mesh) and the *n*-alkane fraction collected by eluting with hexane.

3.2. Lipid quantitation

Lipid identity and abundance were determined at the University of Southern California (USC) using gas chromatography–mass spectrometry (GC/MS) with an Agilent 6890 chromatograph equipped with a Rxi®-5ms (30 m × 0.25 mm, film thickness 0.25 μm) and a split/splitless injector operated in split mode (50:1), connected to an Agilent 5973 MSD mass spectrometer. The *n*-alkanes were assigned by comparison of mass spectra and retention times with library data and authentic standards. Relative abundances were quantified by comparison of integrated peak areas from the GC/MS total ion current (TIC) trace, and were calculated to sum to 100% (Fig. 1).

3.3. Carbon isotope analysis

The carbon-isotopic compositions of individual lipids were analyzed at USC with a Thermo Scientific Trace gas chromatograph connected via an Isolink parallel combustion furnace (at 1030 °C) to a Delta V Plus mass spectrometer. An Rxi®-5ms column (30 m × 0.25 mm, film thickness 1 μm) was used with a PTV injector operated in splitless mode. Samples were injected in triplicate

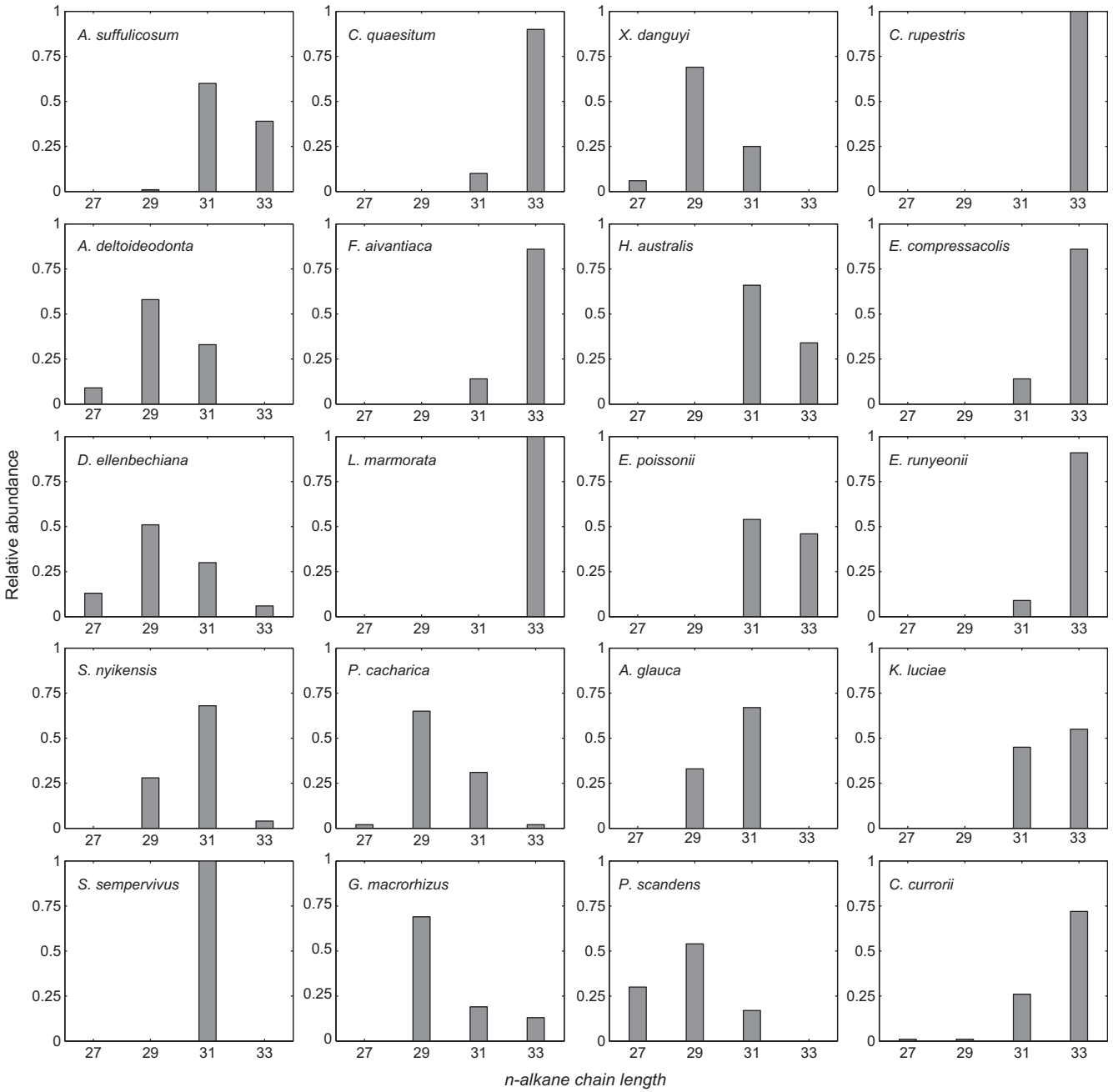

Fig. 1. *n*-Alkane abundance distribution by chain length for individual species.

and were interspersed with external standards of known isotopic composition. Peaks of CO_2 reference gas were co-injected between *n*-alkane peaks during the course of each GC-IRMS (isotope ratio mass spectrometry) run. Two of the peaks were used for standardization of the isotopic analyses, while the remaining peaks were treated as unknowns to assess accuracy. Data were normalized to the Vienna Pee Dee Belemnite (VPDB) isotopic scale by comparison with an external fatty acid methyl ester standard with $\delta^{13}C$ values ranging from −30.92‰ to −23.24‰ (obtained from A. Schimmelmann, Indiana University, Bloomington). The results are reported using conventional notation ($\delta^{13}C$, ‰). Accuracy for replicate analyses of the external standards was 0.5‰ (RMS error, $n = 18$). For co-injected peaks of CO_2 reference gas, precision was typically better than 0.08‰ (1σ). Total uncertainty associated with repeated analyses of individual compounds in different samples is conservatively estimated at <0.6‰.

In addition, the $\delta^{13}C_{bulk}$ values of nine species were analyzed. Samples of ground leaf tissue of ca. 0.6 mg dry wt. were analyzed using a Costech Elemental Analyzer (EA) coupled to a Delta V Plus mass spectrometer at USC. The $\delta^{13}C$ values were calculated by comparison to a CO_2 reference gas with a $\delta^{13}C$ value of −35.2‰. Analytical precision was 0.06‰ (urea standard, $n = 13$) and 0.24‰ (replicate sample aliquots, $n = 5$).

3.4. Hydrogen isotope analysis

The δD values of individual lipids were measured at the California Institute of Technology (Caltech) using a ThermoFinnigan Trace gas chromatograph coupled to a Delta Plus XP mass spectrometer via a pyrolysis interface (GC/TC) operated at 1430 °C. A ZB-5ms column (30 m × 0.25 mm, film thickness 1.0 m) and PTV injector operated in splitless mode were used. Peaks of CH_4 reference gas were co-injected between *n*-alkane peaks during the course of the GC-IRMS run using a home-built system described by Wang and Sessions (2008). Two of the peaks were used for standardization, while the others were treated as unknowns to assess accuracy. Data were normalized to the SMOW/SLAP isotopic scale by comparison with an external standard containing 15 *n*-alkanes (C_{16}–C_{30}; obtained from A. Schimmelmann, Indiana University, Bloomington) with δD values ranging from −41‰ to −256‰ (Sessions et al., 2001).

The accuracy of replicate analyses of the external *n*-alkane standard was 4.1‰ (RMS error, $n = 10$). For the co-injected CH_4 reference peaks, not included as calibration tie points, precision averaged 3.9‰ (1σ). Total uncertainty associated with repeated analysis of individual compounds in different samples is conservatively estimated at <8‰.

The results are reported using conventional notation (δD‰). We report the net (or apparent) fractionation between measured δD values of leaf wax and supplied water as enrichment factors ($\varepsilon_{C31/w}$) defined as:

$$\varepsilon_{C31/w} = \alpha_{C31/w} - 1 = \frac{\delta_{C31} + 1}{\delta_w + 1} - 1 \quad (1)$$

Enrichment factors and δ values are commonly reported in permil, which implies a factor of 1000 that can then be left out of Eq. (1) (Cohen et al., 2007). δD_{C31} is the δD value of the plant leaf wax lipid, in this case C_{31} *n*-alkane, and δD_w is the δD of the source water, in this case irrigation water of constant isotopic composition.

3.5. Water isotope analysis

Water collected from the water supply in the greenhouse, fed by the on-site well, was sampled on October 17, 2006 and sealed into glass ampoules on the same day. Isotopic analysis was conducted with a Los Gatos Research Liquid Water Isotope Analyzer at Caltech on February 18, 2007. The isotopic composition was determined from three injections of 0.8 μl water, relative to a daily calibration with three standards of known isotopic composition established relative to the SMOW/SLAP isotopic scale. The precision of replicate injections during the period when the sample was analyzed averaged 0.7‰ (1σ, $n = 144$), with replicate analyses reproducible to within 1.3‰ (1σ, $n = 7$).

4. Results and discussion

4.1. Leaf wax composition

Succulent species afforded odd numbered, long chain *n*-alkanes restricted to the C_{27}–C_{33} range. The distribution varied between species (Fig. 1), with average chain length (ACL) ranging between 27.6 and 32.2 and maximum carbon number (C_{max}) between C_{27} and C_{33}, both with an average of C_{31} (Table 2). Similar ranges are reported for other CAM species (Bi et al., 2005; Chikaraishi and Naraoka, 2003; Feakins and Sessions, 2010). Plant leaf waxes generally contain chain lengths >C_{21} (Eglinton and Hamilton, 1967) and this study supports a tendency to longer chain length in drought-adapted species, consistent with reports for species in other high temperature and arid environments (Huang et al., 2000; Rommerskirchen et al., 2003; Schefuss et al., 2003). Chain length distribution can also be related to phylogeny. Three species of the *Aizoaceae* family and four of the *Crassulaceae* family display a consistency in chain length distribution, with abundance of $C_{33} > C_{31}$. However, no such consistency is observed for the other families, indicating that phylogenetic affinity is not the only control on leaf wax lipid composition.

4.2. Carbon isotopic compositions

Comparison between $\delta^{13}C_{bulk}$ and $\delta^{13}C_{C31}$ gave $R^2 = 0.33$ ($n = 4$). We note that the carbon-isotopic compositions of *n*-alkanes cover a similar range to $\delta^{13}C_{bulk}$ but $\delta^{13}C_{alkane}$ values are ca. 6‰ more negative. The $\delta^{13}C$ values of leaf wax *n*-alkanes range from the least negative (−18.54‰), and likely most CAM-enabled, *Crassula rupestris*, to the most negative (−32.47‰), or C_3-like values in *Antheriricum suffulicosum*. Similar values occur across the C_{29}, C_{31} and C_{33} *n*-alkanes. They averaged −28.09 ± 5.25‰ (1σ, $n = 3$) for C_{29}, −27.22 ± 3.86‰ (1σ, $n = 11$) for C_{31}, and −25.74 ± 4.47‰ (1σ, $n = 9$) for C_{33} *n*-alkane (Table 2). Correlation of the values for C_{29} and C_{31} *n*-alkanes produces a slope of 0.907 with $R^2 = 0.822$, $n = 3$, p, 0.277. Correlation of the values for C_{31} and C_{33} gives a slope of 0.959 with $R^2 = 0.920$, $n = 7$, $p < 0.001$. These data are consistent with minimal net isotopic fractionation during chain elongation.

We measured plants with $\delta^{13}C_{bulk}$ values from −26.66‰ to −17.64‰. CAM bulk tissue $\delta^{13}C$ values have been reported in the range −13.5 to −27‰ in growth experiments (Osmond et al., 1973), and an overlapping range of −13.3 to −28.3‰ for 12 species of CAM from a humid, tropical environment (Winter et al., 2005). The values for cellulose nitrate similarly fall between −11.7‰ and −29.9‰ for a variety of CAM-intermediate plants grown in a greenhouse in Riverside, California (Sternberg et al., 1984c). Carbon isotope ratios thus indicate that we have captured a wide range of CAM use in our selection of specimens, although no species are expressing full CAM, presumably because of irrigation.

4.3. Hydrogen isotopic compositions

The δD value of irrigation water at the Huntington Botanical Gardens is −45.09 ± 0.24‰. This is consistent with data on the hydrogen isotopic composition of regional precipitation (Friedman et al., 1992; Williams and Rodoni, 1997) and local precipitation and stream water isotopic measurements (Feakins and Sessions, 2010),

Table 2
Molecular and isotopic compositions of leaf wax *n*-alkanes (nd, not determined).

Species	Bulk	$\delta^{13}C$ (‰)				σ (‰)				δD (‰)				σ (‰)				$\varepsilon C_{31}/w$ (‰)	Abundance	
		C_{27}	C_{29}	C_{31}	C_{33}	C_{27}	C_{29}	C_{31}	C_{33}	C_{27}	C_{29}	C_{31}	C_{33}	C_{27}	C_{29}	C_{31}	C_{33}		ACL[a]	C_{max}[b]
A. suffulicosum	−26.57		nd	−31.84	−32.47			0.36	0.12		nd	−193	−190			1.5	1.1	−155	31.8	31
A. deltoideodonta	−19.01	nd	nd	nd						−150	−154	−151		nd	nd	nd		−111	29.5	29
D. ellenbechiana	−24.58	nd	nd	nd	nd					−173	−191	−183	nd	0.6	0.4	2.4		−145	29.6	29
S. nyikensis	−24.55		−31.31	−33.01	nd		0.21	0.30			nd	−168	nd			nd		−129	30.5	31
S. sempervivus	nd			nd								−160				1.9		−121	31.0	31
C. quaesitum	nd			−26.67	−25.32			0.11	0.34			−132	−151			0.9	1.4	−91	32.8	33
F. aivantiaca	nd			−22.08	nd			0.15				−160	−168			0.3	1.5	−120	32.7	33
L. marmorata	nd				−23.90				0.43				−131			1.6	0.8	nd	33.0	33
P. cacharica	nd	nd	nd	nd	nd					nd	−125	−124	nd			0.8	0.7	−83	29.7	29
G. macrorhizus	−26.66		nd	nd	nd						−139	−143	nd		6.2	1.3		−103	29.9	29
X. danguyi	−19.07	nd	nd	nd						nd	−116	nd			1.5			nd	29.4	29
H. australis	−19.75			−28.79	−29.93			0.05	0.14			−153	−148		3.4	0.8		−113	31.7	31
E. poissonii	−21.24			nd	nd							−167	−163			4.5	2.6	−128	31.9	31
A. glauca	nd		−30.93	−28.61			0.55	0.44			−138	−158			3.0	0.0	2.0	−119	30.3	31
P. scandens	nd	nd	−22.03	−23.32	nd		0.07	0.07		nd	−153	−149			0.0	1.2		−109	28.7	29
C. rupestris	nd				−18.54				0.03				−154				2.1	nd	33.0	33
E. compressacolii	nd			−23.38	−23.72			0.20	0.33			−150	−151			0.1	0.9	−110	32.7	33
E. runyeonii	nd			−27.41	−26.70			0.41	0.39			−153	−160			2.8	1.7	−113	32.8	33
K. luciae	nd			−23.11	−21.27			0.04	0.72			−107	−121			0.3	1.1	−65	32.1	33
C. currorii	−17.64	nd	nd	−31.25	−29.82			0.22	0.17	nd	nd	−169	−171			2.1	0.6	−130	32.4	33
Mean	−22.12		−28.09	−27.22	−25.74					−161	−145	−154	−155					−114	31.3	31
σ (interspecies)	3.50		5.25	3.86	4.47						24	21	19					22	1.4	1.7
σ (analytical)	0.24[c]		0.59	0.85	1.07						8	7	5							

nd = not determined.
[a] Average Chain Length (ACL), where $ACL_{(i=27:33)} = \sum C_i{*}i / \sum C_i$.
[b] Modal chain length (C_{max}).
[c] Five replicates of *E. poissonii*.

indicating that the aquifer is recharged mainly by local precipitation.

The δD values of the *n*-alkanes range from −193‰ to −107‰. Average values are −145 ± 24‰ (1σ, n = 7) for C_{29}, −154 ± 21‰ (1σ, n = 17) for C_{31}, and −155 ± 19‰ (1σ, n = 11) for C_{33} (Table 2). Correlation of the C_{29} and C_{31} values gives a slope of 0.905 with R^2 = 0.817, n = 6, p < 0.015; for C_{31} and C_{33} the slope is 0.61 with R^2 = 0.587, n = 12, p < 0.0001. The δD_{C31} values range from the least negative (−107‰) in *Kalanchoe luciae*, to the most negative (−193‰) in *Anthericum suffulicosum*. Interspecies differences in δD_{C31} values result in a large range in calculated $\varepsilon_{C31/w}$ from −65‰ to −155‰, with a mean of −114 ± 22‰ (1σ, n = 17). Species from the class *Liliopsida* have δD_{C31} values more negative (n = 3, −176 ± 22‰) than those of *Magnoliopsida* (n = 14, −150 ± 18‰); however, no other phylogenetic affinity is apparent. We observe a large spread in hydrogen isotopic composition and fractionations despite the controlled greenhouse environment, implying that biotic factors other than phylogeny must influence the magnitude of hydrogen isotopic fractionation between species.

The variance in δD values is consistent with a similar study of greenhouse plants based on cellulose nitrate measurements. That study observed a large range (ca. 120‰) in values of cellulose nitrate between −40‰ and +80‰ (Sternberg et al., 1984c). However, although irrigation water is likely to have been relatively similar (the sites are within 50 miles of each other), the CAM *n*-alkane δD values are notably more negative than cellulose nitrate values (offset >150‰). Thus, there appear to be both significant metabolic controls, between CAM variants, on hydrogen isotopic fractionation (Sternberg et al., 1984c) as well as metabolic fractionation and biosynthetic fractionations, resulting in large offsets between celluloses and the alkanes measured here (Hayes, 2001; Luo and Sternberg, 1991; Sternberg et al., 1984a).

4.4. Carbon and hydrogen isotopic relationships reflect metabolic pathways

There is a positive correlation (R^2 = 0.41, n = 11, p, 0.35; Fig. 2) between measured $\delta^{13}C$ and δD values of the C_{31} *n*-alkane for all specimens, and between $\delta^{13}C_{bulk}$ and δD_{C31} values (R^2 = 0.60 n = 8, p, 0.04; Fig. 2). Assuming that $\delta^{13}C$ in the dataset is controlled mainly by the extent of CAM use, the covariation of carbon and hydrogen isotopes suggests that CAM metabolism also modulates hydrogen isotopic fractionation. Thus, increasing CAM use results in a decrease in the net hydrogen isotopic fractionation between source water and leaf wax *n*-alkanes ($\varepsilon_{C31/w}$).

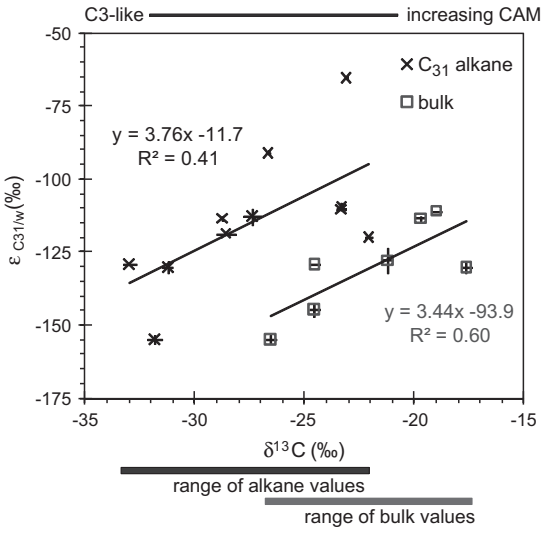

Fig. 2. Comparison of $\delta^{13}C$ values (bulk and C_{31} *n*-alkane) with the δD values of C_{31} *n*-alkane in sampled species.

To explain how CAM modulates net leaf wax hydrogen isotopic fractionation, we consider four possible controls on leaf wax δD values: (i) leaf water δD values, (ii) substrates feeding into biosynthesis (principally NADPH, but also acetate), (iii) fractionation in biosynthesis and (iv) post-synthesis exchange. Transpiration differences [(i)] can be discounted since leaf water data have shown that it is the leaf water of C_3 plants that is relatively enriched, whereas CAM shows very little diurnal enrichment (Sternberg et al., 1986). Hydrogen exchange [(iv)] can be discounted for C–H bonds in lipids at ambient conditions (Sessions et al., 2004), but remains a viable option for carbohydrates. Fractionation during biosynthesis [(iii)] cannot be ruled out, but is unlikely given that CAM plants synthesize lipids via the same route as C_3 plants (Hayes, 2001). This leads us to the conclusion that elevated δD values of leaf waxes in CAM plants originate from D enrichment in the NADPH or acetate substrates feeding biosynthesis.

A similar hydrogen isotopic offset has been noted for cellulose nitrate between CAM and C_3 plants (Sternberg et al., 1984b,c,1986). These studies suggested that D enrichment in CAM plants originates in the glycolysis–gluconeogenesis cycle (Luo and Sternberg, 1991; Yakir and Deniro, 1990), supported by the observation that cellulose (from the cytosol) is more D-enriched than starch (from the chloroplast). Thus, it appears that leaf wax and cellulose D enrichment may originate from common metabolic steps in the cytosol.

Specific metabolic controls on the composition of NADPH are still poorly understood, but the general observation (for bacteria) is that heterotrophy leads to D enrichment relative to photoautotrophy. Zhang et al. (2009) point to isotope effects during the reduction of $NADP^+$ to NADPH in the oxidative pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle as the root cause, but this has not been confirmed. We further note that many CAM plants use malic enzyme (malate dehydrogenase) to release CO_2 from malate for carbon fixation in the Calvin Cycle during the daytime. Oxidation by malic enzyme is coupled to reduction of $NADP^+$, and thus provides a new potential pathway for NADPH generation during autotrophic (CAM) growth. In bacteria, this reaction seems to be associated with D enrichment of lipids (Zhang et al., 2009), so the generation of D-enriched NADPH by malic enzyme during CAM growth would be consistent with our finding of D enrichment with increasing CAM use. A more established mechanism in plants is that increased reliance on stored sugars for metabolism leads to increased δD values (Sessions, 2006; Yakir and Deniro, 1990). We therefore propose that CAM plants are more D enriched because NADPH for lipid biosynthesis is generated more by heterotrophic pathways (probably the PPP) than by photosynthesis. This would be consistent with their slower growth rate during CAM metabolism.

4.5. Implications for sedimentary reconstructions

Sedimentary leaf wax *n*-alkanes are increasingly being used as a proxy for the H isotopic composition of meteoric water. Quantitative reconstructions rely on the existence of a constant biologic offset ('net fractionation') between meteoric water and leaf wax δD values. While differences in this fractionation between specific plant species have been recognized (Chikaraishi and Naraoka, 2003; Feakins and Sessions, 2010; Hou et al., 2007b; Krull et al., 2006; Smith and Freeman, 2006), the hope is that such differences are integrated on the scale of a typical watershed, such that an approximately constant offset can be used for interpretation of proxy data (Hou et al., 2008; Sachse et al., 2006).

Our results for CAM plants indicate a complication for leaf wax δD proxy records in sub-tropical and tropical regions, namely that the ecosystem-scale net fractionation will be influenced both by

the number of CAM-enabled plants within the ecosystem, and by the extent to which CAM-type photosynthesis is employed in these species. The latter effect in particular would be very difficult to detect in ancient sedimentary records. While CAM plants are relatively rare globally, they represent a significant fraction of vegetation in some hot, arid environments, perhaps from as early as the Triassic (Decker and de Wit, 2006). If CAM use becomes more dominant in hotter, drier climates, and if CAM metabolism leads to smaller net fractionations, the combined effect would be an overestimate of climatic drying based on leaf wax δD values.

In contrast, coupled δ^{13}C and δD data for leaf waxes might provide insight into the importance of CAM ecology in ancient environments where the isotopic composition of meteoric water can be independently constrained. The δ^{13}C values alone may not be able to resolve CAM contributions if C_3, C_4 and CAM plants are all present. However, C_3 plants typically produce leaf wax δ^{13}C and δD values that are inversely correlated (Bi et al., 2005; Hou et al., 2007a), a pattern which has been tied to the nature of water use efficiency relationship (Farquhar and Richards, 1984; Hou et al., 2007a), whereas CAM plants have leaf waxes with positively correlated δ^{13}C and δD values. Thus, combined carbon and hydrogen isotopic analyses might provide a first-order test for the dominance of CAM metabolism in ancient ecosystems.

5. Conclusions

Compound-specific isotopic measurements have great power to resolve pathways of metabolism and biosynthesis in succulent plants, which may use CAM to varying degrees. Our approach, sampling 20 species of plants grown in greenhouse conditions, demonstrates that CAM-enabled species have a wide range of carbon-isotopic compositions in both leaf wax lipids and bulk tissue, with leaf waxes being ca. 6‰ more negative than bulk δ^{13}C. Furthermore, CAM flexibility appears to drive a significant amount of variability in hydrogen isotopic composition, based on a positive correlation ($R^2 = 0.60$) between $\delta^{13}C_{bulk}$ and the hydrogen isotopic composition of the C_{31} leaf wax *n*-alkane.

The implications are twofold. First, given the flexible metabolism of CAM-enabled plants, carbon and hydrogen isotope measurements can assess the influence of CAM vs. climatic controls on hydrogen isotopic fractionation. This may be an important consideration for paleoenvironmental reconstruction in those environments where significant fractions of the vegetation are CAM-enabled. Second, compound-specific measurements revealed that the same pattern of hydrogen isotopic enrichment observed in CAM cellulose, compared to that of C_3 plants, is also seen in individual leaf wax compounds (*n*-alkanes) and indicates a common pathway for hydrogen isotopic enrichment.

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