New Phytologist

Review

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Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants

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Received: *31 March 2013*
Accepted: *25 June 2013*

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New Phytologist (2013)
doi: 10.1111/nph.12423

Key words: bundle-sheath leakiness, carbon isotope discrimination, crassulacean acid metabolism (CAM), intercellular carbon dioxide concentration, mesophyll conductance, photosynthetic pathway, water-use efficiency.

Summary

Stable carbon isotope ratios ($\delta^{13}C$) of terrestrial plants are employed across a diverse range of applications in environmental and plant sciences; however, the kind of information that is desired from the $\delta^{13}C$ signal often differs. At the extremes, it ranges between purely environmental and purely biological. Here, we review environmental drivers of variation in carbon isotope discrimination (Δ) in terrestrial plants, and the biological processes that can either damp or amplify the response. For C_3 plants, where Δ is primarily controlled by the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a), coordination between stomatal conductance and photosynthesis and leaf area adjustment tends to constrain the potential environmentally driven range of Δ. For C_4 plants, variation in bundle-sheath leakiness to CO_2 can either damp or amplify the effects of c_i/c_a on Δ. For plants with crassulacean acid metabolism (CAM), Δ varies over a relatively large range as a function of the proportion of daytime to night-time CO_2 fixation. This range can be substantially broadened by environmental effects on Δ when carbon uptake takes place primarily during the day. The effective use of Δ across its full range of applications will require a holistic view of the interplay between environmental control and physiological modulation of the environmental signal.

I. Introduction

It has been known for many decades that the stable carbon isotope ratio ($\delta^{13}C$) of terrestrial plant organic material varies among different kinds of plants and among plants growing in different environments (Nier & Gulbransen, 1939; Wickman, 1952). Pioneering work demonstrated that plant $\delta^{13}C$ relates to different photosynthetic pathways (Bender, 1968) and to leaf gas exchange

characteristics (O'Leary, 1981; Farquhar *et al.*, 1982b). These discoveries have revealed unique insights into the physiology of terrestrial plants.

Plant $\delta^{13}C$ analyses are currently used for a diverse range of applications, which can require rather different types of information from the $\delta^{13}C$ signal. For example, in order to reconstruct paleoclimate using fossil plant or herbivore remains, it would be ideal if the $\delta^{13}C$ signal simply recorded the influence of the environment on plants (Arens *et al.*, 2000; Kohn, 2010). On the other hand, in order to select genotypes that have particular gas exchange characteristics for crop breeding, it would be ideal if the $\delta^{13}C$ signal reflected primarily the intrinsic physiology of the plant, without being overly influenced by vagaries of the growth environment. At times, there is a tension between the desire to assign most of the variation in plant $\delta^{13}C$ to environmental control and the desire to assign it to species- or genotype-specific physiological set points.

Here, we review environmental and physiological sources of variation in carbon isotope discrimination (Δ) in terrestrial plants, and provide an update on recent developments in the field. For C_3 plants, ternary effects have recently been incorporated into a model for Δ, with implications for determining mesophyll conductance from coupled measurements of Δ and leaf gas exchange; for C_4 plants, recent work has led to a more refined understanding of the role of bundle-sheath leakiness in modulating Δ; and for plants with crassulacean acid metabolism (CAM), a number of large species surveys have recently been published, revealing new insights into the distribution and evolutionary history of CAM. Our aim is to provide some guidance for the wide variety of applications that make use of the Δ signal, and to stimulate future research that can clarify some of the less well understood patterns in Δ. Because the C_3, C_4 and CAM photosynthetic pathways show distinct behaviours with respect to Δ, we address each of them separately.

II. Carbon isotope discrimination

Carbon isotope discrimination (Δ) differs from $\delta^{13}C$ in that it describes only that change in isotopic composition induced by the plant, eliminating variation as a result of the starting value of the atmospheric CO_2 used for photosynthesis. Farquhar & Richards (1984) defined Δ as

$$\Delta = \frac{R_a - R_p}{R_p} = \frac{\delta_a - \delta_p}{1 + \delta_p}, \quad \text{Eqn 1}$$

where R_a is the $^{13}C/^{12}C$ ratio of CO_2 in air, and R_p is that of plant carbon. In the second form of Eqn 1, δ_a is $\delta^{13}C$ of CO_2 in air and δ_p is that of plant carbon. The $\delta^{13}C$ is defined with respect to a standard:

$$\delta^{13}C_{sample} = \frac{R_{sample} - R_{std}}{R_{std}}, \quad \text{Eqn 2}$$

where $\delta^{13}C_{sample}$ is that of the sample of interest, R_{sample} is its $^{13}C/^{12}C$ ratio, and R_{std} is the $^{13}C/^{12}C$ ratio of a standard. The internationally accepted standard for expressing stable carbon isotope ratios is PeeDee belemnite (PDB), with a $^{13}C/^{12}C$ of 0.0112372 (Craig, 1957). In order to avoid working with very small numbers, Δ and $\delta^{13}C_{sample}$ are typically multiplied by 1000, and denoted as parts per thousand (‰). When Eqn 1 is multiplied by 1000, this does not affect terms in the denominator. Therefore, if δ_p were 28‰ in the numerator, $1 + \delta_p$ in the denominator would still be 1.028.

Plant biomass provides an estimate of Δ integrated over the period of tissue synthesis. On the other hand, Δ can also be measured instantaneously by combining measurements of leaf gas exchange with online analyses of the carbon isotope ratio of CO_2 entering and leaving the gas exchange cuvette (Evans *et al.*, 1986):

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)}, \quad \text{Eqn 3}$$

where δ_e and δ_o are the $\delta^{13}C$ of CO_2 entering and leaving the cuvette, respectively. The ξ is defined as

$$\xi = \frac{c_e}{c_e - c_o}, \quad \text{Eqn 4}$$

where c_e and c_o are the CO_2 mole fractions, expressed here for dry air, entering and leaving the cuvette, respectively.

Traditionally, stable carbon isotope ratios have been determined by isotope ratio mass spectrometry (IRMS), which can achieve precisions of better than 0.1‰ for $\delta^{13}C$ (Trolier *et al.*, 1996; Vaughn *et al.*, 2004). Recently, absorption spectroscopy techniques, such as tunable diode laser (TDL) spectroscopy and cavity ring-down spectroscopy have become more common. The advantages are high sample throughput, lower cost and suitability for field deployment. The drawback is that analytical precision is often not as good. For TDL, reported precisions for $\delta^{13}C$ for near-ambient CO_2 concentration samples ranged from 0.03 to 4‰ (Bowling *et al.*, 2003; Griffis *et al.*, 2004; Barbour *et al.*, 2007; Ubierna *et al.*, 2013).

When measurements of $\delta^{13}C$ and CO_2 mole fraction are combined in Eqn 3, the measurement error in Δ also depends on ξ. For example, a standard error of 0.2‰ in $\delta^{13}C$ would scale to 3.2‰ in Δ for an ξ of 15, whereas for an ξ of 3, it would scale to 0.6‰. The value of ξ can be reduced by using larger leaf areas and lower flow rates, thereby increasing the drawdown in CO_2 mole fraction in air leaving the leaf cuvette.

III. The C_3 photosynthetic pathway

The most widely applied model of Δ in C_3 plants (Δ_3) is that of Farquhar *et al.* (1982b). The model relates Δ_3 to variation in the ratio of intercellular to ambient CO_2 mole fractions (c_i/c_a), in addition to a number of other parameters. The model has recently been updated to include a ternary correction (Farquhar & Cernusak, 2012). This correction accounts for the influence of transpiration on the diffusion of CO_2 between the atmosphere and the intercellular air spaces. 'Ternary' refers to three interacting gases, in this case CO_2, water vapour and air. The CO_2 molecules diffusing into the leaf collide both with air and water vapour

molecules. When the leaf is transpiring, this causes the intercellular CO_2 mole fraction, c_i, to be lower than it would be in the absence of transpiration (Jarman, 1974; Cowan, 1978; von Caemmerer & Farquhar, 1981). This has recently been confirmed experimentally (Boyer & Kawamitsu, 2011). The ternary correction has been applied in calculations of c_i in commercial gas exchange systems for many years, but was not previously included in the Δ_3 model. Its impact on the latter is on the order of 1–2‰, but varies with leaf-to-air vapour pressure difference (v).

The model for Δ_3, including the ternary correction, is as follows (Farquhar & Cernusak, 2012):

$$\Delta_3 = \frac{1}{1-t}\left(a_b\frac{c_a-c_s}{c_a}+a_s\frac{c_s-c_i}{c_a}\right) + \frac{1+t}{1-t}\left(a_m\frac{c_i-c_c}{c_a}+b\frac{c_c}{c_a}-\frac{\alpha_b}{\alpha_e}e\frac{R_d}{V_c}\frac{c_c}{c_a}-\frac{\alpha_b}{\alpha_f}f\frac{\Gamma^*}{c_a}\right), \quad \text{Eqn 5}$$

where t is the ternary correction factor defined by Eqn 6, a_b is the $^{13}C/^{12}C$ fractionation for CO_2 diffusion across the boundary layer (2.8‰), a_s is that for diffusion through the stomata (4.4‰), and a_m is that for dissolution and diffusion from the intercellular air spaces to the sites of carboxylation in the chloroplasts (1.8‰). The term b is the fractionation associated with carboxylation, mainly by Rubisco in C_3 plants (*c.* 29‰), e is the fractionation during day respiration, and f is the fractionation during photorespiration. The magnitudes of e and f are discussed later. The R_d is the rate of day respiration, V_c is the rate of Rubisco carboxylation, and Γ^* is the CO_2 compensation point in the absence of day respiration. c_a, c_s, c_i, and c_c are the CO_2 mole fractions in the ambient air, at the leaf surface, in the intercellular air spaces and at the sites of carboxylation, respectively. The terms α_b, α_e, and α_f are defined as $1+b$, $1+e$ and $1+f$, respectively. When Eqn 5 is expressed as parts per thousand (‰), only one parameter in each term is multiplied by 1000. Therefore, α_b remains as 1.029, and similarly for α_e and α_f. The ternary correction factor, t, is defined as

$$t = \frac{\alpha_{ac}E}{2g_{ac}}, \quad \text{Eqn 6}$$

where E is transpiration rate, g_{ac} is the combined boundary layer and stomatal conductance to CO_2, and α_{ac} is defined as $1+\bar{a}$, where $\bar{a}$ is the weighted fractionation for diffusion across the boundary layer and stomata in series:

$$\bar{a} = \frac{a_b(c_a-c_s)+a_s(c_s-c_i)}{c_a-c_i}. \quad \text{Eqn 7}$$

The magnitudes of e and f are currently under investigation, and a range of values has been reported. Recent estimates for e range from *c.* 0 to 5‰ (Tcherkez *et al.*, 2004, 2010, 2011b). Recent estimates for f range from *c.* 8 to 16‰ (Gillon & Griffiths, 1997; Lanigan *et al.*, 2008; Evans & von Caemmerer, 2013), with a value of 11‰ suggested on theoretical grounds (Tcherkez, 2006). For instantaneous measurements of Δ_3, the inferred value of f depends on the assumed value for b. Evans & von Caemmerer (2013) recently found that a value for f of 16‰ fit their data for tobacco best when b was assumed to be 29‰.

An apparent respiratory fractionation can occur when Δ_3 is measured instantaneously under an atmosphere with $\delta^{13}C$ differing from that which the plant was exposed to in the hours to days leading up to the measurement. In this situation, the respired CO_2 can have a different isotopic composition than it would have in the steady state, owing to the change in $\delta^{13}C$ of the source CO_2 for photosynthesis. Under these circumstances, the value for e can be replaced by $e_{Rd}+e^*$ to account for the difference between $\delta^{13}C$ of assimilate produced during photosynthesis and that of the likely substrate for respiration (Wingate *et al.*, 2007). Here e_{Rd} would be the fractionation during day respiration and e^* would be $\delta_a-\Delta_{obs}-\delta_{substrate}$, where δ_a is the $\delta^{13}C$ of CO_2 in the cuvette during online measurements, Δ_{obs} is the observed discrimination, and $\delta_{substrate}$ is the $\delta^{13}C$ of likely respiratory substrates.

Online determinations of Δ_3 have proven useful in recent years for estimating mesophyll conductance, g_m, when combined with measurements of leaf gas exchange (Evans *et al.*, 1986; Pons *et al.*, 2009). Observed values of Δ_3 are less than predicted for the case where $c_c=c_i$; this discrepancy forms the basis for estimating g_m. Low values of g_m can significantly constrain photosynthesis (Flexas *et al.*, 2012). Manipulating g_m may therefore provide a means of increasing photosynthesis and water-use efficiency in crop plants (Barbour *et al.*, 2010).

The g_m can be estimated by first defining the predicted Δ_3 when $c_c=c_i$, termed Δ_i, which would apply if g_m were infinite (Evans *et al.*, 1986; Farquhar & Cernusak, 2012):

$$\Delta_i = \frac{1}{1-t}\left[a_b\frac{c_a-c_s}{c_a}+a_s\frac{c_s-c_i}{c_a}\right] + \frac{1+t}{1-t}\left[b\frac{c_i}{c_a}-\frac{\alpha_b}{\alpha_e}e\frac{R_d}{A+R_d}\frac{c_i-\Gamma^*}{c_a}-\frac{\alpha_b}{\alpha_f}f\frac{\Gamma^*}{c_a}\right]. \quad \text{Eqn 8}$$

Eqn 8 differs from Eqn 5 in that c_c has been replaced by c_i, and c_c/V_c has been replaced by $(c_i-\Gamma^*)/(A+R_d)$. The latter substitution is made because A is the term that is actually measured by instantaneous gas exchange, rather than V_c. The difference between Δ_i and the observed Δ_3 (Δ_{obs}) can then be used to estimate g_m:

$$g_m = \frac{1+t}{1-t}\left(\frac{b-a_m-\frac{a_b}{a_e}e\frac{R_d}{A+R_d}}{\Delta_i-\Delta_{obs}}\right)\frac{A}{Pc_a}, \quad \text{Eqn 9}$$

where P is atmospheric pressure. The P enters the equation here because it is preferable to derive g_m from a draw-down in partial pressure rather than mole fraction, so that it is independent of the temperature sensitivity of the solubility of CO_2. The g_m calculated in this way has dimensions of mol m^{-2} s^{-1} bar^{-1}. The ternary correction can have a significant effect on the calculated g_m, particularly at high v (Farquhar & Cernusak, 2012; Evans & von Caemmerer, 2013). The effect of the ternary correction on g_m determinations in tobacco is shown for a range of v in Fig. 1.

The derivation of Eqn 5 assumes that CO_2 is released from mitochondria in the same compartment within the cell as that in which Rubisco is located. The assumption has been justified based

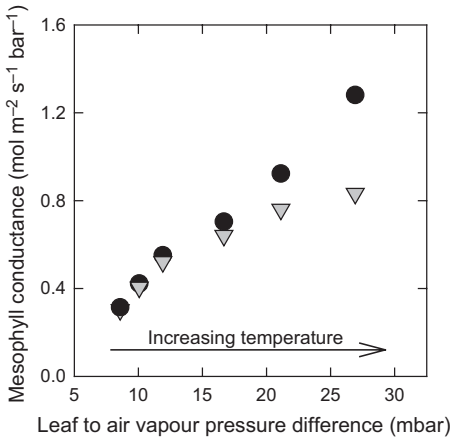

Fig. 1 Mesophyll conductance (g_m) in tobacco calculated without (circles) and with (triangles) the ternary correction plotted as a function of leaf to air vapour pressure difference (v). The source of variation in g_m was leaf temperature, but g_m is plotted here as a function of v to show the sensitivity of the ternary correction to v. (Redrawn from Evans & von Caemmerer (2013), with permission.)

on the observation that chloroplasts generally line mesophyll cell walls adjacent to intercellular air spaces, with mitochondria located deeper inside the mesophyll cells. Thus, CO_2 produced by mitochondria would have to diffuse through chloroplasts to reach the intercellular air spaces. For modelling purposes, this is equivalent to the mitochondrial CO_2 being evolved within the chloroplasts. Recently, it has been suggested that this assumption may not always hold (Tholen & Zhu, 2011; Tholen *et al.*, 2012; Busch *et al.*, 2013). Evans & von Caemmerer (2013) presented an alternative formulation of Eqn 5, which allows the diffusion resistance between mitochondria and the intercellular air space (r_w) to be less than that between the sites of Rubisco and the intercellular air space (r_m). Assuming $r_w = 0.5\,r_m$ in their alternative formulation of Eqn 5 led to an increase in estimated g_m of *c.* 10% at $[O_2]$ of 21% (Evans & von Caemmerer, 2013). The difference between the two estimates of g_m varies as a function of $[O_2]$ because the rate of mitochondrial CO_2 release associated with photorespiration varies with $[O_2]$. To put the issue into context, assuming $r_w = 0.5\,r_m$ in the alternative formulation of Eqn 5 had a similar effect on estimated g_m as assuming $f = 16‰$ vs $f = 11‰$ (Evans & von Caemmerer, 2013).

Eqn 5 predicts Δ_3 during photosynthesis. If the $\delta^{13}C$ of the CO_2 in air is known, then Eqn 5 can be used to estimate the $\delta^{13}C$ of carbon taken up by a C_3 leaf in the light. Some of this carbon will be incorporated into structural plant material, some stored in nonstructural organic compounds, and some respired back to the atmosphere. The metabolic processes that take place after the photosynthetic carbon reduction cycle provide opportunities for fractionation and for an unequal distribution of the $^{13}C/^{12}C$ ratio across different plant organs and metabolic compartments. Considerable research effort is currently directed towards teasing apart the intricate details of these processes, including the roles of dark respiration, phloem loading, anaplerotic CO_2 fixation, and secondary metabolism (Hobbie & Werner, 2004; Tcherkez *et al.*, 2004, 2011a; Badeck *et al.*, 2005; Bowling *et al.*, 2008; Cernusak *et al.*, 2009a; Werner & Gessler, 2011). Post-photosynthetic fractionations can cause Δ as determined from plant biomass to differ from Δ measured on line during photosynthetic carbon uptake.

Eqn 5 can be simplified to a form that omits effects other than those of diffusion through stomata and carboxylation (Farquhar *et al.*, 1982b). While the simplified form is clearly an approximation, it has been sufficient for many applications:

$$\Delta_3 = a + (b - a)\frac{c_i}{c_a}. \qquad \text{Eqn 10}$$

Here a is the diffusional fractionation, taken as 4.4‰, and b is carboxylation fractionation. In this form of the model, b is usually taken as 27‰, which allows for a reduction in fractionation caused by the terms in Eqn 5 that are omitted from Eqn 10, mainly the draw-down in CO_2 mole fraction from c_i to c_c.

Eqn 10 provides a means of estimating c_i/c_a from measurements of δ_p, assuming δ_a is known. For the most part, the CO_2 in the atmosphere is well mixed, and δ_a can be assumed mostly constant, although it may vary in closed canopy forests, glasshouses, and other situations where the turbulent exchange of air between the plant canopy and the free troposphere is impeded. There is a seasonal cycle in tropospheric δ_a with an amplitude approaching 1‰ at high northern latitudes. The amplitude decreases toward the equator and very little seasonal cycle is observed in the southern hemisphere (Trolier *et al.*, 1996). In addition, the δ_a of the troposphere has decreased during the industrial period as a result of the combustion of fossil fuels and the consequent release of ^{13}C-depleted CO_2 into the atmosphere. For analyses of plant organic material formed over a range of time periods, such as in tree rings, this chronological decrease in δ_a should be accounted for. This can be accomplished with data from a combination of direct measurements of the $\delta^{13}C$ of atmospheric CO_2 and measurements of CO_2 in air trapped in ice bubbles (Feng, 1998; McCarroll & Loader, 2004).

Eqn 10 predicts that δ_p should vary as a function of c_i/c_a in C_3 plants if δ_a is relatively constant. This prediction generally holds. Fig. 2 shows the $\delta^{13}C$ of leaf dry matter plotted against instantaneous measurements of c_i/c_a for seedlings of 44 tree species. The dataset includes both conifer and angiosperm species (Orchard *et al.*, 2010). A geometric mean regression through the dataset yields the following equation: $\delta_p(‰) = -12.1\text{–}22.0\ c_i/c_a$. This is close to the predicted relationship assuming $\delta_a = -8‰$, $a = 4.4‰$ and $b = 27‰$, which would be $\delta_p(‰) = -12.4\text{–}22.6\ c_i/c_a$. The seedlings were grown either in the open air or in well-ventilated shade houses under well-watered conditions. Comparisons between plant dry matter $\delta^{13}C$ and instantaneous c_i/c_a can be problematic if ontogeny or a change in environmental conditions causes c_i/c_a at the time of instantaneous measurements to differ from the integral over the course of tissue synthesis. This has been avoided in Fig. 2 by selecting species that have a prolonged vegetative stage (trees) and for which no dramatic change in environmental conditions was imposed. The average standard deviation for individuals within a species across the dataset shown in Fig. 2 was 0.6‰.

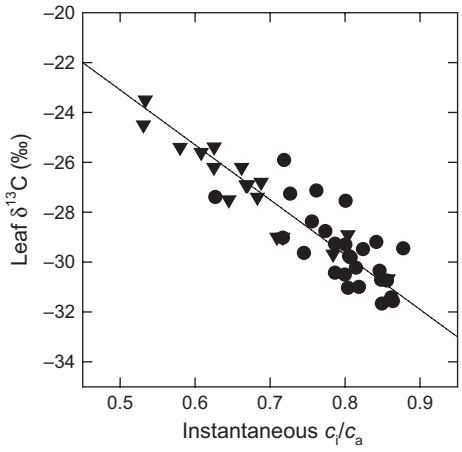

Fig. 2 Leaf carbon isotope ratio ($\delta^{13}C$) plotted against the instantaneous ratio of intercellular to ambient CO_2 mole fractions (c_i/c_a) during photosynthesis. Data are for well-watered tree seedlings (angiosperms, circles; conifers, triangles), grown either outdoors or in well-ventilated shade houses (Brodribb & Hill, 1998; Cernusak *et al.*, 2007, 2008; Orchard *et al.*, 2010). Each data point represents the average of several individuals of one species. Species' identities are detailed in Orchard *et al.* (2010). The solid line is a geometric mean regression: leaf $\delta^{13}C$ (‰) = −12.1–22.0 c_i/c_a.

Although some authors have recently speculated that cross-species comparisons of c_i/c_a based on δ_p could be problematic (Warren & Adams, 2006; Cernusak *et al.*, 2009b; Salmon *et al.*, 2011), Fig. 2 shows that this is really a matter of how broad a view one takes. There are variations around the regression line relating δ_p to c_i/c_a of *c.* 1–3‰ in δ_p for a given c_i/c_a. These could relate to small variations in δ_a, to temporal or spatial variations in c_i/c_a, to the terms in Eqn 5 that are omitted from Eqn 10, or to postphotosynthetic processes not included in Eqn 5. However, it is also clear that δ_p is strongly related to c_i/c_a, with *c.* 80% of among-species variation in δ_p in Fig. 2 explained by c_i/c_a. Whether one should focus on the regression line in Fig. 2 or on the residuals around it depends on the research question being asked.

Measurement of δ_p in C_3 plants is especially powerful for being able to provide a time-integrated estimate of c_i/c_a (Farquhar *et al.*, 1982a). The c_i/c_a is a function of the supply of CO_2 to the leaf intercellular air spaces by stomata and the demand for CO_2 by photosynthetic capacity within the mesophyll. For a given c_a and v, c_i/c_a relates to photosynthetic water-use efficiency (Farquhar & Richards, 1984), defined as the ratio of photosynthesis (A) to transpiration (E):

$$\frac{A}{E} = \frac{c_a\left(1 - \frac{c_i}{c_a}\right)}{1.6v}. \qquad \text{Eqn 11}$$

As a result of the relationship between c_i/c_a and A/E, δ_p can be used, for example, to identify C_3 crop genotypes that potentially have a high water-use efficiency (Rebetzke *et al.*, 2002, 2008; Richards *et al.*, 2002), to assess the water-use efficiency response of trees to rising c_a through measurements of δ_p in tree rings (Francey & Farquhar, 1982; Marshall & Monserud, 1996; Feng, 1999; Loader *et al.*, 2011), and to test models of optimal stomatal behaviour in response to environmental gradients (Farquhar *et al.*, 2002; Wright *et al.*, 2003; Medlyn *et al.*, 2011).

The supply of CO_2 to the leaf intercellular air spaces is mainly controlled by the concentration of CO_2 outside the leaf, c_a, and by stomatal conductance, g_s (Farquhar & Sharkey, 1982). Stomata generally function to allow CO_2 to diffuse into leaves when conditions are favourable for photosynthesis, while at the same time preventing water loss from leaves at rates that would lead to excessive dehydration and impaired photosynthetic capacity. But stomata are not simply open or closed; their degree of opening is a continuous variable that more strongly limits the diffusion of CO_2 into leaves when the supply of soil water is relatively low and when the evaporative demand of the atmosphere is relatively high (Marshall & Waring, 1984). As a result, variation in Δ_3 can be expected in response to the availability of soil moisture and the atmospheric vapour pressure deficit. This can be manifested along precipitation gradients, or, more precisely, along moisture availability gradients that take into account both the local availability of soil water and potential evapotranspiration.

Two meta-analyses have recently summarized Δ_3 responses to mean annual precipitation (Diefendorf *et al.*, 2010; Kohn, 2010). Both found decreasing Δ_3 with decreasing mean annual precipitation, which explained about half the variation in Δ_3 (Fig. 3). Based on these results, it was argued that paleoprecipitation could be reconstructed from ancient plant carbon, preserved, for example, in fossil tooth enamel (Kohn, 2010, 2011). This was disputed on the grounds that such paleoprecipitation estimates would not be meaningful as a result of the high variability in Δ_3 among different species at a given mean annual precipitation (Freeman *et al.*, 2011). Such differences among tree species, when grown under well-watered conditions, are demonstrated in Fig. 2. Ultimately, the utility of $\delta^{13}C$ analyses of ancient plant carbon for reconstructing paleoprecipitation will depend on the degree of uncertainty that one is willing to tolerate (Kohn, 2011).

Nutrient availability can influence c_i/c_a and Δ_3 through effects on photosynthetic capacity. Photosynthetic capacity partly determines

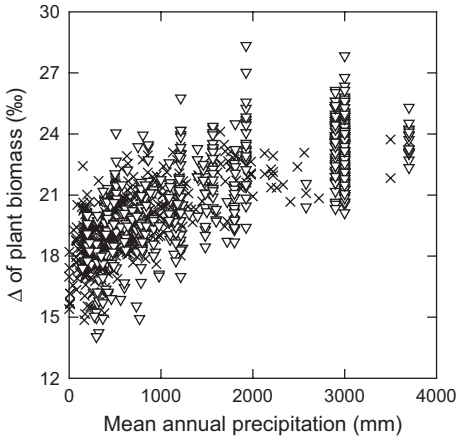

Fig. 3 Carbon isotope discrimination (Δ) of C_3 plant biomass plotted against mean annual precipitation. Data are from two meta-analyses (Diefendorf *et al.*, 2010; Kohn, 2010). For the Diefendorf *et al.* data set (triangles), each data point is a species by site combination. For the Kohn data set (crosses), each data point is a site average.

the demand for CO_2 in the leaf mesophyll. The nutrient that has been best studied in this regard is nitrogen, which has a well-known relationship with photosynthesis (Field & Mooney, 1986). Fig. 4 shows an example of variation in instantaneous c_i/c_a and Δ_3 of plant biomass as a function of leaf nitrogen concentration for seedlings of a tropical pioneer tree, *Ficus insipida*, grown under varying soil fertilities (Cernusak *et al.*, 2007). Leaf δ_p was also observed to vary as a function of leaf nitrogen concentration within the crowns of mature trees of a range of conifer species (Duursma & Marshall, 2006). Other nutrients, such as phosphorus, would be expected to correlate with variation in Δ_3 if they cause variation in photosynthetic capacity (Domingues *et al.*, 2010).

Irradiance can also affect Δ_3. Plants exposed to low photon flux densities show higher c_i/c_a and higher Δ_3 associated with low photosynthesis rates (Ehleringer *et al.*, 1986). At photon flux densities above *c.* 250 μmol m^{-2} s^{-1}, c_i/c_a tends to be independent of irradiance (Wong *et al.*, 1978; Farquhar & Wong, 1984), but Δ_3 may still decrease because of an increasing draw-down from c_i to c_c associated with increasing photosynthesis (Evans *et al.*, 1986; Farquhar *et al.*, 1989).

In addition, c_i/c_a and Δ_3 generally decline with tree height independent of irradiance (Marshall & Monserud, 1996; Ryan & Yoder, 1997; Koch *et al.*, 2004; McDowell *et al.*, 2011), although not always (Barnard & Ryan, 2003). This decline has been attributed to two hydraulic issues: the maintenance of lower water potentials with height above the soil water supply (Koch *et al.*, 2004), and the frictional resistance to water flux, which increases with path length as stems grow taller (Ryan & Yoder, 1997; Becker *et al.*, 2000). Either of these effects would tend to reduce leaf water potential and stomatal conductance, unless there were compensating shifts in leaf area to sapwood area ratio (Barnard & Ryan, 2003). Such potential height effects should be considered when comparing tree ring δ_p among locations and over time (Marshall & Monserud, 1996; McDowell *et al.*, 2011).

The δ_p of C_3 plant biomass generally increases with increasing elevation above sea level at a rate of *c.* 1– 2‰ km^{-1} of elevation gain (Körner *et al.*, 1988, 1991; Marshall & Zhang, 1994; Hultine & Marshall, 2000). This pattern is general, occurring in both herbs (Körner *et al.*, 1988, 1991) and trees (Vitousek *et al.*, 1990; Marshall & Zhang, 1994). The pattern appears to be linked to both decreasing temperature and decreasing atmospheric pressure as elevation increases (Körner *et al.*, 1991). Decreasing oxygen partial pressure reduces c_i/c_a by increasing the carboxylation efficiency of Rubisco (Farquhar & Wong, 1984). Decreasing temperature causes the viscosity of water to increase. This may slow the transport of water from the soil to the evaporative sites in leaves (Roderick & Berry, 2001), thereby decreasing stomatal conductance and the diffusion of CO_2 into leaves, resulting in lower c_i/c_a. There are also changes in leaf morphology with elevation that could contribute to the trend in Δ_3 (Hultine & Marshall, 2000; Zhu *et al.*, 2010).

The preceding discussion demonstrates that Δ_3 responds to environmental controls that influence the balance between supply of CO_2 to carboxylation sites within the leaf and demand for CO_2 by photosynthesis. However, C_3 plants have also been observed to maintain a relatively constant c_i/c_a in the face of a changing growth environment (Wong *et al.*, 1979, 1985; McDowell *et al.*, 2006; Franks *et al.*, 2013). Herein lies the tension between viewing Δ_3 as a universal sensor that responds to, for example, water availability (Prentice *et al.*, 2011) or temperature (Körner *et al.*, 1991) and viewing Δ_3 as a homeostatic set point that differs among species and genotypes (Comstock & Ehleringer, 1992). The two viewpoints both reflect reality, and at the same time oppose one another. Environmental factors modify Δ_3, but internal physiology constrains the response. The balance between environmental forcing on Δ_3 and physiological damping of the response appears to vary among species. This is shown in Fig. 5 for three tropical tree species, which were grown under conditions of high and low water supply, and with and without added fertilizer (Cernusak *et al.*, 2009b). *Swietenia macrophylla* showed a much more pronounced response of Δ_3 to the experimental treatments than did *Tectona grandis* or *Platymiscium pinnatum*.

The response of Δ_3 to environmental gradients can be damped by coordination between stomatal conductance and photosynthetic capacity, which reduces the realized range of variation in c_i/c_a (Wong *et al.*, 1979, 1985; Cernusak & Marshall, 2001; Hetherington & Woodward, 2003; Cernusak *et al.*, 2011). This was the case for several eucalypt species sampled along a rainfall

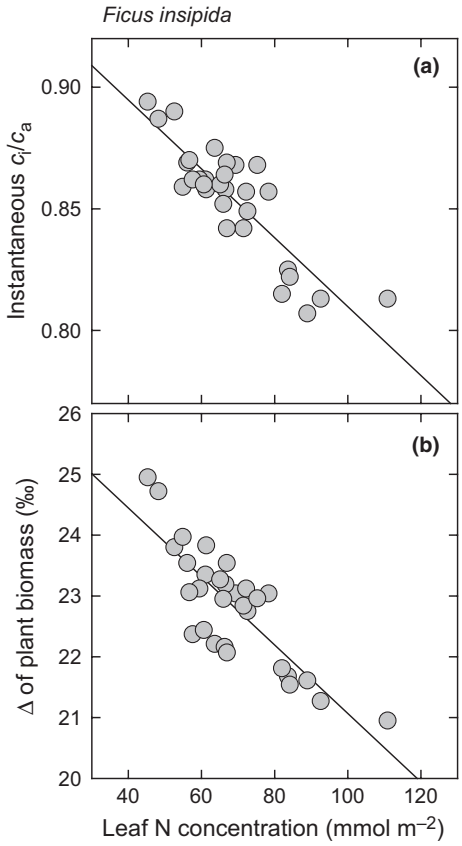

Fig. 4 The ratio of intercellular to ambient CO_2 mole fractions (c_i/c_a) measured instantaneously plotted against leaf nitrogen concentration (a) and carbon isotope discrimination (Δ) of plant biomass plotted against leaf nitrogen concentration (b) for seedlings of *Ficus insipida* grown under varying soil fertility. Solid lines are least-squares linear regressions. (Redrawn from Cernusak *et al.* (2007), with permission.)

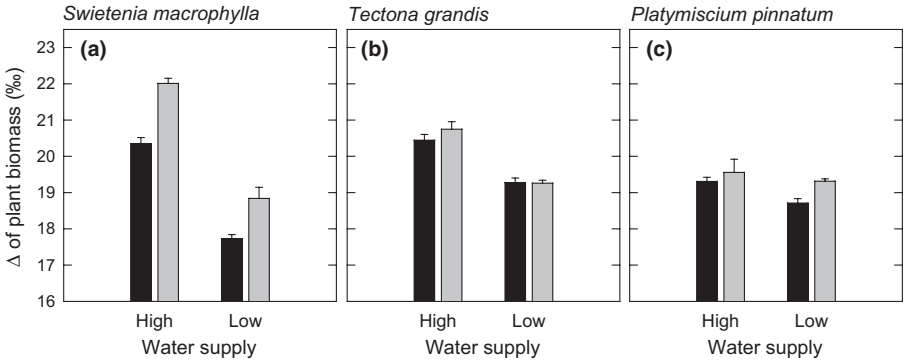

Fig. 5 Carbon isotope discrimination (Δ) of plant biomass for seedlings of three tropical tree species grown under high and low water supply and with (black bars) and without (grey bars) added fertilizer (Cernusak *et al.*, 2009b). Bars are means for five plants and error bars are +1 SE.

gradient in northern Australia (Fig. 6). The dashed line in Fig. 6 shows the expected relationship between c_i/c_a and g_s, if photosynthetic capacity had remained constant across the range of g_s. Instead, photosynthetic capacity decreased with decreasing g_s, such that c_i/c_a showed only a weak relationship with g_s. Although such close coordination between g_s and photosynthetic capacity has been known for some time (Wong *et al.*, 1978, 1979, 1985), the mechanisms through which it is achieved are still not well understood (Jarvis *et al.*, 1999; von Caemmerer *et al.*, 2004).

A second process that can damp the response of c_i/c_a and Δ_3 to environmental gradients, particularly of water availability, is adjustment of the ratio of leaf area to water-conducting tissue (Farquhar *et al.*, 2002). An example of this type of adjustment occurs along the north Australian tropical transect, a rainfall

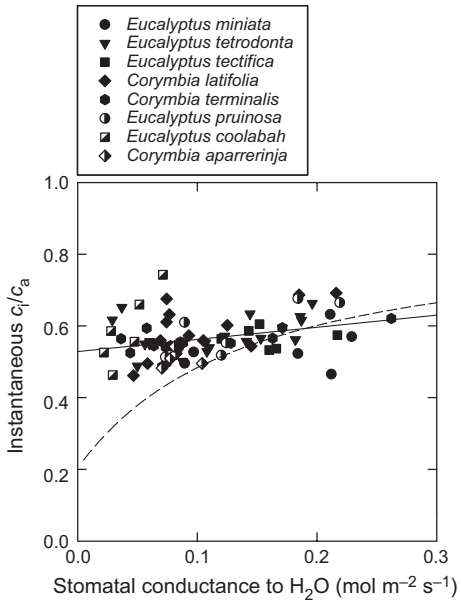

Fig. 6 The ratio of intercellular to ambient CO_2 mole fractions (c_i/c_a) plotted against stomatal conductance for several eucalypt species growing along the north Australian tropical transect. The solid line is a least-squares linear regression through the data. The dashed line is the predicted relationship between the two parameters if the maximum Rubisco carboxylation velocity, the electron transport rate and the mesophyll conductance had remained constant over the range of stomatal conductance. (The figure is from Cernusak *et al.* (2011), with permission.)

gradient in northern Australia (Hutley *et al.*, 2011). Little change was observed in the Δ_3 of plant biomass in trees along the transect (Schulze *et al.*, 1998; Miller *et al.*, 2001; Cernusak *et al.*, 2011), in contrast to what would be expected based on the global meta-analyses shown in Fig. 3 and observations along other rainfall gradients (Stewart *et al.*, 1995; Midgley *et al.*, 2004; Prentice *et al.*, 2011). Instantaneous gas exchange measurements confirmed that c_i/c_a changes little along the transect, even towards the end of the dry season when the north–south gradient in water availability is most pronounced (Cernusak *et al.*, 2011). Eucalypts along the transect reduce their leaf area to sapwood area ratios from wet to dry seasons, thereby reducing the total leaf area for a given amount of water transporting tissue, allowing for maintenance of relatively constant c_i/c_a (Eamus *et al.*, 2000; Cernusak *et al.*, 2011). This leaf area adjustment may be possible owing to the highly predictable nature of the wet season, and this could explain why the northern Australian rainfall gradient is associated with much less variation in Δ_3 than other rainfall gradients associated with more stochastic precipitation regimes.

Manipulative experiments provide further evidence for the role of leaf area adjustment in constraining variation in c_i/c_a and Δ_3 (Cernusak & Marshall, 2001; McDowell *et al.*, 2006). In one example, *Pinus ponderosa* stands were thinned to seven densities and maintained over a 40 yr period. The ratio of leaf area to sapwood area increased as a result of decreased basal area in thinned stands. There was an initial increase in Δ_3 after thinning. However, after one decade, Δ_3 returned to control values in thinned stands as the increase in leaf area to sapwood area ratio compensated for increased water availability associated with reduced basal area (McDowell *et al.*, 2006).

It is clear that there are elements of both environmental control and genetically determined physiological set points in Δ_3 (Ehleringer, 1993a,b; Zhang & Marshall, 1994; Ehleringer & Cerling, 1995). This can present a challenge for interpretation, depending on the application for which Δ_3 measurements are employed. Research priorities for gaining further insight into this interplay should be to unravel the mechanisms that lead to coordination between g_s and photosynthetic capacity, to determine how the statistics of rainfall regimes influence the response of Δ_3 to water availability, including the role of leaf area adjustment, and to determine the specific phenotypic traits associated with genotypic variation in Δ_3 (Masle *et al.*, 2005; Liang *et al.*, 2010).

IV. The C_4 photosynthetic pathway

The C_4 photosynthetic pathway is more recently derived than the ancestral C_3 pathway (Edwards *et al.*, 2010; Sage *et al.*, 2012). It occurs mainly in grasses, in some dicotyledonous herbs, shrubs, and in a small number of trees (Pearcy & Troughton, 1975; Sage *et al.*, 2011). The C_4 pathway concentrates CO_2 around Rubisco, thereby greatly reducing photorespiration relative to C_3 plants. C_4 plants often have Kranz anatomy (Haberlandt, 1914), meaning that Rubisco is compartmentalized within the bundle-sheath cells, although C_4 photosynthesis can also occur within a single cell (Voznesenskaya *et al.*, 2001).

C_4 plants have lower Δ (Δ_4) than C_3 plants. The difference can be used to identify species that employ the C_4 photosynthetic pathway (Bender, 1968). The lower Δ_4 is a consequence of the processes and enzymes involved in C_4 photosynthesis. Initially, bicarbonate is fixed in the mesophyll cells by PEP (phosphoenolpyruvate) carboxylase to form four-carbon acids (Hatch *et al.*, 1967). Bicarbonate is enriched in ^{13}C compared with CO_2 and discrimination against ^{13}C by PEP carboxylase is much less than by Rubisco, the primary carboxylating enzyme in C_3 plants. The C_4 acids fixed by PEP carboxylase then move to the bundle-sheath cells where they are decarboxylated, raising the [CO_2] around Rubisco. Some of the carbon that is fixed by PEP carboxylase leaks out of the bundle-sheath cells, with the proportion termed leakiness (ϕ). The extent of ϕ is determined by the bundle-sheath conductance to CO_2, which depends on physical properties, such as the presence of a suberized lamella (Hattersley, 1982; Hatch *et al.*, 1995), and on the [CO_2] gradient between the bundle-sheath and mesophyll cells, which in turn depends on the balance between PEP carboxylase and Rubisco activities (Peisker & Henderson, 1992). The ϕ plays an important role in determining Δ_4 variations in C_4 plants, as it controls the extent to which Rubisco fractionation is expressed.

A model to describe Δ_4 was first published by Farquhar (1983) and has recently been updated to include ternary effects (Farquhar & Cernusak, 2012):

$$\Delta_4 = \frac{1}{1-t}\left[a_b\frac{c_a-c_s}{c_a}+a_s\frac{c_s-c_i}{c_a}\right] + \frac{1+t}{1-t}\left[a_m\frac{c_i-c_m}{c_a}+\frac{b_4+\phi\left(\frac{b_3 c_{bs}}{c_{bs}-c_m}-s\right)}{1+\frac{\phi c_m}{c_{bs}-c_m}}\frac{c_m}{c_a}\right], \quad \text{Eqn 12}$$

where b_4 is the effective fractionation by PEP carboxylase (−5.7‰ at 25°C), b_3 is the fractionation by Rubisco (*c.* 29‰), and s is fractionation during diffusion of CO_2 out of the bundle-sheath cells (1.8‰). The c_m and c_{bs} are the CO_2 mole fractions in the mesophyll cytosol and in the bundle-sheath, respectively. Elaborations to take into account day respiration, photorespiration and incomplete equilibration between CO_2 and bicarbonate can also be included (Farquhar, 1983).

As with Eqn 5, Eqn 12 can be simplified to a more manageable form (Farquhar, 1983):

$$\Delta_4 = a\frac{c_a-c_i}{c_a}+[b_4+\phi(b_3-s)]\frac{c_i}{c_a}. \quad \text{Eqn 13}$$

Eqn 13 neglects ternary effects, assumes CO_2 is in equilibrium with bicarbonate in the mesophyll cytoplasm, assumes negligible fractionation during photorespiration and dark respiration, assumes g_m is infinite, and assumes the CO_2 partial pressure inside the bundle-sheath cells is much larger than in the mesophyll cells. The impact of these simplifications should be carefully considered when the formulation is used to calculate ϕ (Ubierna *et al.*, 2011).

Variation in Δ_4 in response to environmental and genetic drivers is small but significant. Differences among plants are often in the range of 1–3‰, much smaller than for C_3 plants (Fig. 2). Interpreting the variation in Δ_4 is challenging because it cannot be attributed to a single major factor, as is generally the case with c_i/c_a in C_3 species. In C_4 species, even in the simplest case scenario presented in Eqn 13, Δ_4 depends on both c_i/c_a and ϕ (Fig. 7a). There is either a positive or negative correlation between Δ_4 and c_i/c_a, depending on whether ϕ is larger or smaller than *c.* 0.37 [$\approx (a-b_4)/(b_3-s)$]. Moreover, the linear relationship between Δ_4 and c_i/c_a described by Eqn 13 is a simplification, and the full model relating Δ_4 to c_i/c_a, expressed in Eqn 12, has some curvature (Fig. 7b).

In C_4 species, δ_p of leaf dry matter has been shown to range from −9.2‰ to −19.3‰ (Hattersley, 1982), but most values are concentrated in a narrow band between −11‰ and −14‰, with a global average of *c.* −12.5‰ (Cerling *et al.*, 1997). For comparison, the global average for C_3 plants is *c.* −28‰ (Kohn, 2010). C_4 grasses are grouped into three subtypes depending on the major enzyme used to decarboxylate the C_4 acids: NAD-ME (NAD-malic enzyme), NADP-ME (NADP-malic enzyme) and PEPCK (phosphoenolpyruvate carboxykinase). When plants were grown under controlled conditions to minimize environmental variation, leaf δ_p spanned *c.* 3‰, with small but significant differences across subtypes: NAD-ME, −12.7‰; PEPCK, −12.0‰; NADP-ME, −11.4‰ (Hattersley, 1982).

Currently, there is no satisfactory explanation for differences in Δ_4 across subtypes. As described earlier, Δ_4 depends on both ϕ and c_i/c_a. The ϕ has been shown to differ across subtypes, with variation related to morphological features such as the presence of suberized lamellae around the bundle-sheath cells. The ϕ was estimated to be higher in the NAD-ME subtype that lacks suberized lamellae (Hattersley, 1982; Hatch *et al.*, 1995). The lack of suberized lamellae in the NAD-ME subtype might be counterbalanced by other morphological features; for example, this subtype has elongated chloroplasts surrounding the mitochondria (Hattersley & Browning, 1981). Furthermore, the presence of suberized lamellae cannot be the only factor accounting for isotopic differences among subtypes, as both NADP-ME and PEPCK have suberized lamellae and yet differed in δ_p (Hattersley, 1982). The NADP-ME subtype has reduced grana in the bundle-sheath chloroplasts (Hatch, 1987; Pfündel & Neubohn, 1999), and consequently less photosystem II activity and O_2 evolution than the other subtypes (Yoshimura *et al.*, 2004; Gowik & Westhoff, 2011). Photorespiration is not always an unwanted process; under stress conditions it serves as an energy sink to prevent overreduction of the photosynthetic electron transport chain (Osmond & Grace, 1995). Interestingly, the NADP-ME subtype, with reduced O_2 evolution from the bundle-sheath cells, occurs in mesic environments,

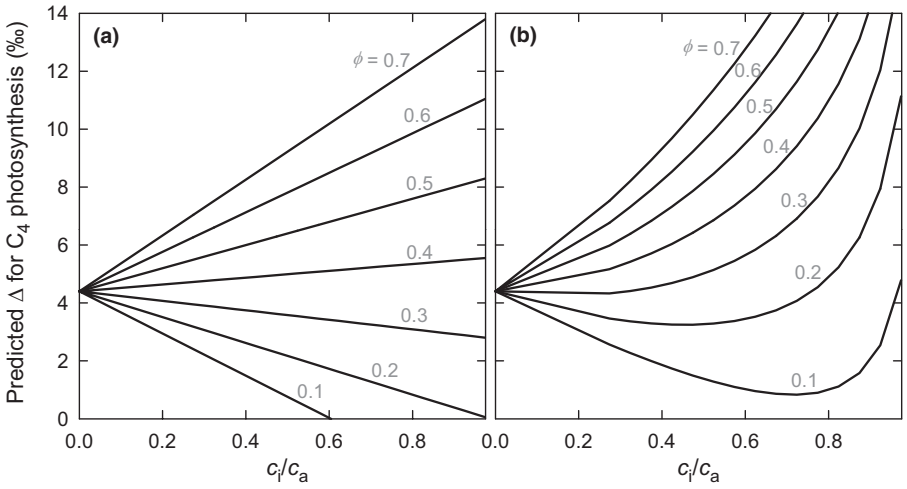

Fig. 7 Predicted carbon isotope discrimination (Δ) in C_4 plants as a function of the ratio of intercellular to ambient CO_2 mole fractions (c_i/c_a) during photosynthesis. Different lines represent the relationship for different values of leakiness (ϕ). Relationships in (a) are for the simplified model presented in Eqn 13, and relationships in (b) for the nonsimplified model presented in Eqn 12. Full details of the calculations are provided in Ubierna *et al.* (2011). Bundle-sheath conductance was assumed to be 0.01 mol m^{-2} s^{-1} bar^{-1}.

whereas the NAD-ME subtype occurs in drier areas (Hattersley, 1982; Schulze *et al.*, 1996). Cousins *et al.* (2008) showed that NAD-ME leaves had lower δ_p than did NADP-ME leaves, but instantaneous Δ_4 did not differ between subtypes. This observation suggests that postphotosynthetic fractionations might play a role in determining δ_p.

The response of Δ_4 to drought can be positive, negative, or insignificant, depending on ϕ, which contrasts with the consistent directional response in C_3 plants. Under most environmental conditions, ϕ is < 0.37, and Δ_4 is expected to increase with decreasing water availability as a result of decreasing c_i/c_a (Fig. 7). Buchmann *et al.* (1996) and Ghannoum *et al.* (2002) reported increases in Δ_4 of < 1‰, with increasing drought stress in all biochemical subtypes. Increased Δ_4 in leaf dry matter with increasing drought stress has also been found at large geographical scales (Tieszen & Boutton, 1989; Liu *et al.*, 2005; Murphy & Bowman, 2009) and throughout different microhabitats (Wang *et al.*, 2005). There are also contrasting observations; for example, no relationship between δ_p of leaf dry matter and rainfall was reported across several sites in Africa (Swap *et al.*, 2004).

As in C_3 species, C_4 plants exhibit a relationship between Δ_4 and water-use efficiency, although the trend is generally muted and in the opposite direction to that for C_3 plants. Ghannoum *et al.* (2002) showed that increases in water-use efficiency with drought translated into significantly more depleted δ_p of leaf dry matter (0.5‰) for 18 grasses from the NAD-ME and NADP-ME subtypes. There was a negative correlation between water-use efficiency and δ_p of leaf dry matter in 30 lines of *Sorghum bicolor* (Henderson *et al.*, 1998). Depleted δ_p values in C_4 grasses have also been correlated with increased photosynthesis, growth and yield (Bowman *et al.*, 1989; Hubick *et al.*, 1990; Meinzer *et al.*, 1994; Buchmann *et al.*, 1996).

The largest reported variations in Δ_4 are in response to light. The increase in observed Δ_4 from high to low irradiance was *c.* 3‰ in *Zea mays* (Kromdijk *et al.*, 2010; Ubierna *et al.*, 2013) and *Miscanthus giganteus* (Kromdijk *et al.*, 2008; Ubierna *et al.*, 2013), and as much as 8‰ in *Flaveria bidentis* (Pengelly *et al.*, 2010; Ubierna *et al.*, 2013). Buchmann *et al.* (1996) found that in all C_4 subtypes, reduced light during growth resulted in about a 2‰ increase in Δ_4. The large Δ_4 at low irradiances has been interpreted as high ϕ (0.6–0.9) and inefficient functioning of the C_4 photosynthetic pathway (Cousins *et al.*, 2006; Tazoe *et al.*, 2006, 2008; Kromdijk *et al.*, 2008, 2010; Pengelly *et al.*, 2010). Recently, Ubierna *et al.* (2011, 2013) demonstrated that these reports of large ϕ might have resulted from the simplifications made to the theoretical model of Δ_4. If Eqn 13 was used to solve for ϕ, it resulted in very large values at low irradiances. However, when no simplifying assumptions were included in the Δ_4 calculations (Eqn 12), ϕ only slightly increased from high to low irradiance.

In fact, if ϕ is mostly constant and insensitive to environmental variation, then Δ_4 depends largely on c_i/c_a, which can be related to environmental variables in a predictable way. Unfortunately, ϕ cannot be measured directly, which has resulted in somewhat contradictory reports on its values and sources of variation. Leakiness has been estimated using ^{14}C labelling (Hatch *et al.*, 1995), mathematical modelling based on observations of quantum yield, O_2 inhibition of photosynthesis, or the inorganic carbon pool size (Farquhar, 1983; Jenkins *et al.*, 1989; He & Edwards, 1996), and coupled measurements of Δ_4 and leaf gas exchange (Evans *et al.*, 1986; von Caemmerer *et al.*, 1997). The latter is the most commonly used method, especially since the development of laser technologies for isotopic measurements. Initial studies used δ_p of leaf dry matter as a proxy for Δ_4 during photosynthesis. This approach resulted in differences in ϕ with environmental parameters such as drought (Saliendra *et al.*, 1996), across subtypes (Buchmann *et al.*, 1996), and overall larger ϕ values (0.3–0.5) than when it was derived from online Δ_4 measurements (0.2–0.3, Henderson *et al.*, 1992). Given the generally small δ_p variations among C_4 plants, using δ_p of leaf dry matter to estimate ϕ could be problematic in light of possible postphotosynthetic fractionations. The problem is further complicated by different integration times between leaf tissues and instantaneous gas fluxes.

The more reliable estimates of ϕ from instantaneous measurements of gas exchange and Δ_4 show it to be relatively constant over different CO_2 concentrations and temperatures (Henderson *et al.*, 1992), light intensities (Ubierna *et al.*, 2011, 2013), moderate drought stress (Williams *et al.*, 2001), C_4 subtypes (Cousins *et al.*, 2008), and genotypes (Henderson *et al.*, 1998). Thus as technology for making online determinations of Δ_4 has improved and

calculations have been refined, the emerging trend is for a relatively small (< 0.3) and constant ϕ under a wide range of conditions. Under these circumstances, Δ_4 is primarily influenced by c_i/c_a (Fig. 7).

C_4 grasses contribute significantly to the production of both food and biofuels, and therefore play an important role in human society. Determination of Δ_4 provides a tool to probe C_4 photosynthetic performance in relation to environmental and genotypic variation. To make full use of this tool, future research should investigate the mechanisms that cause variation between Δ_4 measured online and that measured in leaf dry matter, sources of variation of ϕ, and the extent to which and under what conditions Δ_4 of leaf dry matter reflects c_i/c_a. More studies using combined measurements of instantaneous gas exchange and Δ_4, together with fully parameterized models for Δ_4, should be useful for describing ϕ under varying conditions.

V. Crassulacean acid metabolism

Crassulacean acid metabolism (CAM) is a water-conserving mode of photosynthesis exhibited by *c.* 6% of vascular plant species (Smith & Winter, 1996). The CAM cycle involves the uptake of atmospheric CO_2 via PEP carboxylase in the dark and the overnight storage of the carbon in malic acid. During the following light period, the malic acid is decarboxylated and the CO_2 is refixed by Rubisco (Borland *et al.*, 2011). In addition to the CAM cycle, most plants with CAM can also fix atmospheric CO_2 directly via C_3 photosynthesis in the light. The relative proportion of CO_2 fixation in the dark and the light is species dependent, plant- and tissue-age dependent, and is modulated by the environment.

Carbon isotopic discrimination in CAM plants (Δ_{CAM}) is the result of this combination of C_4 and C_3 photosynthetic processes. The daytime fixation into carbohydrates of nocturnally produced malic acid would not introduce any further ^{13}C discrimination if all the malic acid were to be consumed and all the liberated CO_2 refixed by Rubisco. CAM plants can also take up atmospheric CO_2 during the day, especially in the afternoon when the nocturnally fixed malic acid pool has been consumed (Osmond, 1978). This CO_2 is usually fixed by Rubisco, although PEP carboxylase can also be involved (Griffiths *et al.*, 1990).

The Δ_{CAM} can be calculated as a photosynthesis-weighted average of nocturnal and daytime CO_2 uptake (Farquhar *et al.*, 1989):

$$\Delta_{CAM} = \frac{\int^D A\Delta_4 dt + \int^L A\Delta_3 dt}{\int^D A dt + \int^L A dt}, \quad \text{Eqn 14}$$

where A is photosynthesis rate, $\int^D dt$ and $\int^L dt$ are the time integrals in the dark and in the light, respectively, and Δ_4 and Δ_3 are carbon isotope discriminations during the C_4 and C_3 phases of carbon fixation, respectively. An expression for Δ_4 can be derived from Eqn 12 when $\phi = 0$:

$$\Delta_4 = \frac{1}{1-t}\left(a_b\frac{c_a - c_s}{c_a} + a_s\frac{c_s - c_i}{c_a}\right) + \frac{1+t}{1-t}\left(a_m\frac{c_i - c_m}{c_a} + b_4\frac{c_m}{c_a}\right). \quad \text{Eqn 15}$$

An expression for Δ_3 can be obtained from Eqn 5, with b now representing the flux-weighted average discrimination of ribulose bisphosphate and PEP carboxylations (Farquhar *et al.*, 1989). Several simplifications can be applied to these formulae, namely negligible ternary effects, large boundary layer and mesophyll conductances, and negligible respiratory and photorespiratory fractionations. This results in the following simplified expression (Farquhar *et al.*, 1989):

$$\Delta_{CAM} = a + \frac{\int^D A(b_4 - a)\frac{c_i}{c_a} dt + \int^L A(b - a)\frac{c_i}{c_a} dt}{\int^D A dt + \int^L A dt}. \quad \text{Eqn 16}$$

Elaborations can be made to these equations to account for processes such as continuing decarboxylation of malic acid in the afternoon when stomata open, and leakage of CO_2 during the middle of the day, when CO_2 released from malic acid is refixed (Griffiths *et al.*, 2007). It is not known to what extent these processes may influence Δ_{CAM} in the field.

In contrast to C_3 and C_4 plants, δ_p of plants with CAM can vary by > 20‰ (Silvera *et al.*, 2005). The range is broad, owing to the variable contributions of light and dark fixation, in addition to other environmental and internal influences that affect fractionation (Bender *et al.*, 1973; Osmond *et al.*, 1973). Any factor that alters the contributions of light and dark CO_2 uptake to carbon gain or alters the relative limitations of carboxylation and diffusion to CO_2 uptake, for example stomatal or mesophyll conductance, will influence the isotopic signal (Farquhar *et al.*, 1989).

Whole-tissue δ_p cannot be used *a priori* to distinguish plants in which CAM is weakly expressed from C_3 plants, or strong CAM plants from C_4 plants. Where CAM δ_p values overlap with values typical for C_3 or C_4 plants, supplementary characteristics such as nocturnal acidification and leaf gas exchange are required to confirm the operation of the CAM cycle (Fig. 8a).

The extensive range in δ_p of plants with CAM was illustrated in a study of orchids from Panama (Silvera *et al.*, 2005). The 87 species with increases in nocturnal acidity characteristic of CAM exhibited δ_p values between −11.8 and −32.3‰, a span of 20.5‰ (Fig. 8b). The least negative isotopic values were observed in plants with high capacities for nocturnal acidification. Forty of the species with low but significant amounts of nocturnal acidification had isotopic values that overlapped with those of 86 C_3 orchid species that lacked nocturnal acidification.

In a detailed analysis of the processes that shape Δ_{CAM} in succulent tissues of CAM plants, Griffiths *et al.* (2007) highlighted the importance of g_m. This constraint increases Δ_4 during nighttime PEP carboxylation and decreases Δ_3 during daytime CO_2 uptake, consistent with observations that short-term Δ_4 in the dark is more negative than predicted by models based on stomatal and carboxylation limitations alone (Holtum *et al.*, 1983; O'Leary *et al.*, 1986; Griffiths *et al.*, 1990; Roberts *et al.*, 1997; Winter & Holtum, 2005). Discrimination within the mesophyll probably contributed 1.4–2.5‰ to the nocturnal online Δ_4 signal in three massively succulent columnar cacti, *Trichocereus atacamensis*, *Carnegiea gigantea* and *Stenocereus thurberi*, which exhibited internal conductances among the lowest values reported for vascular plants (Williams *et al.*, 2012).

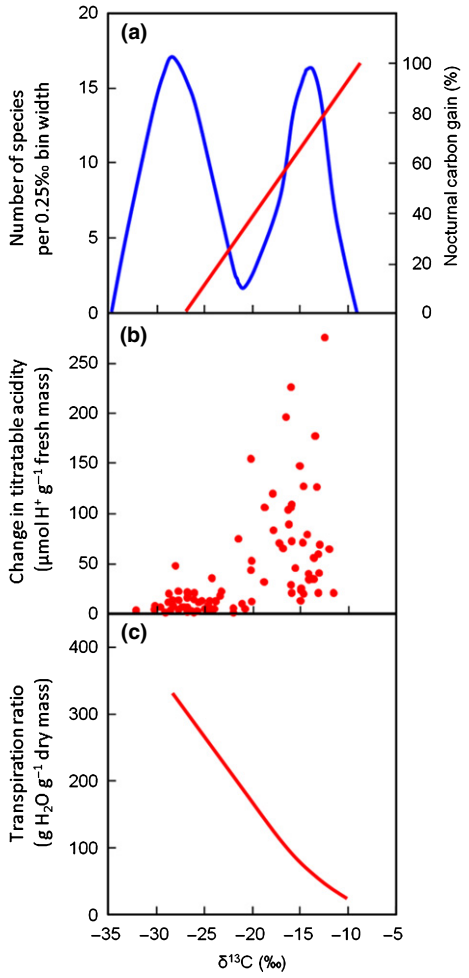

Fig. 8 (a) Bimodal distribution of $\delta^{13}C$ values (blue line) derived from 506 species from nine plant families containing C_3 and crassulacean acid metabolism (CAM) species. The blue line represents a histogram with 0.25‰ bin widths that has been smoothed (see Winter & Holtum, 2002, for sources). The red line indicates the relationship between $\delta^{13}C$ of plant carbon and the contribution of nocturnal CO_2 fixation to the production of this carbon (after Winter & Holtum, 2002, with permission). (b) The $\delta^{13}C$ values of 87 orchid species from Panama that exhibited statistically significant nocturnal increases in titratable acidity, based on data from Silvera *et al.* (2005). (c) The relationship between gravimetrically determined transpiration ratio and $\delta^{13}C$ for C_3 and CAM plants growing at a tropical outdoor research facility in Panama (from Winter *et al.*, 2005 and Cernusak *et al.*, 2007, with permission).

In spite of the complexities of diffusional and enzymatic influences on Δ_{CAM}, it is remarkable that δ_p values in plants with CAM correlate linearly with the proportion of CO_2 fixed during the day and night (O'Leary, 1988; Winter & Holtum, 2002), such that each 10% contribution of dark fixation results in about a 1.8‰ less negative δ_p signal (Fig. 8a). Measurements of whole-tissue δ_p for well-watered plants growing inside a gas-exchange chamber indicated a range from −8.7‰ for strong CAM plants that obtained 100% of their carbon from dark fixation, to −26.9‰ for plants that obtained 100% of their carbon during the light (Winter & Holtum, 2002).

This span of 18.2‰ for the full photosynthetic spectrum from 0 to 100% CAM is smaller than the range of δ_p observed *in situ* in species-rich taxa that contain both C_3 and CAM plants. In 1873 species of C_3 and CAM bromeliads, δ_p varied by 28.2‰, from −8.9 to −37.1‰ (Crayn *et al.*, 2004), and δ_p of 1002 species of C_3 and CAM orchids varied by 25.1‰, from −11.4 to −36.5‰ (Silvera *et al.*, 2010). In these surveys, the most ^{13}C-depleted specimens showed values considerably more negative than demonstrated by Winter & Holtum (2002) for plants that exclusively fix CO_2 during the light. Such extreme values are often associated with plants from shaded and humid environments where the diffusional limitation of CO_2 uptake is reduced and the source CO_2 is ^{13}C-depleted. On the other hand, C_3 plant material collected from certain arid environments, where CO_2 uptake is strongly diffusion-limited, may have δ_p values as high as −19.8‰ (Ehleringer *et al.*, 1998). A similarly high value was reported for a high-altitude C_3 species of the Rapataceae (Crayn *et al.*, 2001). The large variation in the isotope signal of confirmed C_3 plants suggests that the slope of the relationship between δ_p and the relative contributions of dark and light fixation is sensitive to the environment (Winter & Holtum, 2005). The least negative values observed in bromeliads and orchids coincide with the value of −8.9‰ predicted for 100% CAM in Fig. 8(a), demonstrating that the isotopic signal representing full CAM is less sensitive to the environment than is the C_3 isotopic signal, or that the environments inhabited by plants with full CAM share similarities.

An example of the different sensitivities of C_3- and CAM-type isotopic signals to the environment can be seen in bromeliads collected from different altitudes. Between sea level and 5000 m, δ_p of bromeliads with values more negative than −20‰ became less negative by 1.4‰ km^{-1} (D. Crayn *et al.*, unpublished). By contrast, bromeliads with CAM-type δ_p (less negative than −20‰) exhibited no significant δ_p change with increasing altitude.

The distribution of δ_p values in taxa known to contain C_3 and CAM species is bimodal, with a peak containing strong CAM species *c.* −12 to −16‰, a peak containing C_3 and weak-CAM species *c.* −24 to −32‰, and only few species in between (Kluge *et al.*, 1991; Pierce *et al.*, 2002; Winter & Holtum, 2002; Crayn *et al.*, 2004; Silvera *et al.*, 2005, 2010), as shown in Fig. 8(a). This bimodality implies that both strong CAM and weak CAM are selected for and that intermediate behaviour is not favoured. Bimodality may reflect biochemical optimization and anatomical tradeoffs between the C_3 and CAM pathways (Nelson & Sage, 2008; Borland *et al.*, 2011). Since δ_p is a time-integrated signal, the isotopic value alone does not distinguish between the continuous contribution of a weak CAM signal and the short-term contributions of strong CAM to a mainly C_3-type signal. The latter pattern could occur in some tropical species of *Clusia* with facultative CAM (Holtum *et al.*, 2004; Winter *et al.*, 2008, 2009). A wide spectrum of δ_p is observed in annuals such as *Mesembryanthemum crystallinum* (Winter *et al.*, 1978) and *Calandrinia polyandra* (Winter & Holtum, 2011), which undergo a gradual seasonal shift from C_3 to CAM. In *M. crystallinum*, δ_p ranges from *c.* −27 to −14‰ (Winter *et al.*, 1978; Bloom & Troughton, 1979). The least negative value probably underestimates the contribution of dark fixation to carbon gain at the end of the growing season as a result of dilution by previously fixed carbon with more negative $\delta^{13}C$.

Overall, the information embedded in the Δ_{CAM} signal is rich. Isotopic surveys have demonstrated that the CAM pathway is much more widespread among species than was considered previously (Rundel *et al.*, 1979; Winter, 1979; Winter *et al.*, 1983; Earnshaw *et al.*, 1987; Kluge *et al.*, 1991; Smith & Winter, 1996; Zotz & Ziegler, 1997; Silvera *et al.*, 2005, 2010). Δ_{CAM} has provided characters for phylogenetic analyses (Crayn *et al.*, 2004; Silvera *et al.*, 2009), established the contributions of CO_2 fixation in the light and dark, and provided information about transpiration ratios (Fig. 8c) and palaeoclimates (English *et al.*, 2007). Measurements of instantaneous and short-term Δ_{CAM} have been fundamental in establishing our physiological and biochemical concepts of CAM (O'Leary & Osmond, 1980; Holtum *et al.*, 1983, 1984; O'Leary *et al.*, 1986; Griffiths *et al.*, 1990, 2007; Griffiths, 1992; Roberts *et al.*, 1997). Future research should focus on further understanding the processes that may influence the relationship between Δ_{CAM} and the relative engagement of the CAM cycle; for example, the extent to which leakage of CO_2 from photosynthetic CAM tissues during the daytime occurs and may alter Δ_{CAM}.

VI. Conclusion

Stable carbon isotope ratios of terrestrial plants have the potential to provide unique insights into physiological processes and interactions between plants and the environment. Continued research into environmental and physiological determinants of Δ will further increase this potential. For C_3 plants, a priority should be to understand the mechanisms that lead to coordination between photosynthetic capacity and stomatal conductance, thereby muting the response of Δ_3 to the environment under some conditions. This is also important for engineering plants that have both high water-use efficiency and high photosynthetic capacity. For C_4 plants, future research should focus on understanding the interactions between ϕ and c_i/c_a, and when and to what extent Δ_4 reflects these determinants. For CAM plants, future studies on how growth environment influences the relationship between δ_p and the relative contribution of dark and light CO_2 fixation will improve the interpretation of δ_p signals in the field. Insights will likely emerge as a more refined understanding of Δ_{CAM} is pursued, for example the role of daytime leakage of nocturnally fixed CO_2 in influencing Δ_{CAM}. The enhanced capacity of laser systems for high-throughput analyses of online Δ will continue to advance our understanding of the more subtle determinants of Δ, for example, the distribution of mitochondria in mesophyll cells in relation to chloroplasts in C_3 plants (Tholen *et al.*, 2012; Busch *et al.*, 2013). Across the full range of applications that employ Δ measurements, it will be helpful to maintain a holistic view of how both the environment and internal physiology influence the Δ signal.

Acknowledgements

L.A.C. was supported by a Future Fellowship from the Australian Research Council (FT100100329). N.U. and G.D.F. also acknowledge support from the Australian Research Council (DP1097276).

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