Effects of intraleaf variations in carbonic anhydrase activity and gas exchange on leaf $C^{18}OO$ isoflux in *Zea mays*

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Summary

- Variation in the $C^{18}OO$ content of atmospheric CO_2 ($\delta^{18}O_a$) can be used to distinguish photosynthesis from soil respiration, which is based on carbonic anhydrase (CA)-catalyzed ^{18}O exchange between CO_2 and ^{18}O-enriched leaf water ($\delta^{18}O_w$).
- Here we tested the hypothesis that mean leaf $\delta^{18}O_w$ and assimilation rates can be used to estimate whole-leaf $C^{18}OO$ flux (isoflux), ignoring intraleaf variations in CA activity and gas exchange parameters.
- We observed variations in CA activity along the leaf (> 30% decline from the leaf center toward the leaf ends), which were only partially correlated to those in $\delta^{18}O_w$ (7 to 21‰), $\delta^{18}O$ and $\delta^{13}C$ of leaf organic matter (25 to 30‰ and −12.8 to −13.2‰, respectively), and substomatal CO_2 concentrations (intercellular CO_2 concentrations, c_i, at the leaf center were ~40% of those at the leaf tip).
- The combined effect of these variations produced a leaf-integrated isoflux that was different from that predicted based on bulk leaf values. However, because of canceling effects among the influencing parameters, isoflux overestimations were only ~10%. Conversely, use of measured parameters from a leaf segment could produce large errors in predicting leaf-integrated $C^{18}OO$ fluxes.

Key words: carbonic anhydrase, intraleaf variations, isoflux, ^{18}O, ^{18}O-CO_2, ^{18}O-leaf water, *Zea mays*.

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Introduction

Natural variations in the ^{18}O content ($\delta^{18}O$) of CO_2 provide a useful tracer for photosynthetic activity as a consequence of a sequence of events: first, $\delta^{18}O$ of chloroplast water is high because of evaporative effects; secondly, in the chloroplasts, exchange of oxygen between CO_2 and H_2O is catalyzed by carbonic anhydrase (CA); thirdly, a large fraction, proportional to the assimilation flux, of the ^{18}O-labeled CO_2 diffuses from the chloroplast back to the atmosphere (see Yakir, 1998 for a review). At the leaf scale, this 'retroflux' of ^{18}O-enriched CO_2 from the leaf back to the atmosphere is observed as discrimination against $C^{18}OO$ associated with leaf assimilation, $^{18}\Delta$ (Farquhar & Lloyd, 1993). The effect of $^{18}\Delta$ on atmospheric CO_2 above plant canopies was used to partition net CO_2 fluxes into ecosystem photosynthesis and respiration, taking advantage of the enrichment in evaporation of leaf water as compared with the less enriched soil water (Yakir & Wang, 1996; Bowling *et al.*, 2003). This effect is also observed at the global scale as latitudinal and seasonal changes in the $\delta^{18}O$ of atmospheric CO_2, reflecting global-scale plant productivity (Francey & Tans, 1987; Farquhar *et al.*, 1993; Ciais *et al.*, 1997). The quantitative use of the ^{18}O-CO_2 signal, however, still critically depends on better understanding of processes influencing $^{18}\Delta$ (Gillon & Yakir, 2000a,b, 2001). This must include considerations of the large heterogeneity in the isotopic composition of leaf water, which has been repeatedly observed (Yakir *et al.*, 1989; Luo & Sternberg, 1992; Luo & Sternberg, 1992; Wang & Yakir, 1995; Helliker & Ehleringer, 2000; Gan *et al.*, 2002, 2003, with Luo & Sternberg (1992)

referring to variations in δD), and possible heterogeneity in other factors that can influence the $\delta^{18}O$ of CO_2, including CA activity as well as leaf internal CO_2 concentration, which has not yet been considered in this context.

The primary control on the $\delta^{18}O$ of CO_2 is the $\delta^{18}O$ of the liquid water with which it was last in contact. CO_2 equilibrates isotopically with water according to the following reaction:

$$H_2{}^{18}O_{(l)} + CO_{2(g)} \rightleftharpoons H^+ + (HCO_2{}^{18}O)^-_{(aq)} \rightleftharpoons H_2O_{(l)} + C^{18}O_{(g)} \quad \text{Eqn 1}$$

(l, liquid; g, gas; aq, aqueous) which involves a temperature-dependent equilibrium fractionation between the oxygen in the CO_2 and in water (Brenninkmeijer *et al.*, 1983). In the presence of CA, which is ubiquitous in leaves, equilibrium can be reached nearly instantaneously, with a turnover rate of up to 10^6 s^{-1} (Silverman, 1982), and typical rates of 100–1400 μmol CO_2 m^{-2} s^{-1} on a leaf area basis (Gillon & Yakir, 2001). The quantity of water usually involved in the CO_2–water interaction is many orders of magnitude greater than the quantity of CO_2 present, so that isotopically equilibrated CO_2 takes on the oxygen isotopic ratio of the water in which it is dissolved, plus the temperature-dependent equilibrium fractionation, regardless of its initial $\delta^{18}O$ value. However, full isotopic equilibrium is not always attained, depending on CA activity and internal CO_2 concentrations (Gillon & Yakir, 2000a,b). Furthermore, leaf water is not well mixed and, as noted above, large heterogeneity in the isotopic composition of bulk leaf water ($\delta^{18}O_w$) has been repeatedly demonstrated. Farquhar & Gan (2003) recently provided a mathematical basis for describing the progressive enrichment in leaves, based on the string of lakes approach of Gat & Bowser (1991) and an internal Péclet effect (Farquhar & Lloyd, 1993; Barbour *et al.*, 2004).

The overall discrimination against ^{18}O during leaf CO_2 assimilation, $^{18}\Delta$, can be described as in Farquhar & Lloyd (1993), modified in Gillon & Yakir (2001):

$$^{18}\Delta = \frac{R_a}{R_l} - 1 = \frac{\bar{a} + \xi[\theta_{eq}(\delta_l - \delta_a)/(\delta_a/1000+1) - (1-\theta_{eq})(\bar{a}/\xi+1)]}{1 - \frac{\xi}{1000}[\theta_{eq}(\delta_l - \delta_a)/(\delta_a/1000+1) - (1-\theta_{eq})(\bar{a}/\xi+1)]} \approx \bar{a} + \xi[\theta_{eq}(\delta_l - \delta_a) - (1-\theta_{eq})(\bar{a}/\xi+1)] \quad \text{Eqn 2}$$

where R_a and R_l, the oxygen isotope ratios of CO_2 in the air and CO_2 in equilibrium with water at the site of exchange; $\bar{a}$, the weighted average fractionation during diffusion of CO_2 from the atmosphere to the chloroplast (8.8‰ in stagnant air and 0.8‰ in solution, with the weighted average taken as 7.4‰; Gillon & Yakir, 2001); $\xi = c_{cs}/(c_a - c_{cs})$, with c_a and c_{cs} the CO_2 concentrations in the atmosphere and at the site of oxygen exchange between CO_2 and water in the leaves (assumed to be near the 'chloroplast surface'), respectively (ξ represents the retro-diffusion flux back to the atmosphere after ^{18}O exchange with leaf water); δ_a and δ_l, the $\delta^{18}O$ values of CO_2 in the atmosphere and in equilibrium with leaf water (strictly, the estimated $\delta^{18}O$ of water in the chloroplast), respectively; $\delta = (R_{sample}/R_{standard} - 1)10^3$‰ (the standards are V-PDB, Vienna peedee belemnite, for CO_2 and V-SMOW, Vienna standard mean ocean water, for water samples); θ_{eq}, the extent of isotopic equilibrium between CO_2 and water, where $\theta_{eq} = 1$ at full equilibrium. (Note that, unlike the primary effect on ^{13}C, the secondary effect on ^{18}O during carboxylation by the photosynthetic enzyme RUBISCO is expected to cause negligible discrimination.)

To assess the effects of leaf discrimination on CO_2 in the air above the leaf, both at physiological and at larger scales, an 'isoflux' term is often used. The ^{18}O assimilation isoflux in this case refers to the net assimilation flux of $C^{18}OO$:

$$^{18}ISOFLUX_A = -A \cdot R_A \cong A(\delta_a - {}^{18}\Delta) \quad \text{Eqn 3}$$

where A, assimilation flux; R_A, the molar ratio $^{18}O/^{16}O$ in the assimilated CO_2. $^{18}ISOFLUX_A$ has units of μmol m^{-2} s^{-1} ‰ and use of the δ notation (a measured quantity) makes it conceptually equivalent, but not identical, to the actual $C^{18}OO$ flux (cf. Bowling *et al.*, 2003; note that we use the convention in atmospheric studies of negative fluxes out of the atmosphere, resulting in positive isofluxes).

The objective of this study was to examine the effect of the large variations in $\delta^{18}O_w$ observed in leaves on the integrated leaf $^{18}ISOFLUX_A$, and to test the hypothesis that mean leaf $\delta^{18}O_w$ can be used to estimate whole-leaf $^{18}ISOFLUX_A$. This hypothesis must be based on the assumption that other influencing parameters, primarily CA activity and c_{cs}, are either constant or would vary in concert with $\delta^{18}O_w$ and would therefore not influence estimates of $^{18}ISOFLUX_A$. Accepting or rejecting this hypothesis can have important consequences for the use of ^{18}O in CO_2 in physiological, ecological, and large-scale studies using the ^{18}O approach, and to interpretations of variations in the $\delta^{18}O$ of atmospheric CO_2.

Materials and Methods

Gas exchange

All parameters were measured in 10-cm segments (segments were numbered so that segment 1 was the base of the leaf) along at least three corn (*Zea mays* L.) leaves in field-grown plants in the southern coastal plains of Israel at around midday (11:00–13:00 h). The incident light intensity (I) was measured with a LiCor PAR sensor (LiCor, Lincoln, NE, USA) along the leaves before and after each gas exchange measurement and averaged. Gas exchange parameters (net assimilation rate, A, stomatal conductance, g_s, and intercellular CO_2 concentration c_i) were measured in attached leaves using a portable gas exchange system (Li-6400; LiCor) with light supplied by the instrument LED array and adjusted to match the sunlight intensity measured just before measurement (this provided near-ambient but more stable conditions during measurements). In some cases, light response curves were obtained in attached leaves under field

conditions by stepwise changes of the light intensity of the instrument (first increasing I from ambient values to saturation, and then decreasing back to low values). The observed gas exchange parameters were also used to estimate the extent of ^{18}O exchange between CO_2 and water (θ_{eq}; see the Results section).

Isotopic analysis

Leaf water was extracted by vacuum distillation at 80°C. $\delta^{18}O$ values were determined by equilibration of 0.5 ml of water with CO_2 for 24 h at 29°C followed by cryogenic purification of a CO_2 aliquot. $\delta^{18}O$ values of the CO_2 were measured by dual inlet isotope ratio mass spectrometer (IRMS; MAT250; Finigan, Bremen, Germany). Values were calibrated on the V-SMOW scale by simultaneously measuring an internal water standard (having a $\delta^{18}O$ value of −4.5‰ periodically calibrated to the international V-SMOW standard obtained from the International Atomic Energy Agency, Vienna, Austria).

Ground whole dry leaf segments were analyzed for $\delta^{13}C$ by a conventional online combustion elemental analyzer (EA1109 CHN-O; Carlo Erba Instuments, Milan, Italy) connected to IRMS (Optima; Micromass, Manchester, UK). Leaf organic $\delta^{18}O$ measurements were obtained by pyrolysis on graphite, using the same elemental analyzer, followed by IRMS measurement of the CO produced (Saurer *et al.*, 1998).

Carbonic anhydrase activity, θ_{eq} and c_{cs}

Carbonic anhydrase (CA) was extracted, within 2 h of collecting leaves, from leaf discs (1.7 cm^2) taken at 10-cm intervals along the leaves. Extraction and activity measurement assay were performed using the method described by Gillon & Yakir (2000a,b). CA was extracted by grinding leaf discs in extraction buffer (50 mM HEPES-NaOH, pH 8.3, 0.5 mM EDTA, 10 mM dithiothreitol, 10% glycerol, and 1% triton X-100) at 4°C. The *in vitro* CA activity assay was performed at 2°C by adding saturated aqueous CO_2 into an assay buffer (20 mM Na-barbitol, pH 8.3) containing the enzyme extract, and the rate of pH decrease yielded CA_{assay}. The *in vivo* CA activity at leaf temperature and saturating light (CA_{leaf}) was then estimated as CA_{assay} converted to leaf conditions: $CA_{leaf} = F\,CA_{assay}\,[(17.5 + K_m)/17.5]\,[c_{cs}/(c_{cs} + K_m)]$, where F is a temperature correction factor, $F = Q_{10}^{(T\text{leaf}-T\text{assay})/10}$, assuming $Q_{10} = 2$ (Burnell & Hatch, 1988), and K_m is the CO_2 concentration at half maximal activity, taken as 2.8 mM (Hatch & Burnell, 1990). The CO_2 concentration at the chloroplast surface (c_{cs}) was estimated from $A = g_w(c_i - c_{cs})$, using light-saturated A and c_i values and where g_w, the internal wall conductance, is assumed to be 1 mmol m^{-2} s^{-1} (Gillon & Yakir, 2000b). The extent of isotopic equilibrium between CO_2 and water (θ_{eq}) was determined as $\theta_{eq} = 1 - e^{-k\tau/3}$, where $k\tau = CA_{leaf}/F_{in}$ and $F_{in} = A[c_{cs}/(c_a - c_{cs}) + 1]$ (Gillon & Yakir, 2000a).

Results

The light intensity (I) incident along the leaf in its natural orientation under field conditions was variable. I values were maximal, ~700 µmol m^{-2} s^{-1}, around the mid-leaf sections and declined toward both the tip and the base to ~400 µmol m^{-2} s^{-1} (Fig. 1a). Gas-exchange parameters, measured in 10-cm segments along the leaf, were generally consistent with leaf orientation toward the sun and for most parameters reflected the two main parts of the leaf: the upward section from the base to approximately mid-leaf, and the downward section from mid-leaf to the tip (Fig. 1).

A light response curve measured at mid-leaf (Fig. 1e) indicated that A was light saturated at light intensities of *c.* 1100 µmol m^{-2} s^{-1}. Hence, A increased, together with I,

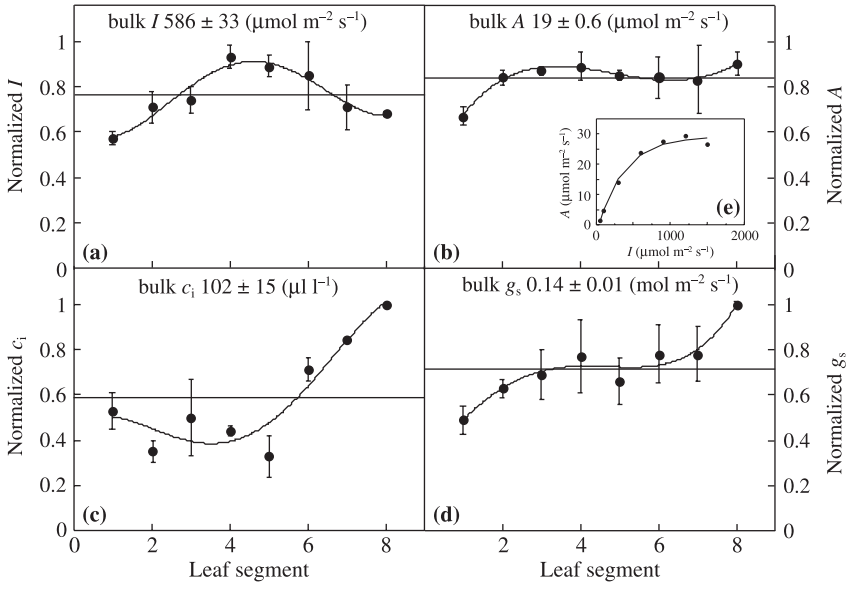

Fig. 1 (a) Light intensity (I), (b) net assimilation rate (A), (c) intercellular CO_2 concentration (c_i) and (d) stomatal conductance (g_s), measured at 10-cm intervals along corn (*Zea mays*) leaves. Leaf segments (10 cm each) are labeled from the base (lowest number) to the tip of the leaf. A, g_s, c_i were measured under artificial light equivalent to the natural light intensity incident on the leaf, as measured shortly before the gas exchange measurements. To account for leaf-to-leaf variations in absolute values, gas exchange parameters in each leaf were normalized to the maximal value of each parameter before averaging. The horizontal line indicates the normalized mean value along the leaf, with the actual bulk leaf value given at the top of each panel. The light response curve in (e) was measured in the middle of one leaf.

from 15 ± 0.8 μmol m^{-2} s^{-1} (mean ± standard error; n = 3) at the leaf base to 20 ± 3 μmol m^{-2} s^{-1} at mid-leaf (Fig. 1b). A was relatively constant, however, from mid-leaf to the tip (19 ± 0.8 μmol m^{-2} s^{-1}; n = 12), in spite of the decrease in I. Stomatal conductance (g_s) increased along the leaf from 0.11 ± 0.02 mol m^{-2} s^{-1} (n = 3) at the leaf base to 0.21 ± 0.01 mol m^{-2} s^{-1} (n = 3) at the tip, with a small decrease at mid-leaf (Fig. 1d). Although modest, variations in g_s were consistent with changes in A from the leaf base to mid-leaf, resulting in relatively constant values of c_i (81 ± 8 μl l^{-1}; n = 12). This was followed by an increase in c_i values from mid-leaf to 184 ± 9 μl l^{-1} (n = 3) at the tip (Fig. 1c), which was correlated with the increase in g_s but relatively constant A. As the ambient CO_2 concentration (c_a, recorded before each gas exchange measurement) did not vary much, the ratio c_i/c_a varied in a similar manner to the variations in c_i from the leaf base to mid-leaf (0.23 ± 0.04; n = 12) and to the tip (0.53 ± 0.03; n = 3).

The ratio c_i/c_a also influences the $\delta^{13}C$ of leaf organic matter ($\delta^{13}C_{org}$) which on average decreased from −12.8 ± 0.1‰ (n = 4) at the base to −13.2 ± 0.1‰ (n = 4) at the tip of the leaves (Fig. 2b). However, in the first half of the leaf, from base to mid-leaf, this ^{13}C depletion was mainly a consequence of the first segment being relatively enriched (−12.8 ± 0.1‰; n = 4), whereas segments 2–4 did not greatly change (−12.9 ± 0.06‰; n = 12). Overall, therefore, high c_i values were correlated with more depleted $\delta^{13}C_{org}$ in leaf organic matter. Note, however, that the correlation between c_i and $\delta^{13}C_{org}$ may be fortuitous as these parameters represent different time scales and likely also spatial scales, as most organic matter is produced at the leaf base during leaf development.

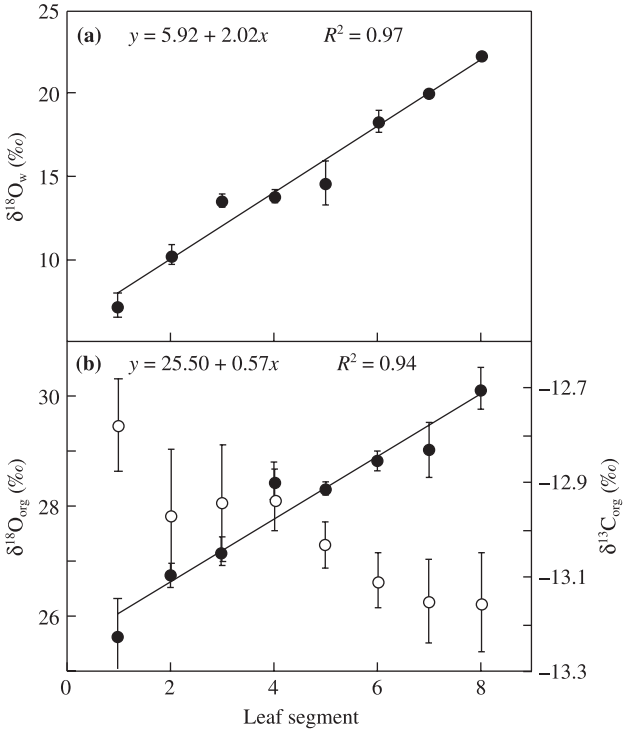

Fig. 2 Isotopic composition along corn (*Zea mays*) leaves: (a) the oxygen isotope composition of leaf water ($\delta^{18}O_w$); (b) the oxygen ($\delta^{18}O_{org}$; closed circles) and carbon ($\delta^{13}C_{org}$; open circles) isotope composition of leaf organic matter. The equation denotes the curve fit for $\delta^{18}O$ only. Values are mean ± standard error for four leaves. Leaf segments (10 cm each) are labeled from the base (lowest number) to the tip of the leaf.

The oxygen isotopic composition of leaf water ($\delta^{18}O_w$) showed pronounced enrichment along the leaf, from 7.2 ± 0.7‰ (n = 4) at the base to 21.2 ± 0.6‰ (n = 4) at the tip (Fig. 2a). Stem water in these plants was −1.9 ± 0.1‰. A similar trend of enrichment along the leaf, although less pronounced, was observed in the oxygen isotopic composition of leaf organic matter ($\delta^{18}O_{org}$; Fig. 2b). $\delta^{18}O_{org}$ values increased from 25.7 ± 0.7‰ (n = 3) at the leaf base to 30.1 ± 0.4‰ at the tip. This resulted in a good correlation between $\delta^{18}O_w$ and $\delta^{18}O_{org}$, with a best-fit line of $\delta^{18}O_{org} = 23.96 + 0.27\delta^{18}O_w$ (R^2 = 0.92), indicating an enrichment trend along the leaf that was only *c.* 30% of that in $\delta^{18}O_w$. Note that, as for ^{13}C discussed above, the isotopic signals in water and organic matter represent different temporal and possibly also spatial scales. During cellulose synthesis in the leaf base, extensive exchange with stem water can occur (Farquhar *et al.*, 1998; Yakir, 1998; Roden *et al.*, 2000).

The activity of the enzyme carbonic anhydrase (CA_{assay}) measured in 10 leaves showed a similar pattern to that in incident light intensity along the leaf (although large leaf-to-leaf variations were observed). On average, CA_{assay} increased from 136 ± 17 μmol m^{-2} s^{-1} (n = 10) at the leaf base to 175 ± 22 μmol m^{-2} s^{-1} at mid-leaf and decreased to 122 ± 38 μmol m^{-2} s^{-1} at the tip (Fig. 3a). The mean bulk leaf CA_{assay} was 160 ± 18 μmol m^{-2} s^{-1}.

A similar pattern was observed when the potential *in vivo* activity of carbonic anhydrase (CA_{leaf}, the measured assay activity converted to leaf conditions) was estimated using the gas exchange parameters (A and c_i) at saturating light intensity (Fig. 1e). CA_{leaf} increased from 173 μmol m^{-2} s^{-1} at the leaf base to 228 μmol m^{-2} s^{-1} at mid-leaf and decreased to 158 μmol m^{-2} s^{-1} at the tip of the leaf (Fig. 3b). CA_{leaf} was used to calculate the extent of isotopic equilibrium between CO_2 and water (θ_{eq}) using measured light-saturated assimilation rates as a constant basis. Complete isotopic equilibrium (θ_{eq} = 1) was never reached in the corn leaves (Fig. 3c) and θ_{eq} generally varied according to variation in CA activity, but with a smaller range, from 0.86 at the leaf base to 0.92 at mid-leaf and 0.83 at the tip.

The combined effect of variations along the leaf in $\delta^{18}O_w$, gas exchange parameters and θ_{eq} on the leaf $^{18}ISOFLUX_A$ was estimated using calculated values of leaf discrimination against ^{18}O, $^{18}\Delta$, according to Eqns 2 and 3. δ_a was taken as −0.2‰ (the average value measured at the month in which leaves were sampled in our Negev station of the National Oceanic and Atmospheric Administration's Climate Monitoring and Diagnostic Laboratory, NOAA-CMDL, network;

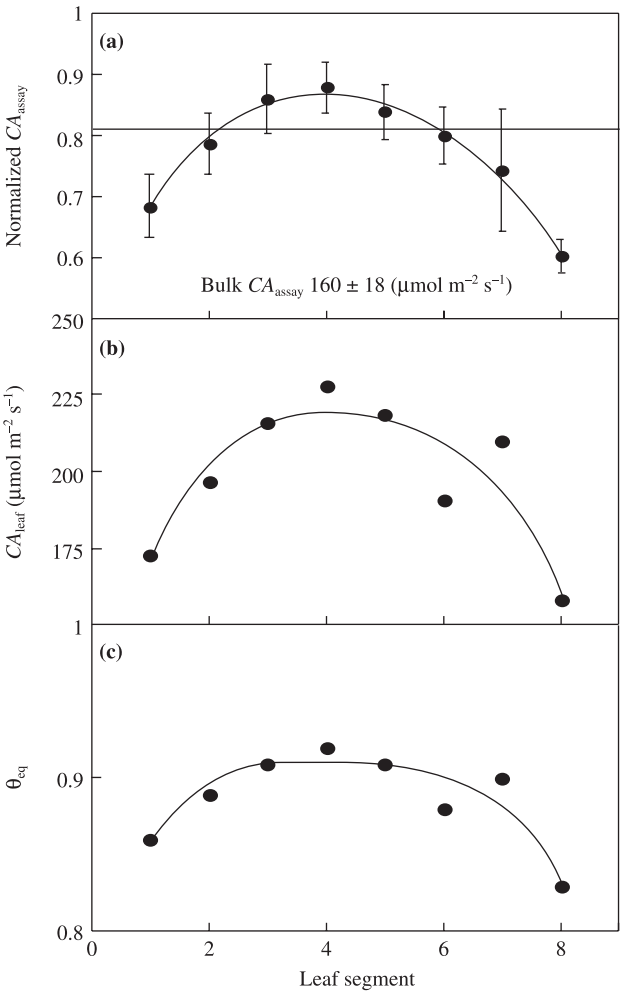

Fig. 3 Rate of activity of carbonic anhydrase (CA) along corn (*Zea mays*) leaves: (a) measured activity of leaf extracts in assay conditions (CA_{assay}) at 10-cm intervals along the leaf. To account for leaf-to-leaf variations in absolute values, CA_{assay} values were normalized to maximal activity in each leaf. The horizontal line indicates the normalized mean value along the leaf, with the actual bulk leaf value indicated at the bottom of the panel. Values are mean ± standard error for 10 leaves, with leaf segments (10 cm each) labeled from the base (lowest number) to the tip of the leaf. (b) CA activity under *in vivo* conditions (CA_{leaf}) estimated as in Gillon & Yakir (2000b) using leaf segment CA_{assay} values (mean of 10 leaves), measured leaf temperature and intercellular CO_2 concentration (c_i) at saturating light intensity. (c) Extent of isotopic equilibrium between CO_2 and water (θ_{eq}) calculated using CA_{leaf} and the light-saturated assimilation rate (full equilibrium at $\theta_{eq} = 1$).

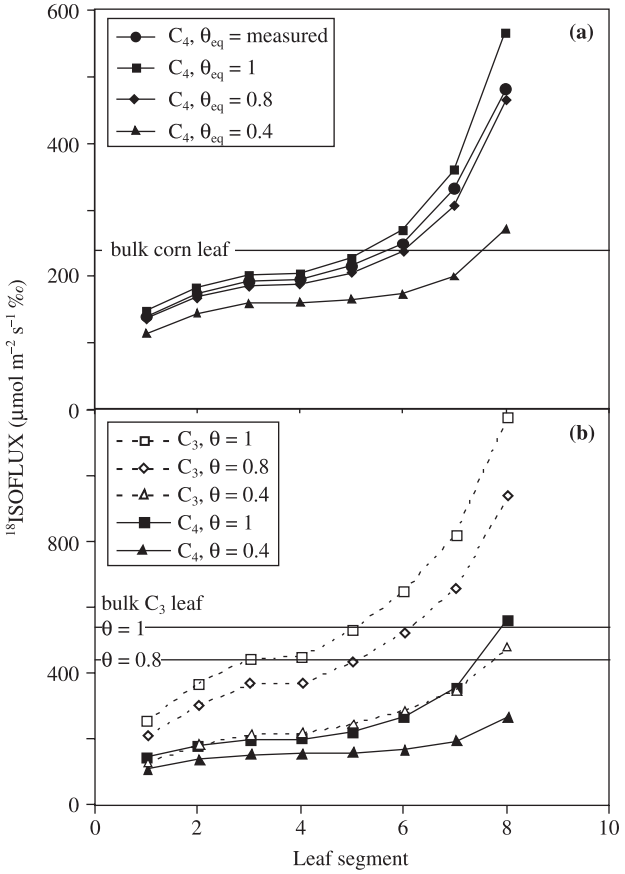

Fig. 4 The ^{18}O assimilation flux calculated as $^{18}ISOFLUX_A = -A(\delta_a - {}^{18}\Delta)$ for (a) C_4 plants and (b) simulated C_3 plants assuming $c_a - c_i$ drawdown in a C_3 grass to be 0.53 of that measured in corn (*Zea mays*), where c_i is the intercellular CO_2 concentration and c_a is the CO_2 concentration in the atmosphere. δ_a denotes the $\delta^{18}O$ value of atmospheric CO_2. $^{18}\Delta$, the overall discrimination against ^{18}O during leaf CO_2 assimilation, was estimated using the measured extent of isotopic equilibrium (θ_{eq}) along corn leaves (closed circles in panel a), or using a range of possible θ_{eq} values typical of C_4 grasses ($\theta_{eq} = 0.4$, triangles), C_3 grasses ($\theta_{eq} = 0.8$, diamonds) or dicots ($\theta_{eq} = 1$, squares). The measured assimilation rate (A) along the leaf (mean of three leaves) was used in $^{18}ISOFLUX_A$ estimates. Closed and open symbols denote C_4 and C_3 values, respectively. The horizontal lines indicate whole-leaf mean $^{18}ISOFLUX_A$ values. Leaf segments (10 cm each) are labeled from the base (lowest number) to the tip of the leaf.

www.cmdl.noaa.gov/ccgg/index.htm). $^{18}\Delta$ increased slightly from 8.9‰ at the leaf base to 9.4‰ at mid-leaf and then sharply to 24.2‰ at the tip. In the lower half of the leaf, the small change in $^{18}\Delta$ likely reflected the slight decrease in c_i and calculated c_{cs} values, balanced by an increase in $\delta^{18}O_w$. The sharp increase in $^{18}\Delta$ in the upper half of the leaf likely reflected the combined increase in c_{cs} and $\delta^{18}O_w$. Average $^{18}\Delta$ for the leaf, calculated using mean values for the entire leaf in Eqns 2 and 3, was 11.6‰. As a result of the increase in A from the leaf base to mid-leaf (Fig. 1b), $^{18}ISOFLUX_A$ increased from 137 to 194 μmol m^{-2} s^{-1} ‰. A sharper increase to 480 μmol m^{-2} s^{-1}‰ was observed from mid-leaf to the tip, reflecting the increase in $^{18}\Delta$, in spite of the constant A (Fig. 4). $^{18}ISOFLUX_A$ calculated with mean parameters for the entire leaf was 222 μmol m^{-2} s^{-1} ‰.

Discussion

The primary objective of this study was to evaluate the effect of the large variations in $\delta^{18}O_w$ observed in leaves (Yakir *et al.*,

1989; Luo & Sternberg, 1992; Wang & Yakir, 1995; Helliker & Ehleringer, 2000; Gan *et al.*, 2002, 2003) on the integrated leaf $^{18}ISOFLUX_A$ (Eqn 3). A reasonable hypothesis is that $^{18}ISOFLUX_A$ varies along leaves in concert with $\delta^{18}O_w$, and consequently the mean $\delta^{18}O_w$ value of bulk leaf water can serve as a good predictor of the whole-leaf $^{18}ISOFLUX_A$. Rejection of this hypothesis complicates estimation of $^{18}ISOFLUX_A$, and can result in a very different leaf $^{18}ISOFLUX_A$ from that estimated based on the relatively easy to predicted mean leaf $\delta^{18}O_w$ value. For example, a case in which $\delta^{18}O_w$ increases along the leaf but CA_{leaf} and c_{cs}, the primary controls of leaf $^{18}ISOFLUX_A$ (cf. Eqns 2,3), are constant along the leaf would produce an $^{18}ISOFLUX_A$ that simply reflected the bulk, or mean, $\delta^{18}O_w$ value; however, this could be very different in a case were the contribution to the $^{18}ISOFLUX_A$ of the very high $\delta^{18}O_w$ observed in approaching the leaf tip was diminished as a result, say, of low c_{cs} and/or low CA activity in this part of the leaf.

The results show the expected large progressive enrichment in $\delta^{18}O_w$ along corn leaves, but also variable patterns of other parameters influencing the transfer of ^{18}O from leaf water to CO_2. Some parameters, such as I, showed bimodal patterns along the leaf, consistent with the light regime under natural field conditions. Other parameters, such as CA_{leaf}, A and c_i (which was used to estimate c_{cs}), showed secondary response patterns producing a nonlinear progression along the leaves. As a result, the leaf $^{18}ISOFLUX_A$ did not have a similar pattern to $\delta^{18}O_w$ along the leaf and reflected instead the balance of the various patterns. We therefore had to reject our hypothesis. However, as discussed below (see The leaf $^{18}ISOFLUX_A$), contrasting effects, such as high CA_{leaf} associated with low c_{cs}, reduced the effects of the complex gradients along the leaf on the total leaf isoflux, thus reducing the potential error introduced by the conventional use of leaf bulk $\delta^{18}O_w$ to predict $^{18}ISOFLUX_A$.

The water component

The observed large progressive enrichment in $\delta^{18}O_w$ (measured as the difference between the $\delta^{18}O$ of segment water and that of the source, stem, water was linearly related to the distance from the base of the leaf and was similar to that observed previously (Helliker & Ehleringer, 2000, 2002a). This progressive enrichment process was described mathematically for a chain of lakes by Gat & Bowser (1991) and was recently adapted to leaves by making it continuous and incorporating a Péclet effect, to account for the competing effects of advection and diffusion to and from the site of evaporation (Farquhar & Gan, 2003). While these models describe the increasing enrichment along leaves, the integrated $\delta^{18}O$ value of the entire system (i.e. the mean bulk leaf water) predicted by the models approaches the value predicted by the original evaporation model of Craig & Gordon (1965; cf. Flanagan, 1993), which was developed for one well-mixed water pool. This simpler bulk leaf model cannot, however, be used to accurately estimate the leaf $^{18}ISOFLUX_A$ if intraleaf variations in other relevant parameters are not constant (or vary in concert with $\delta^{18}O_w$ along the leaf).

It is well established that the $\delta^{18}O$ of leaf organic matter, $\delta^{18}O_{org}$, is linked to $\delta^{18}O_w$ and can provide a time-integrated record of $\delta^{18}O_w$ (e.g. Yakir, 1992). Indeed, there was a high linear correlation between $\delta^{18}O_{org}$ and $\delta^{18}O_w$ along the corn leaves, as observed in other plants (e.g. Helliker & Ehleringer, 2002a). However, the slope of ~0.3 indicated a more moderate enrichment along the leaf for $\delta^{18}O_{org}$ than for $\delta^{18}O_w$. This is expected because of the exchange of organically bound oxygen with water pools other than the segment water represented by our $\delta^{18}O_w$ values (Yakir, 1992; Saurer *et al.*, 1997; Farquhar *et al.*, 1998). Calculating the exchange parameters recently proposed by Barbour & Farquhar (2000), we obtained a mean $P_{ex}P_x$ value (representing, respectively, the proportion of exchangeable oxygen atoms during synthesis of cellulose, and the proportion of xylem water present in the cells where cellulose, the dominant organic species in the leaf material, is synthesized) of 0.73 with an ε_0 value (representing the ^{18}O discrimination factor between carbonyl oxygen and water) of 25.4‰. This $P_{ex}P_x$ estimate is much higher than the values of 0.25–0.38 observed in short grasses (Helliker & Ehleringer, 2002a; Barbour *et al.*, 2004). The structural organic material, which comprises most of the material in our samples, is formed primarily in the leaf-base meristem. This suggests that, while $\delta^{18}O_{org}$ values are initially determined in the leaf region with high rates of assimilation, they are consequently influenced by exchange with the water at the leaf-base site during leaf elongation (Helliker & Ehleringer, 2002a,b; Barbour *et al.*, 2004). Therefore, a leaf-segment $\delta^{18}O_{org}$ is likely to be influenced by the temporal progression in the $\delta^{18}O$ of water during leaf development (including variations in longitudinal Péclet effect in the leaf as it increases in length). Such complicating factors must be considered before $\delta^{18}O_{org}$ can be used as a direct record of $\delta^{18}O_w$ along leaves.

From water to CO_2

The transfer of the ^{18}O signal from leaf water to CO_2 depends on the exchange of oxygen between the leaf water and CO_2 (Eqn 1). Because of the short residence time of CO_2 inside the leaves (normally less than 1 s) and the relatively slow noncatalyzed CO_2 hydration rate, this exchange critically depends on the concentration of CO_2 at the site of CO_2–H_2O exchange (c_{cs}) and on the rate of CA activity. The value of c_{cs} should be intermediate between that at the substomatal cavities, c_i, and that at the site of the photosynthetic enzyme RUBISCO (Gillon & Yakir, 2000a; see Materials and Methods). Leaf-scale physiology therefore strongly influences the evolution of the leaf $C^{18}OO$ flux.

Clearly, the physiological parameters measured here varied along the corn leaf independent of the linear enrichment in $\delta^{18}O_w$ (Figs 1, 2). The dominant factors influencing intraleaf

physiology were probably incident light (Fig. 1) and developmental stage (young at the base and senescing at the tip; not examined here). In response to variations in these dominant parameters, g_s, c_{cs} and CA_{leaf} seem to have varied to produce relatively stable A and θ_{eq} across most of the leaf (Figs 1, 3). Most prominently, the pattern in incident light, which was maximal at mid-leaf, was largely paralleled by the patterns in c_{cs} and CA_{leaf} and all three parameters changed by 30–50% along the leaves. In contrast, both θ_{eq} and A varied by less than 10% along the leaf (excluding one data point for A). Optimization of A and CA activity (which determines θ_{eq}) probably helps to maximize leaf productivity, while it is θ_{eq} and c_{cs} that dominate $^{18}\Delta$ and the leaf $^{18}ISOFLUX_A$ (Eqns 2,3).

The activity of carbonic anhydrase in the present study was significantly higher than the mean value estimated previously for C_4 plants (Gillon & Yakir, 2000b, 2001), although, in these studies too, cultivated corn had the highest CA activity among the C_4 plants. Therefore, while C_4 plants were reported to have a mean θ_{eq} value of ~0.4, here we obtained for cultivated corn plants θ_{eq} values of ~0.9 (Fig. 3c). In estimating leaf isoflux we therefore also simulated the effects of the observed intraleaf variations when mean θ_{eq} is 0.4 (see The leaf $^{18}ISOFLUX_A$).

As for ^{18}O, the $\delta^{13}C$ of leaf organic matter, $\delta^{13}C_{org}$, can potentially provide a long-term integrated record of c_i (Farquhar *et al.*, 1982, 1998) and therefore of c_{cs}, and possibly of their variations along the leaf. Indeed, the organic ^{13}C record along the leaf showed a similar pattern to that of c_i (excluding one data point at the base of the leaf; cf. Fig. 1c vs Fig. 2b). The general trend of ^{13}C depletion along the leaf in corn and sugar cane (*Saccharum* spp.) was observed previously and could also be linked to c_i (Sasakawa *et al.*, 1989; Meinzer & Saliendra, 1997). However, different patterns of incident light along the leaf led to different trends in c_i, with decreasing values along the sugar cane leaves (Meinzer & Saliendra, 1997) and increasing values in the corn leaves. These contrasting patterns are consistent with the differences in estimated bundle sheath leakiness. Leakiness was estimated at ~0.40 in corn, while a value of 0.32 was calculated in sugar cane (Meinzer & Saliendra, 1997). These differences in leakiness are significant; the theoretical model for C_4 discrimination (Farquhar, 1983) predicts that, at a leakiness of ~0.35, the pattern of ^{13}C vs c_i/c_a is inverted, with an inverse correlation below this value and a direct correlation above this value. However, as noted above for $\delta^{18}O_{org}$, leaf organic matter (mostly cellulose) is formed in the leaf-base meristem and not at the particular segment sampled. As discussed for $\delta^{18}O_{org}$, $\delta^{13}C_{org}$ too is likely to be influenced by the temporal progression in c_i during leaf development. For example, the tip of the leaves likely contained cellulose formed in the base meristem of the young leaves when vapor pressure deficit and temperature were lower than during the sampling time at peak season, consistent with the more depleted $\delta^{13}C_{org}$ observed. Note that, while such temporal effects would also apply to $\delta^{18}O_{org}$, they would be overshadowed by the oxygen exchange with tissue water discussed above, which should not influence $\delta^{13}O_{org}$. However, for both $\delta^{13}O_{org}$ and $\delta^{18}O_{org}$, the complicating factors involved must be considered before these parameters can be used as a direct record of c_i or $\delta^{18}O_w$ along leaves.

The leaf $^{18}ISOFLUX_A$

The effects of $\delta^{18}O_w$ and leaf physiology were integrated by estimating $^{18}\Delta$ (Eqn 2) and $^{18}ISOFLUX_A$ (Eqn 3). Note that $^{18}ISOFLUX_A$ was dominated by $^{18}\Delta$ as A and δ_a did not vary much along the leaf. In the bottom half of the leaf, a relatively small change in $^{18}\Delta$ and $^{18}ISOFLUX_A$ was observed, reflecting the slight decrease in c_i and the calculated c_{cs} values, balanced by an increase in $\delta^{18}O_w$ (Fig. 4a). In the top half of the leaf, the sharp increase in $^{18}\Delta$ and greater $^{18}ISOFLUX_A$ reflected the combined increase in c_{cs} and $\delta^{18}O_w$ (Fig. 4a).

The sensitivity of the patterns in $^{18}ISOFLUX_A$ to θ_{eq} values was examined by using $\theta_{eq} = 0.4$, which is typical for C_4 plants (Gillon & Yakir, 2000b). This resulted in only slight changes in the bottom part of the leaf but considerably greater $^{18}ISOFLUX_A$ toward the leaf tip (480 μmol m^{-2} s^{-1} ‰ using the observed θ_{eq} and 270 μmol m^{-2} s^{-1} ‰ with $\theta_{eq} = 0.4$). Using $\theta_{eq} = 1$, $^{18}ISOFLUX_A$ varied along the leaf, from 146 μmol m^{-2} s^{-1} ‰ at the leaf base to 201 μmol m^{-2} s^{-1} ‰ at mid-leaf to 563 μmol m^{-2} s^{-1} ‰ at the tip. This variable sensitivity to θ_{eq} values demonstrated the importance of c_i (and, when available, c_{cs}), which controls the gross, one-way retro-diffusion flux from the leaf back to the atmosphere. Therefore, high c_i (and c_{cs}) results in high ^{18}O-labeled retro-diffusion flux and consequently high $^{18}\Delta$. Thus, a combined effect of θ_{eq} and c_i (or c_{cs}) strongly impacts the leaf $^{18}ISOFLUX_A$. Such an effect can be expected in C_3 leaves when θ_{eq} is near 1 and c_i values are generally much higher than in C_4 leaves.

For such a comparison, we simulated C_3 grass leaves (e.g. wheat, Triticumaestivum) by maintaining the same patterns observed along corn leaves, but assuming the stomatal drawdown in CO_2 concentration (from c_a to c_i) in a C_3 leaf to be 0.53 of observed values in corn, yielding a mean c_i value of 219 μl l^{-1}, compatible with a typical ratio of 2.1 for c_i values between C_4 and C_3 leaves (Lloyd & Farquhar, 1994). This allowed a comparison, as a first approximation, of the expected $^{18}ISOFLUX_A$ along leaves between C_3 plants and C_4 monocots. As expected, with both θ_{eq} and c_i values high in the 'C_3 leaf', $^{18}ISOFLUX_A$ was much greater than in typical C_4 leaves (Fig. 4b). High c_i also makes the leaves more sensitive to variations in θ_{eq} values. For $\theta_{eq} = 1$, which is typical for C_3 dicots, or $\theta_{eq} = 0.8$, which is typical for C_3 grasses (Gillon & Yakir, 2001), $^{18}ISOFLUX_A$ at the tip of the leaf was 1178 and 940 μmol m^{-2} s^{-1} ‰, respectively (Fig. 4b).

Finally, we compared the weighted average leaf $^{18}ISOFLUX_A$, obtained by following the variations along the leaves, with that obtained by using a single bulk leaf value for $\delta^{18}O_w$ and a mean value for each of the physiological parameters (Table 1). The errors introduced in this case ranged between 7% (for C_4

Table 1 $^{18}ISOFLUX_A$ estimates using different values for the extent of isotopic equilibrium between CO_2 and water (full equilibrium at $\theta_{eq} = 1$)

	C_4 $^{18}ISOFLUX_A$ (μmol m^{-2} s^{-1} ‰)			C_3 $^{18}ISOFLUX_A$ (μmol m^{-2} s^{-1} ‰)		
	$\theta_{eq} = 1$	$\theta_{eq} = 0.8$	$\theta_{eq} = 0.4$	$\theta_{eq} = 1$	$\theta_{eq} = 0.8$	$\theta_{eq} = 0.4$
Base	146	135	112	257	215	130
Mid	201	187	159	450	373	221
Tip	563	464	270	1178	940	480
Bulk	238	212	161	538	440	247
Average	267	235	172	588	479	266
Error (%)	10.9	9.8	6.6	8.4	8.1	6.9

For C_4, $^{18}ISOFLUX_A$ is calculated from measured gas exchange parameters and the $\delta^{18}O$ of leaf water ($\delta^{18}O_w$) in corn (*Zea mays*). For C_3, gas exchange parameters are simulated assuming $c_a - c_i$ drawdown in a C_3 grass to be 0.53 of that measured in corn. Values are given for the base of the leaf, the mid-leaf and the tip based on measurements in 10-cm leaf segments. 'Average' refers to the weighted average obtained from leaf isofluxes estimated for each leaf segment. 'Bulk' refers to the whole-leaf $^{18}ISOFLUX_A$ calculated using the leaf mean values for $\delta^{18}O_w$ and gas exchange parameters. Errors were estimated as error = 100(1 – bulk/average) and refer to estimating leaf $^{18}ISOFLUX_A$ without the weighting of isotopic and physiological parameters involved in calculating the $^{18}ISOFLUX_A$ of leaf segments (see Eqn 3).

assuming $\theta_{eq} = 0.4$) and 11% (for C_4 assuming $\theta_{eq} = 1$) and were *c.* 7–8% for simulated C_3 grass leaves. The error would of course be much greater if an arbitrary segment of a leaf was used. In this case, the range would be between 35 and 56% underestimation when using segments from the leaf base (for C_4, $\theta_{eq} = 0.4$ and for C_3, $\theta_{eq} = 1$, respectively) and between 57 and 111% overestimation when using segments near the leaf tip (for C_4, $\theta_{eq} = 0.4$ or 1, respectively). Although the patterns of c_i and CA activity along the leaf did not co-vary with $\delta^{18}O_w$, leading us to reject our initial hypothesis of a direct correlation between $^{18}ISOFLUX_A$ and $\delta^{18}O_w$, the results showed that the overestimation of $^{18}ISOFLUX_A$ caused by ignoring these variations and using instead the bulk leaf values is relatively small. Therefore, under current levels of uncertainty in modeling leaf and ecosystem isofluxes, it is probably still reasonable to use bulk leaf values to estimate the total leaf $^{18}ISOFLUX_A$, but not values from any specific part of the leaf.

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