

On the effect of heavy water (D₂O) on carbon isotope fractionation in photosynthesis

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Abstract. Internal conductance to carbon dioxide is a key aspect of leaf photosynthesis although is still not well understood. It is thought that it comprises two components, namely, a gas phase component (diffusion from intercellular spaces to cell walls) and a liquid phase component (dissolution, diffusion in water, hydration equilibrium). Here we use heavy water (D₂O), which is known to slow down CO₂ hydration by a factor of nearly three. Using ¹²C/¹³C stable isotope techniques and *Xanthium strumarium* L. leaves, we show that the on-line carbon isotope discrimination ($\Delta^{13}\text{C}$, or Δ_{obs}) associated with photosynthesis is not significantly decreased by heavy water, and that the internal conductance, estimated with relationships involving the deviation of $\Delta^{13}\text{C}$, decreased by 8–40% in 21% O₂. It is concluded that in typical conditions, the CO₂-hydration equilibrium does not exert an effect on CO₂ assimilation larger than 9%. The carbon isotope discrimination associated with CO₂ addition to ribulose-1,5-bisphosphate by Rubisco is slightly decreased by heavy water. This effect is proposed to originate from the use of solvent-derived proton/deuteron during the last step of the catalytic cycle of the enzyme (hydration/cleavage).

Introduction

Leaf photosynthesis is driven by the gradient of CO₂ mole fraction between the atmosphere and the intercellular spaces, by stomatal conductance and also by the internal gradient of CO₂ from intercellular spaces to carboxylation sites, which depends on internal conductance. Internal conductance is not infinite, so can be seen as a photosynthetic limitation (Evans *et al.* 1986). Many studies investigating the components and the origin of the internal conductance can be found in the literature (for a recent example see Warren and Dreyer 2006). Briefly, it is assumed that leaf internal conductance is made of two parts, a gas phase and a liquid phase component. Much uncertainty remains regarding the nature and the contribution of the components of the liquid part of the internal conductance.

Ever since carbonic anhydrases (CAs) were found in plants (Day and Franklin 1946; Bradfield 1947), their involvement as a component of the liquid phase conductance has been hypothesised. Much debate nevertheless remains on whether CAs, which catalyse the reversible interconversion of CO₂ and HCO₃⁻, are essential to C₃ photosynthesis. Chloroplastic CA has been assumed to be a key regulator of CO₂ supply to Rubisco carboxylation by favouring hydration of CO₂ in the peripheral stroma, thus, facilitating the diffusion of inorganic carbon within the chloroplast and balancing carboxylation by peripheral *v.* central Rubisco molecules in the chloroplast (Enns 1967; Arakelyan *et al.* 1993). In addition, correlations between the photosynthesis rate or the growth rate and CA activity have been detected (Khan 1994, 2002; Tiwari *et al.* 2006). Further, under Zn deficiency (which induces a decrease in CA activity) internal CO₂

transfer conductance is considerably decreased (Sasaki *et al.* 1998). However, Price *et al.* (1994) found that tobacco plants with low CA activity (2% of the wild-type value) showed nearly no effect on CO₂ assimilation and only a slight effect on carbon isotope discrimination. Williams *et al.* (1996) showed that CA-antisense plants had only a small decrease in photosynthetic carbon isotope discrimination (of around 1.5 ‰) and a drop in the (CO₂) oxygen isotope discrimination. As CA activity is responsible for oxygen exchange between CO₂ and leaf water (which is ¹⁸O-enriched because of transpiration), the latter result provides evidence that CA activity is decreased *in planta*. Therefore, it was concluded by those authors that CA suppression has a modest effect only on photosynthetic CO₂ transfer. With pea protoplasts, the addition of the soluble CA inhibitor acetazolamide influenced neither the photosynthetic rate nor electron transport (Ignatova *et al.* 2001). Thus, no clear picture emerges from the literature; it remains plausible that CA is involved in CO₂ transfer resistance, although other roles such as pH-buffering might be emphasised (Oja *et al.* 1999).

In the present paper, we take advantage of the effect of heavy water (D₂O) on photosynthesis of *Xanthium strumarium* (Asteraceae) leaves, to investigate the importance of the liquid phase components of internal conductance. In D₂O, all the components of the CO₂ conductance in the liquid phase are affected (Table 1). However, the CA-catalysed interconversion of CO₂ and bicarbonate is the most reduced, with a H₂O/D₂O isotope effect on both k_{cat} (turn-over rate) of hydration and K_{e} (equilibrium constant) – although there is nearly no $k_{\text{cat}}/K_{\text{m}}$ isotope effect. The value of the observed isotope effect

Table 1. The H₂O/D₂O isotope effects (α) considered in the present study

Reaction	α	Reference
<i>Carboxylation of RuBP by Rubisco (pH 7.5)</i>		Van Dyk and Schloss (1986)
k_{cat} isotope effect	2.8	
$k_{\text{cat}}/K_{\text{m}}$ isotope effect	2.3	
<i>Diffusion of CO₂ in water (calculated[†])</i>	0.995	Bearman and Jolly (1984)
<i>Interconversion CO₂/HCO₃⁻ by CA</i>		
K_{e} equilibrium isotope effect	2.7	Silverman and Vincent (1983)
k_{cat} isotope effect of CO ₂ hydration	3.8	
$k_{\text{cat}}/K_{\text{m}}$ isotope effect of CO ₂ hydration	1.0	
K_{e} equilibrium isotope effect	3.2	Pocker and Bjorkquist (1977)
k_{cat} isotope effect of CO ₂ hydration	3.3	
$k_{\text{cat}}/K_{\text{m}}$ isotope effect of CO ₂ hydration	1.1	
<i>Uncatalysed hydration of CO₂</i>		
Kinetic isotope effect	1.8	Pocker and Bjorkquist (1977)
<i>Dissolution of CO₂ in water</i>	0.994	Wilhelm <i>et al.</i> (1977)

[†]The calculation of the isotope effect assumes that water molecules aggregates involve six molecules, as suggested by O'Leary (1984).

depends upon the kinetic status of the reaction (close to or far from equilibrium). CO₂ hydration and dehydration are likely to be nearly at equilibrium in the chloroplast: the rate constants of the enzyme-catalysed reaction in both forward and backward directions are very high (in the 10⁵–10⁶ molecules site⁻¹ s⁻¹ range; Pocker and Ng 1973; Silverman and Vincent 1983). The observed isotope effect of a reversible reaction has been mathematically developed by Tcherkez (2004) and Tcherkez and Farquhar (2005) for aldolase. By strict analogy, assuming hydration is a simple reversible step, the net rate of bicarbonate production is a rational function of first-order kinetics (exponential terms). Applying the equation to CO₂ hydration (that is, CO₂ + H₂O → H⁺ + HCO₃⁻ and CO₂ + D₂O → D⁺ + DCO₃⁻), the isotope effect as a function of time is as follows (see Appendix by Tcherkez and Farquhar 2005 for the evidence):

$$\alpha_{\text{obs}} = \frac{1-u_{\text{H}}}{1-u_{\text{D}}} \times \frac{u_{\text{D}} + 1/K_{\text{eD}}}{u_{\text{H}} + 1/K_{\text{eH}}}, \quad (1)$$

where α_{obs} is the H/D observed isotope effect and u_{H} is given by (similarly for u_{D}): and

$$u_{\text{H}} = \exp\left(-k_{\text{H}}\left(1 + \frac{1}{K_{\text{eH}}}\right)t\right), \quad (2)$$

where k_{H} is the rate of the forward reaction (hydration), K_{eH} and K_{eD} are equilibrium constants, and t is time. With the kinetic values $k = k_{\text{cat}}/K_{\text{m}}$ and K_{e} by Silverman and Vincent (1983) and a site concentration of ~0.1 mmol L⁻¹, the isotopic equilibrium

(that is, the equilibrium isotope effect value) is reached in nearly 5 μ s (at pH 8 and an initial amount of 10 μ mol L⁻¹ CO₂). Such a delay is very short considering the time needed for carboxylation by Rubisco (the overall Rubisco-catalysed carboxylation turnover is near: eight sites per Rubisco molecule \times 1 mmol L⁻¹ \times 4 s⁻¹ site⁻¹ = 32 s⁻¹, which gives near 31 ms, for a physiological Rubisco amount about 10 times higher than that of CA). In other words, the CO₂/HCO₃⁻ conversion catalysed by carbonic anhydrase is very rapidly at isotopic equilibrium, and the thermodynamic isotope effect of nearly three applies (Table 1). This also accords with the investigation of the ¹⁸O abundance of leaf CO₂ efflux, which suggested that CO₂ fully exchanged its oxygen with chloroplastic water in the chloroplast in the light (Farquhar *et al.* 1993; Yakir *et al.* 1994; Cernusak *et al.* 2004).

Thus, the comparison of the (total) internal conductance in natural and heavy water provides a simple way to see whether a 3-fold change of carbonic anhydrase activity influences photosynthesis. We also recognise that the carboxylation rate by Rubisco is affected by deuterium (with a H/D isotope effect of around two, Table 1); nevertheless, the specificity factor $S_{\text{c/o}}$ is not modified (Kent and Tomany 1984): $S_{\text{c/o}}$ is simply the ratio of the rate constant of the carboxylation step to that of the oxygenation step (that is, k_6/k_3 ratio in the nomenclature by Farquhar 1979), both being water-independent (Lorimer 1981). Thus, the compensation point in the absence of day respiration (Γ^*) is not affected by heavy water, and neither is the carboxylation to oxygenation ratio, for a given CO₂-to-O₂ concentration ratio. The carbon isotope discrimination by Rubisco, however, is thought to be decreased by deuterium (Roeske and O'Leary 1984; Table 2).

In the following, we use ¹²C/¹³C isotopic techniques to measure internal conductance in H₂O and D₂O (in which CA activity is slowed down). We show that a difference of internal conductance arises indeed in heavy water, and we also explore the effects of heavy water on other processes associated with gas exchange of leaves.

Materials and methods

Plant material and growth conditions

Xanthium strumarium L. plants were grown (in the greenhouse) from seeds in 100-mL pots of potting mix and transferred to 3-L pots after 2 weeks. Minimum PPFD during a 16-h photoperiod was maintained at ~400 μ mol m⁻² s⁻¹ by supplementary lighting. Temperature and vapour pressure deficit were maintained at ~25.5/18.5°C and 1.4/1.2 kPa day/night, respectively. The carbon isotope composition ($\delta^{13}\text{C}$) of CO₂ in the greenhouse air was $-9.5 \pm 0.3\%$. The third or fourth leaves (from the apical bud) were used for all measurements. Heavy water (99.8% D₂O) was from Eurisotop (Saint-Aubin, France). For experiments using heavy water, leaves were cut (under water) at the end of the previous light period and the petiole placed in D₂O for a whole night period of 13 h to renew leaf water before starting gas-exchange measurements.

Gas-exchange measurements

The measurements were made as by Tcherkez *et al.* (2005). Briefly, the assimilation chamber was connected to the sample air hose of the LI-6400 (Li-Cor Inc.). The chamber was made of aluminium and clear plexiglass. Two fans placed in the chamber

Table 2. The H₂O/D₂O isotope effect on the ¹²C/¹³C isotope effects involved in water-dependent steps of C₃ photosynthesis

Step	¹² C/ ¹³ C isotope effect		Ratio	Reference
	in H ₂ O	in D ₂ O		
Carboxylation	1.029	1.021 ^A	1.008	Roeske and O'Leary (1984)
<i>Diffusion of CO₂ in water</i>				
Experimental	1.0007	–	–	O'Leary (1984)
Calculated	1.0009	1.0009	1	Bearman and Jolly (1984)
<i>Dissolution of CO₂ in water</i>				
Experimental	1.0011	–	–	O'Leary (1984)
Experimental	1.00107	–	–	Vogel <i>et al.</i> (1970)

^AValue obtained on spinach Rubisco in H₂O with deuterated ribulose-1,5-bisphosphate as a substrate.

gave a boundary layer conductance to water of ~6.7 mol m⁻² s⁻¹. Leaf temperature was controlled at 21°C with circulating water from a cooling water bath to the jacket of the leaf chamber, and was measured with a copper-constantan thermocouple plugged into the thermocouple sensor connector of the LI-6400 chamber/infrared gas analyser (IRGA). Ingoing air was dried (to ~1 mmol H₂O mol⁻¹) and passed through the chamber at a rate of 1 L min⁻¹, monitored by the LI-6400. Mole fractions of CO₂ were measured with the IRGA of the LI-6400. Light was supplied by a 500-W halogen lamp (Massive NV, Kontich, Belgium). Inlet CO₂ was obtained from a gas cylinder (Air Liquide, Grigny, France) with a δ¹³C of -50.2 ± 0.2‰. The outlet air of the chamber was regularly shunted and was sent to the loop to measure its isotope composition and, thus, the on-line carbon isotopic discrimination (Δ_{obs}). The gas inside the loop was introduced into the EA for GC as described previously (Tcherkez *et al.* 2003). Δ_{obs} during photosynthesis was measured following the method described by Evans *et al.* (1986). For internal conductance measurements, the CO₂ mole fraction in the chamber was maintained at 400 μL L⁻¹ and the relative humidity was maintained around 80%. The different A/c_a(A/c_i) values were obtained through varying the light level. Artificial air with 2% O₂ was supplied by a cylinder (Crystal gas mixture, 2% O₂ in N₂, Air Liquide).

Corrections for D₂O transpiration

When leaves transpired D₂O, no change was detected in water vapour pressure simply because the IRGA of the LI-6400 uses wavelengths (~2.6–2.8 μm) that are not absorbed by heavy water. Transpiration was measured microvolumetrically each 5 min (that is, Δt = 300 s) by monitoring the volume (denoted as *v*) needed to adjust the water level to an index (on the tube in which the peduncle was placed). Transpiration is then given by:

$$\Phi = \frac{v}{s \times \Delta t \times V_M}, \quad (3)$$

where *s* is leaf surface area and *V_M* the molar volume of D₂O (18.116 10⁻³ L mol⁻¹).

It should be noted that (providing changes in leaf water content are negligible) Φ is a net transpiration flux, which takes into account both diffusion of heavy water out of the leaf and diffusion of natural water into the leaf when inlet humidity is composed of natural water (H₂O). In other words, when natural

water (H₂O) was used in the inlet air, H₂O molecules may have entered the leaf while D₂O evaporated and, thus, may have had an effect on D₂O-transpiration. Although the effect is small, additional calculations have been made to correct for it (see Appendix II).

By contrast, when inlet water was D₂O, the net consumption flux Φ was equal to the loss of D₂O through transpiration, and no additional corrections were needed. The inlet D₂O vapour pressure was fixed with a Li-Cor dew-point generator Li-610, fed with heavy water. The difference in saturation vapour pressure between natural and heavy water was taken into account. The equations used to derive the conductance and *c_i* are then classical, after correcting for the diffusion ratio between D₂O and CO₂ (see Appendix II).

Isotopic analyses

The δ¹³C of inlet and outlet CO₂ were measured using the mass spectrometer 'Optima' (Micromass, Villeurbanne, France) coupled to an elemental analyser to separate CO₂ by chromatography (continuous flow), as described by Tcherkez *et al.* (2005). As heavy water may also be enriched in ¹⁸O, a property that would modify reaction rates further, the oxygen isotope composition of source water was measured. Source water δ¹⁸O (minimum water quantity of 0.1 mL) was measured after 48 h equilibration at 21°C with 500 μmol mol⁻¹ CO₂ in tight glass flasks; the δ¹⁸O of CO₂ was measured by introducing the gas sample with pure N₂ (N₂ Alphagaz, Air Liquide) into the loop of the elemental analyser described above. The isotope ratio of CO₂ is equal to that of the water (the molar ratio H₂O/CO₂ is more than 2 × 10³) corrected for the equilibrium isotope effect of CO₂-water exchange, that is, 1.0419 for H₂O (Brenninkmeijer *et al.* 1983) and 1.0236 for D₂O (Majzoub 1966). In the present paper, the δ-values are given with respect to PDB (carbon) and SMOW (oxygen).

The carbon isotope composition of night-respired CO₂ and the night-respiration rate were measured using a closed system coupled to the mass spectrometer through the elemental analyser, as described by Tcherkez *et al.* (2003).

¹²C/¹³C isotopic theory

The isotopic theory developed in the following has been described elsewhere (Farquhar *et al.* 1989) and the main equations only are recalled here. The 'theoretical' form of the carbon isotope fractionation associated with photosynthesis

(denoted as Δ_i), which neglects (photo) respiratory fractionation and the boundary layer resistance and assumes an infinite internal conductance, is:

$$\Delta_i = a + (\bar{b} - a) \frac{c_i}{c_a}, \quad (4)$$

where the diffusional fractionation a is the fractionation associated with diffusion and \bar{b} is the fractionation associated with carboxylation. c_a and c_i are the outlet and internal mole fractions of CO_2 . Two different uses of the above equation should be distinguished: that one used for regression analyses (conducted in Fig. 1) and that one used for calculating the deviation of the observed discrimination from the theoretical value (see below). In the first case, the fitted \bar{b} value integrates internal conductance effects (and so is generally less than 29‰). In the second case, \bar{b} is purely related to carboxylation.

The more complete expression of the isotope discrimination, usually assumed to explain the ‘observed’ photosynthetic fractionation (Δ_{obs}), is given by:

$$\Delta_{\text{obs}} = a_b \frac{c_a - c_b}{c_a} + a \frac{c_b - c_i}{c_a} + (a_1 + e_s) \frac{c_i - c_c}{c_a} + b \frac{c_c}{c_a} - \frac{eR_d/k + f\Gamma^*}{c_a}, \quad (5)$$

where the subscripts a, b, i and c refer to atmospheric, leaf surface, intercellular and carboxylation site CO_2 , respectively. Here, a_b is the gaseous diffusional fractionation associated with the boundary layer (2.9‰), and a_1 (1.1‰) and e_s (0.7‰) are

the fractionations associated with diffusion in the liquid phase and with CO_2 dissolution, respectively. Also, e and f are the fractionations associated with day respiration (rate R_d) and photorespiration, respectively, k is the carboxylation efficiency and Γ^* the CO_2 -compensation point in the absence of day respiration, and b is the carbon isotope fractionation associated with Rubisco-catalysed carboxylation. Under the assumption that $b = \bar{b} = 29\text{‰}$ and $a_b \frac{c_a - c_b}{c_a}$ is negligible, it can then be shown that the internal CO_2 -conductance (denoted as g_m) is such that (Evans *et al.* 1986):

$$\Delta_i - \Delta_{\text{obs}} = \frac{b - e_s - a_1}{g_m} \times \frac{A}{c_a} + d, \quad (6)$$

where A is net assimilation and $d = (eR_d/k + f\Gamma^*)/c_a$. A linear regression can be used only if d is constant, that is, if c_a is maintained constant during experiments (in the present study, it was $400 \mu\text{mol mol}^{-1}$). Such a linear model is used in Fig. 2A (see below).

The internal conductance can also be obtained with the following relationship, which does not assume similar values for b and \bar{b} (von Caemmerer and Evans 1991):

$$(\Delta_i - \Delta_{\text{obs}}) \frac{c_a}{c_i} = \frac{b - e_s - a_1}{g_m} \times \frac{A}{c_i} + (\bar{b} - b) + d \frac{c_a}{c_i} \quad (7)$$

If d is small (say, 1‰), the intercept of this relationship is close to $\bar{b} - b$. This linear model (with such an assumption) is used in Fig. 2B (see below).

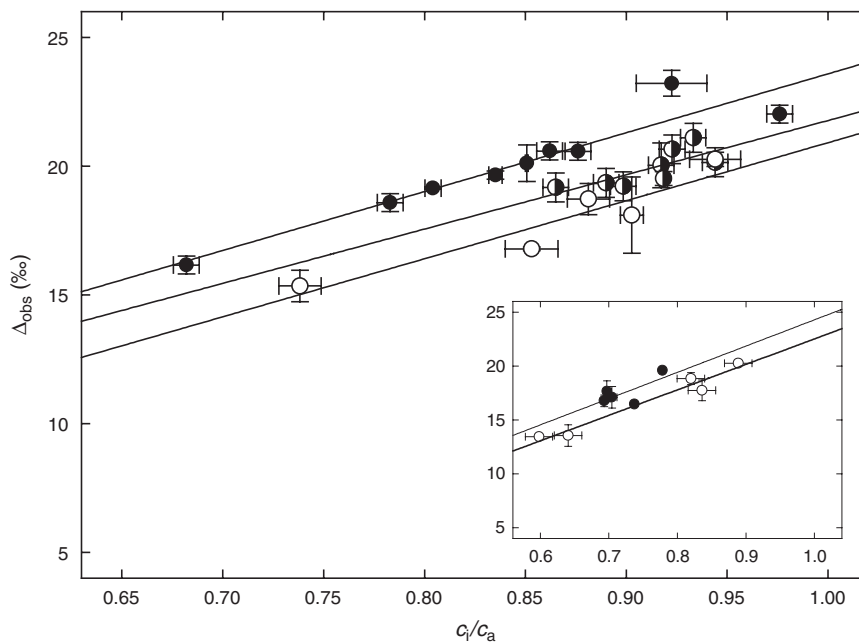


Fig. 1. The relationship between the on-line carbon isotope discrimination (Δ_{obs}) and c_i/c_a of detached leaves in H_2O (closed circles) or D_2O with either H_2O or D_2O in the inlet air (semi-closed and open circles, respectively). Straight lines are linear type II regressions. They are significant ($F = 66.4$, $P < 0.0001$; $F = 7.9$, $P < 0.05$; $F = 20.7$, $P < 0.02$ for H_2O , D_2O and $\text{D}_2\text{O} + \text{D}_2\text{O}$ inlet, respectively). Slopes are $22.9 \pm 2.8\text{‰}$ and $21.1 \pm 3.0\text{‰}$ for H_2O and D_2O , respectively. Intercepts are $0.72 \pm 2.3\text{‰}$ (H_2O), $0.69 \pm 3.2\text{‰}$ ($\text{D}_2\text{O} + \text{inlet H}_2\text{O}$) and $-1.6 \pm 4.2\text{‰}$ ($\text{D}_2\text{O} + \text{inlet D}_2\text{O}$). (Inset) same as in the main panel, in 2% O_2 . Slopes are $22.5 \pm 4.8\text{‰}$ and $23.6 \pm 2.8\text{‰}$ for H_2O and D_2O , respectively. Intercepts are $-1.62 \pm 4.2\text{‰}$ (H_2O) and $-1.12 \pm 2.2\text{‰}$ (D_2O). Both regressions are significant ($F = 20.7$, $P < 0.02$; $F = 66.8$, $P < 0.005$). Data represent means (\pm s.e.) on three measurements made with independent leaves.

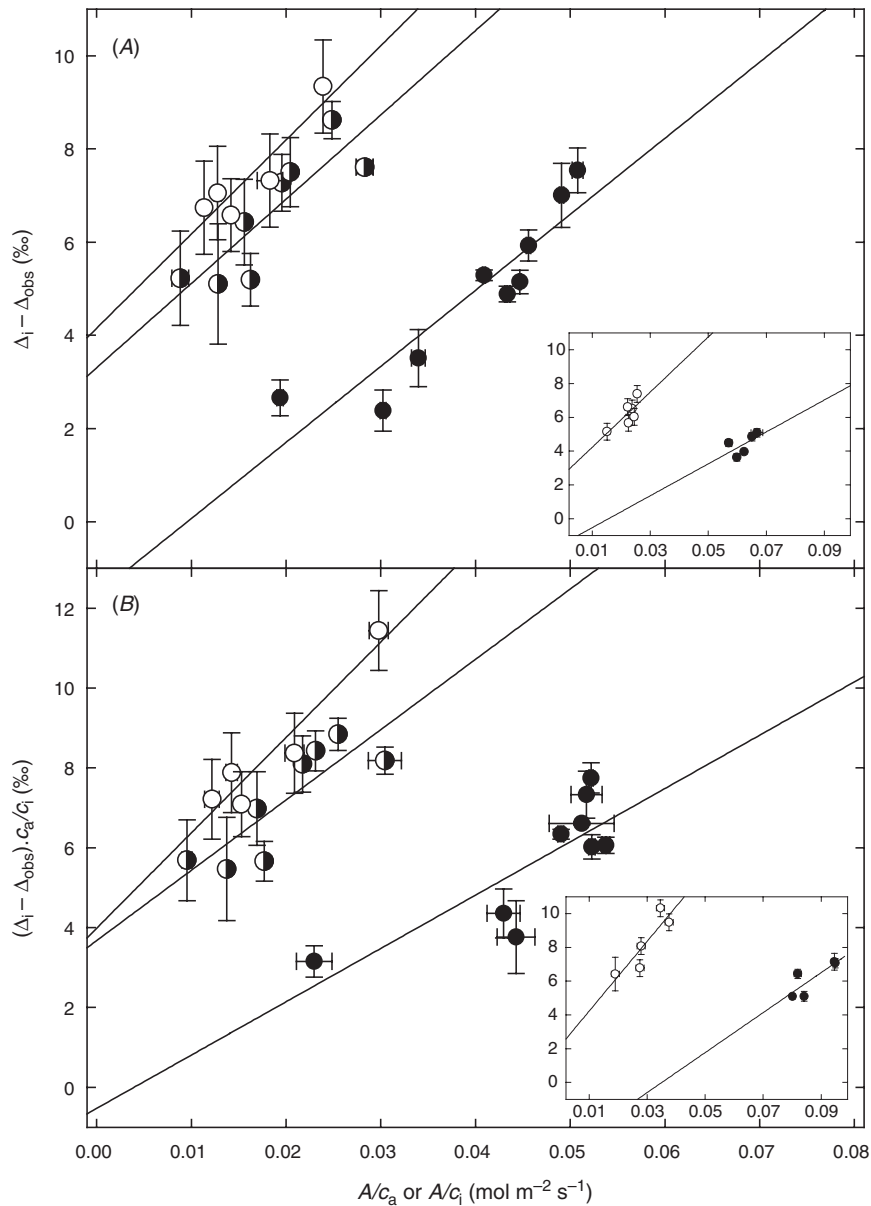


Fig. 2. (A) The relationship between the deviation $\Delta_i - \Delta_{\text{obs}}$ of the on-line carbon isotope discrimination from the theoretical value and A/c_a . It assumes a Rubisco-catalysed $^{12}\text{CO}_2/^{13}\text{CO}_2$ discrimination of 29‰. Data are on detached leaves in both H_2O (closed circles) and D_2O with either H_2O or D_2O in the inlet air (semi-closed and open circles, respectively). A/c_a is varied through the light level, at constant c_a of $400 \mu\text{mol mol}^{-1}$. Straight lines are linear type II regressions. They are all significant ($F = 16.5, P < 0.01$; $F = 37.7, P < 0.001$; $F = 16.4, P < 0.03$ for H_2O , D_2O , and $\text{D}_2\text{O} + \text{inlet D}_2\text{O}$, respectively). Slopes and intercepts are 163 ± 26 , 181 ± 40 and $202 \pm 50 \text{‰ mol}^{-1} \text{ m}^2 \text{ s}$, and -1.6 ± 1.0 , $+3.3 \pm 0.9$ and $+4.1 \pm 0.8 \text{‰}$, respectively. (Inset) same as in (A), in 2% O_2 . Slopes are $95 \pm 71 \text{‰ mol}^{-1} \text{ m}^2 \text{ s}$ and $163 \pm 66 \text{‰ mol}^{-1} \text{ m}^2 \text{ s}$ for H_2O and D_2O , respectively. Intercepts are $-1.47 \pm 4.4 \text{‰}$ (H_2O) and $2.6 \pm 1.5 \text{‰}$ (D_2O). The D_2O -regression is significant ($F = 6.0, P < 0.07$) but that associated with H_2O is not with a typical threshold less than 0.1 ($F = 1.8, P < 0.28$). (B) Same data replotted as $(\Delta_i - \Delta_{\text{obs}}) \cdot c_a/c_i$ v. A/c_i . Linear regressions are significant ($F = 15.1, P < 0.01$; $F = 13.2, P < 0.01$; $F = 30.6, P < 0.02$ for H_2O , D_2O and $\text{D}_2\text{O} + \text{inlet D}_2\text{O}$, respectively); slopes and intercepts are 133 ± 36 , 176 ± 45 and $239 \pm 43 \text{‰ mol}^{-1} \text{ m}^2 \text{ s}$, and -0.5 ± 1.7 , $+3.7 \pm 0.9$ and $+3.9 \pm 0.8 \text{‰}$, respectively. (Inset) same as in (B), in 2% O_2 . Slopes are $118 \pm 47 \text{‰}$ and $206 \pm 54 \text{‰}$ for H_2O and D_2O , respectively. Intercepts are $-4.1 \pm 4.2 \text{‰}$ (H_2O) and $2.2 \pm 1.6 \text{‰}$ (D_2O). Both regressions are significant ($F = 6.2, P < 0.08$; $F = 14.1, P < 0.02$). Each datum represents the mean (\pm s.e.) on three measurements made on independent leaves.

It should be noted that the two linear models described above may be considered as two extreme cases: in Eqn 6, the b values are considered similar so that the offset that may occur between H_2O and D_2O would be attributed

to the (photo)respiratory component, and in Eqn 7, such an offset is attributed to a shift in the b value. Both models are then used here to give a range of b and d values.

Results

Photosynthesis values in heavy water

In heavy water, the net assimilation rate, measured in conditions similar to those during growth, was reduced nearly 2-fold (Table 3), a value close to the k_{cat}/K_m isotope effect (2.3) of Rubisco (Table 1). The inhibition of the photosynthesis rate did not come from the considerable ^{18}O -enrichment in D_2O that might occur because of the D_2O production techniques such as distillation (Malkov 1959): source D_2O had a near-natural ^{18}O -abundance, with a $\delta^{18}\text{O}$ value of +27.1‰ (Table 3). In addition, neither the photosynthesis rate nor the isotope discrimination changed significantly when H_2O was changed to D_2O in the inlet of the open gas-exchange system (Table 3), showing that, with inlet H_2O , the possible dilution of D_2O by H_2O was very small (see also the Appendix I).

In contrast to the assimilation rate, the carbon isotope discrimination was unchanged in D_2O , with values of around 20‰ (Table 3), indicating that $^{12}\text{C}/^{13}\text{C}$ isotopic properties of photosynthesis might not be influenced by heavy water, unless compensating mechanisms occurred (see below).

The respiration rate was very slightly (not significantly) affected by heavy water, with an additional ^{13}C -depletion of 2.1‰ of night-respired CO_2 after 13 h in the dark (Table 3).

The relationship between $\Delta^{13}\text{C}$ and c_i/c_a in natural and heavy water

The plot of the on-line carbon isotope discrimination ($\Delta^{13}\text{C}$, also usually denoted as Δ_{obs}) v. the internal-to-external CO_2 ratio (c_i/c_a) is shown in Fig. 1. Clearly, there was no drastic difference in the slope of the relationship so that the \bar{b} value of the (simplified, with infinite internal conductance g_m) model by Farquhar *et al.* (1982) was only slightly influenced by heavy water: in heavy water, \bar{b} was $25.5 \pm 3.0\text{‰}$ (H_2O in the inlet) and $22.5 \pm 4.9\text{‰}$ (D_2O in the inlet) and it was $27.2 \pm 2.8\text{‰}$ in natural water. In 2‰ O_2 , the \bar{b} value was similar in H_2O and D_2O (Fig. 1, inset). In both 21 and 2‰ O_2 , the difference between \bar{b} values obtained in D_2O and H_2O was not significant; in other words, at this stage of the analysis, it seems that the carbon isotope effect associated with carboxylation is unaffected by heavy water (but see below).

The internal conductance in natural and heavy water

The relationship between the deviation $\Delta_i - \Delta_{\text{obs}}$ of the carbon isotope discrimination from the value at infinite conductance with zero (photo)respiratory fractionation (Δ_i) as a function of A/c_a is shown in Fig. 2A. Not surprisingly, data for heavy water are on the left hand side because of low A values (lower A/c_a values).

Further, the heavy and light datasets did not appear to be on the same relationship; however, the slopes were quite similar. Using a carboxylation discrimination value of 29‰ for both D_2O - and H_2O -experiments (see 'Isotope theory'), we found internal conductance values of 0.166 in H_2O , and 0.150 (inlet H_2O) and 0.134 (inlet D_2O) $\text{mol m}^{-2} \text{s}^{-1}$ in D_2O . The difference between conductance values so obtained in H_2O and D_2O was not statistically significant (Table 4). By contrast, the intercept was larger in D_2O (+3.3 and +4.1‰) compared with H_2O (−1.5‰). In the framework of the linear model of Fig. 2A, the intercept value accounts for the (photo)respiratory fractionations. As the specificity factor of Rubisco does not change in heavy water, one might assume that the offset came from the larger $^{12}\text{C}/^{13}\text{C}$ isotope effects associated with (photo)respiratory decarboxylations. It could be so if these decarboxylations were slowed down and became more rate-limiting in heavy water (see also Discussion).

However, in 2‰ O_2 , the results were similar, in that the deviation $\Delta_i - \Delta_{\text{obs}}$ was still larger in D_2O compared with H_2O , with a $\sim 3\text{‰}$ difference in the intercept (Fig. 2A, inset); the slopes were slightly but non-significantly different (Fig. 2A, legend). This simply indicates that the photorespiratory fractionation was not likely to be responsible for the offset between the H_2O and the D_2O lines of Fig. 2A.

When plotting the discrimination deviation times c_a/c_i , a difference also emerged in the intercept (which is equal to $\bar{b} - b + d \times c_a/c_i$) (Fig. 2B) and this difference did not change to a large extent in 2‰ O_2 (Fig. 2B, inset). The (photo) respiratory contribution to the intercept, thus, seems to be (very) small. Now neglecting the photorespiratory term d , the b -difference would be -0.5 in H_2O and $+3.7$ in D_2O (and -4.1 and $+2.2$ in 2‰ O_2). In other words, this would indicate a lower carbon isotope fractionation within the range 25–26‰ in heavy water [that is, 25.3‰ (inlet H_2O) and 25.0‰ (inlet D_2O) in 21‰ O_2 and 26.8 in 2‰ O_2] compared with that in natural water (29.5‰ in 21‰ O_2 and 33.1‰ in 2‰ O_2) (Table 4). Using these values of b , the slopes in 21‰ O_2 were such that the internal conductance was significantly lower ($P < 0.05$) in heavy water: 0.205 ± 0.048 in H_2O and 0.144 ± 0.020 (inlet H_2O)/ 0.114 ± 0.025 (inlet D_2O) $\text{mol m}^{-2} \text{s}^{-1}$ in D_2O (Table 4). The same trend occurs in 2‰ O_2 (0.280 ± 0.092 in H_2O and 0.130 ± 0.043 $\text{mol m}^{-2} \text{s}^{-1}$ in D_2O , Table 4) although the difference is not statistically significant.

Discussion

Although the importance of internal conductance to CO_2 transfer (g_m) has been well recognised, studies about it are frequently subject to technical difficulties (Warren 2006), so

Table 3. Photosynthetic and respiratory properties of *Xanthium strumarium* detached leaves in natural water (H_2O) and in heavy water (D_2O)

The respiration rate (R_n) and the isotope composition of night-respired CO_2 ($\delta^{13}\text{C}$) are indicated. Photosynthesis (A), stomatal conductance to CO_2 (g_s) and $^{12}\text{C}/^{13}\text{C}$ isotope discrimination are average values in $400 \mu\text{mol mol}^{-1} \text{CO}_2$, $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 21°C and 21‰ O_2 (conditions similar to growth conditions). Mean \pm s.e. ($n = 4$). Between brackets: photosynthesis-related values in D_2O with inlet D_2O conditions in the light

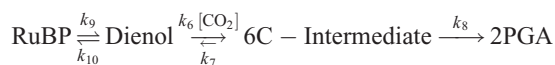
Source water ($\delta^{18}\text{O}$, ‰)	R_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	$\delta^{13}\text{C}$ (‰)	A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	$\Delta^{13}\text{C}$ (‰)
H_2O (−21.2 \pm 1.8)	0.73 \pm 0.06	−27.3 \pm 0.5	16.5 \pm 0.5	0.23 \pm 0.01	19.6 \pm 0.1
D_2O (+27.1 \pm 0.5)	0.69 \pm 0.05	−29.4 \pm 0.4	9.6 \pm 0.5 (8.7 \pm 0.9)	0.21 \pm 0.03 (0.20 \pm 0.08)	20.0 \pm 0.1 (20.6 \pm 0.6)

that the magnitude of the components of g_m are not well known. Here we took advantage of the slowing effect of heavy water on $\text{CO}_2\text{-HCO}_3^-$ equilibrium and carboxylation to investigate their roles in photosynthesis. In fact, heavy water has negligible effects on other steps (dissolution, diffusion) (see Appendix I). Although the effect of D_2O on electronic (primary) photosynthetic reactions has been extensively studied (see Soriano and Cramer (2001) and Flores *et al.* (2006) as recent examples), to our knowledge it is the first time the effect of D_2O on both photosynthetic gas exchange and $^{12}\text{C}/^{13}\text{C}$ discrimination has been studied.

H₂O/D₂O isotope effect associated with carboxylation

Heavy water caused an isotope effect of nearly two on the assimilation rate (Table 3), and this is consistent with the H/D (k_{cat}/K_m) isotope effect associated with carboxylation (by Rubisco) of 2.3 (Table 1). The effect of heavy water on the $^{12}\text{C}/^{13}\text{C}$ fractionation by Rubisco can be seen with (i), a plot showing $\Delta_{\text{obs}} v. c_i/c_a$, the slope of which is $\bar{b} - a$ (but in such a plot, \bar{b} integrates internal conductance effects, see 'Theory' section) (ii), the intercept of the plot showing $(\Delta_i - \Delta_{\text{obs}})c_a/c_i v. A/c_i$. In 21% O_2 , the estimated fractionation associated with carboxylation seems to be lowered by a few permil, as revealed by the slightly smaller slope in Fig. 1 and the larger intercept of Fig. 2B. This is also true in 2% O_2 (in low-oxygen non-photorespiratory conditions, the (photo)respiratory component $d = (eR_d/k + f\Gamma^*)/c_a$ decreases to eR_d/kc_a which is very small). The drop would be nevertheless small, in the range 1.7–4.2‰ (compared with the *in vitro* Rubisco 29‰ value), so that the relative decrease of the fractionation is between 5 and 14%.

The effect of D_2O on the $^{12}\text{C}/^{13}\text{C}$ fractionation can be understood in the light of the mechanism of the catalytic cycle. This has been formalised as (Farquhar 1979; Tcherkez and Farquhar 2005):



where RuBP stands for ribulose-1,5-bisphosphate and PGA for phosphoglyceric acid. Under the assumption that k_7 (rate constant associated with decarboxylation) is negligible, the observed $^{12}\text{C}/^{13}\text{C}$ isotope effect associated with CO_2 addition is $^{12}k_6/^{13}k_6$. As a result, it is D_2O -independent because heavy water slows down enolisation (that is, k_9 and k_{10} rate constants) and, perhaps, hydration/cleavage (rate constant k_8) (Tcherkez and Farquhar 2005). This view is in agreement with the variation of the carbon isotope effect in a phylogenetic range of Rubiscos, which is interpreted as a change in the intrinsic isotope effect (Tcherkez *et al.* 2006) rather than (i), a change in other limiting steps or (ii), in the prevalence of decarboxylation, which has been found to be negligible in assay conditions (Pierce *et al.* 1986; and see McNevin *et al.* 2007; for a discussion).

However, we find here a reduction of a few permil and accordingly, Roeske and O'Leary (1984) also found an effect of deuterated RuBP on the carbon isotope fractionation by Rubisco (Table 2). Such an effect probably comes from a slower hydration/cleavage rate in heavy water, which would artificially induce the reverse reaction of carboxylation (that is,

decarboxylation k_7) to occur. It would be so because the energy barrier for hydration is higher in heavy water, making decarboxylation more likely. As decarboxylation probably fractionates against ^{13}C , it would compensate for the isotope effect of CO_2 addition by decarboxylating ^{13}C -depleted CO_2 . This would explain the diminution of the carbon isotope discrimination by deuterium.

There are several reasons to consider such a scenario as reasonable, if not likely. First, there is a higher H/D isotope effect on k_{cat} ($= k_8$, the rate constant of hydration and cleavage) than on k_{cat}/K_m (~overall rate) (2.8 as compared with 2.3, Table 1). Second, this would agree with the catalytic mechanism proposed by Cleland *et al.* (1998) and the hypothesis of Mauser *et al.* (2001) that the carbamylated Lysine of the active site (Lys 201), which is involved in proton abstraction during ribulose-1,5-bisphosphate enolisation, is also involved in the hydration of the 6-carbon intermediate formed by CO_2 addition (in other words, deuteration of the Lys residue would be responsible for slowing the k_8 step, which in turn may promote decarboxylation of the 6C intermediate). Parenthetically, this would explain why D_2O and deuterated RuBP have similar effects on $^{12}\text{C}/^{13}\text{C}$ fractionation. Third, in the tobacco L335V line in which there is a leucine-to-valine mutation in loop 6 of the large subunit, both the k_{cat} and the $^{12}\text{C}/^{13}\text{C}$ fractionation are lower than in the wild type, and this has been interpreted as a change in the probability of decarboxylation (McNevin *et al.* 2007).

H₂O/D₂O isotope effect on photorespiratory fractionation

The y-intercepts of Fig. 1 give the value of a (diffusional fractionation) minus the (photo) respiratory fractionation d . In 21% O_2 , the intercept is 0.72‰ in H_2O v. 0.69‰ in D_2O + inlet H_2O and -1.6 ‰ in D_2O + inlet D_2O . They are statistically identical, and although a change to low O_2 conditions did change the estimated intercepts, there was no significant difference between H_2O and D_2O conditions. However, there was a clear offset between the relationship of Fig. 2 in H_2O and that in D_2O in air (main panel), and again in 2% O_2 (inset). In 2% O_2 , the offset was slightly decreased by 1–1.5‰. Neglecting day respiration (which is indeed small) and assuming a realistic Γ^*/c_a value of around 0.1, this 1–1.5‰ decrease (in the offset) would indicate an increase of the isotope effect associated with glycine decarboxylation from 1.020 (Tcherkez 2006) to 1.030–1.045. This corresponds to what would be observed if decarboxylation became more limiting, so that the isotope effect approaches the intrinsic limit (1.060, Tcherkez 2006). We also note that the (photo)respiratory fractionation value obtained in H_2O is negative (Fig. 2A). This might come from the large ^{13}C -enrichment of growth CO_2 (-9.5 ‰) (and so, of decarboxylated CO_2) compared with inlet CO_2 (-50.2 ‰).

H₂O/D₂O isotope effect on CO₂ evolved in darkness

There is an effect of D_2O on dark-respired CO_2 (that is, CO_2 evolved in the night-time) as a 2.1‰ ^{13}C -depletion of the night-respired CO_2 was observed in heavy water (Table 3). The yeast thiamine diphosphate-dependent pyruvate decarboxylase (EC 4.1.1.1) undergoes a (small) $\text{H}_2\text{O}/\text{D}_2\text{O}$ solvent isotope effect

near unity (Wang *et al.* 2001) and similarly, little isotope effect may be assumed for the respiratory enzyme pyruvate dehydrogenase (EC 1.2.4.1, also thiamine diphosphate dependent) so that the weak effect of D₂O on the respiratory rate comes as no surprise (Table 3). We also note that the H/D isotope effect associated with the yeast pyruvate decarboxylase is larger on k_{cat} than on k_{cat}/K_m (Wang *et al.* 2001). In other words, the CO₂ production step of the catalytic cycle (the rate constant of which is k_{cat}) becomes limiting in heavy water, because of the slower protonation of the α -carbon of pyruvate. Consequently, a larger ¹²C/¹³C isotope effect associated with CO₂ production is expected and there is indeed a ¹³C-depletion of evolved CO₂ in heavy water (Table 3).

H₂O/D₂O isotope effect on internal conductance

The effect of heavy water on the internal conductance, given by the slope of the deviation $\Delta_i - \Delta_{\text{obs}}$, is negligible in the present study when a common $b = 29\%$ discrimination for carboxylation is assumed (Fig. 2A). When a difference in the b value (Table 4) is considered to account for the difference in intercepts of $(\Delta_i - \Delta_{\text{obs}}) \times c_a/c_i$, a difference occurs (Fig. 2B), which is significant (with $P < 0.06$) in 21% O₂: 0.205 in H₂O v. 0.144 and 0.114 mol m⁻² s⁻¹ in D₂O (Table 4). In the usual conditions (our plant growth conditions, Table 3), this decrease in internal conductance would lead to a drop of ~30 $\mu\text{mol mol}^{-1}$ in intracellular CO₂ mole fraction (c_c) when leaves are subjected to D₂O. We may calculate what the effect on CO₂ assimilation would be when CA activity is slowed down (regardless of the H/D isotope effect on Rubisco) in an electron transport limited mode, if c_c drops by 30 $\mu\text{mol mol}^{-1}$ from 200 $\mu\text{mol mol}^{-1}$. Using $(c_c - \Gamma^*) / (c_c + 2\Gamma^*)$ as a scaled estimate of A , we get $(200 - 40) / (200 + 80) = 160/280 = 0.57$ with full CA-activity. If CA activity goes down and $c_c = 170 \mu\text{mol mol}^{-1}$, we have $(170 - 40) / (170 + 80) = 130/250 = 0.52$. The resulting difference in A is near 9%. Although other effects such as reallocation of nitrogen to Rubisco synthesis and larger stomatal conductances occurred (though they were statistically insignificant) in the study by Price *et al.* (1994), our study is in accord with the findings of these authors, who observed little change in the assimilation rate when CA activity was

reduced. Our results are also consistent with the theoretical study by Cowan (1986), in which the rate of CO₂ fixation with optimal partitioning of nitrogen (between Rubisco and CA) is only ~5% greater than it would be if the same amount of nitrogen were invested in Rubisco alone.

The elasticity value of internal conductance with respect to CA activity is, at most,

$$1 - \frac{\frac{g_m(\text{D}_2\text{O})}{g_m(\text{H}_2\text{O})}}{1 - \frac{K_c(\text{D}_2\text{O})}{K_c(\text{H}_2\text{O})}} = \frac{1 - \frac{0.114}{0.205}}{1 - \frac{1}{2.7}} \approx 0.7 \quad (8)$$

In other words, a 3-fold reduction of the CA equilibrium constant has a modest effect (elasticity less than 1) on the CO₂ transfer resistance in the typical conditions of the present study (21% O₂, 400 $\mu\text{mol mol}^{-1}$ CO₂, 21°C). Extremely low (chloroplastic) CA plants exhibit a slight reduction of the carbon isotope discrimination – and a larger decrease of the oxygen isotope discrimination because of the strong alteration of the CO₂/H₂O equilibration efficiency – (Price *et al.* 1994; Williams *et al.* 1996). The 3-fold reduction in the present study did not induce a measurable effect on $\Delta^{13}\text{C}$ (Table 3). This is so because CO₂ assimilation was lower, and thus c_i/c_a values were higher, compensating for the smaller fractionation associated with carboxylation. This is also explained by the very high catalytic rates of the enzyme (k_{cat} values indicate that the enzyme is nearly limited by diffusion only) that make the CO₂/H₂O equilibration very efficient.

In plants where CA activity reduction is caused by Zn deficiency, Sasaki *et al.* (1998) found a decrease of the internal conductance that is comparable to our observations, although it could be related to other additional side effects of Zn deficiency. Parenthetically, we emphasise the fact that all cellular CAs are slowed down in our study, indicating that the minor effect of the deficiency in chloroplastic CA on photosynthesis (found by Price *et al.* 1994; Williams *et al.* 1996; Sasaki *et al.* 1998) can be generalised to cell CA activity as a whole.

Our study, which is in agreement with papers using genetic manipulations of CA, indicates that CA enzymatic activity is a limitation to C₃ photosynthesis but not a major one. Similarly, in C₄ plants, it has been recently shown that a reduced CA activity also has little effect on photosynthesis (while the CO₂-H₂O oxygen exchange was reduced, as revealed by $\Delta^{18}\text{O}$ values), unless CA activity was suppressed by antisense manipulation (Cousins *et al.* 2006a, 2006b). The modest effect of the catalytic activity of CAs on facilitating photosynthetic CO₂ fixation raises the question of the biological significance of these enzymes, and the cost (in terms of energy for synthesis and nitrogen requirement) they may represent. Nevertheless, as suggested by Cowan (1986), an enhancement ~5% of photosynthesis is not negligible as far as evolution is concerned, particularly when the proteins involved account for no more than 2% of leaf proteins. In addition, while CAs might be involved in PSII-catalysed O₂ generation (Villarejo *et al.* 2002; but see McConnell *et al.* 2007), we recognise that CAs may have other important roles, such as contributing to the structure of the thylakoid membrane (e.g. Rudenko *et al.* 2007).

Table 4. Internal conductance (g_m) values (in mol m⁻² s⁻¹) obtained in the present study, from regressions based on Fig. 2 data

Between brackets: b values (in %) from the intercept of Fig. 2B. In 21% O₂, the two assumptions about b did not significantly affect the g_m values ($P = 0.05$). n.s., regression non-significant. The b values are significantly different between H₂O and D₂O treatments in both O₂ conditions ($P < 0.05$)

Assumption	g_m in H ₂ O	g_m in D ₂ O	g_m in D ₂ O + inlet D ₂ O
<i>Data in 21% O₂</i>			
$b = 29\%$	0.166 ± 0.038	0.150 ± 0.049	0.134 ± 0.026
b values from regression	0.205 ± 0.048 (29.5 ± 1.8)	0.144 ± 0.020 (25.3 ± 1.0)	0.114 ± 0.025 (25.0 ± 0.8)
<i>Data in 2% O₂</i>			
$b = 29\%$	n.s.	–	0.166 ± 0.056
b values from regression	0.280 ± 0.092 (33.1 ± 4.2)	–	0.130 ± 0.043 (26.8 ± 1.6)

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Appendix I. Validity of the present D-enrichment method

It might be argued that D₂O is strongly diluted at the evaporative sites because of the exchange with H₂O from the inlet air of the gas-exchange system. However, because the isotope ratio of source water is very high (99.8% D₂O, that is, D/H = 499), the dilution by inlet water is small, as we explain below. Accordingly, the experiments with H₂O or D₂O in inlet air do not differ significantly (Fig. 2, semi-closed and open symbols and Tables 3 and 4). In other words, the modest extent of the effect of heavy water on internal conductance (as discussed in the main text) is not caused by an artefactual depletion in deuterium in the leaf when H₂O is used in the inlet.

Leaf water isotope composition

In the case of strong D-enrichment, the relationship giving the isotopic abundance at the evaporative sites uses proportions instead of delta-values. If R_S is the D/H ratio of source water, the proportion of D is $P_D = R_S/(1+R_S)$ and that of H is $P_H = 1/(1+R_S)$. In transpired water,

$$P_D = \frac{g_D(d_i - d_a)}{g_H(w_i - w_a) + g_D(d_i - d_a)} \quad \text{and} \quad P_H = \frac{g_H(w_i - w_a)}{g_H(w_i - w_a) + g_D(d_i - d_a)}$$

where g_D and g_H are D₂O and H₂O conductance, d_i and w_i are D₂O- and H₂O-mole fractions at the evaporative sites, and R_S is the D/H ratio of source water. In the steady-state, the proportion of D (P_D) of source water equals that of transpired water, and this gives (both denominators $(1 + R_S)$ and $g_D(d_i - d_a) + g_H(w_i - w_a)$ simplify):

$$R_S = \frac{g_D(d_i - d_a)}{g_H(w_i - w_a)}$$

This equation is identical to the Craig-Gordon model applied to leaf water (Flanagan *et al.* 1991). Re-arranging, that relationship gives the D/H isotope ratio of water at the evaporative sites (R_e) as follows:

$$R_e = \alpha^+ \left(\alpha_k R_S (1-h) + R_v h \right), \quad (\text{A1})$$

where R_v is the D/H ratio of atmosphere water, respectively. Here, h is relative humidity and α^+ (1.116) and α_k (1.040) are the isotope effects associated with the liquid-vapour equilibrium and diffusion in air, respectively. The mass-balance equation applied to heavy water gives:

$$u_e R_{in} w_e + s E R_E = \left(u_e + s(E + R_E E) \right) R_v w_a, \quad (\text{A2})$$

where u_e is the entering flow, R_{in} the D/H isotope ratio of inlet water; s is the leaf surface area and E is H₂O-transpiration. w_e and w_a are the water partial pressures of inlet air and outlet air, respectively. R_E is the D/H isotope ratio of transpired water, which is equal, in the steady-state, to R_S . Eqn A1 may be re-arranged to:

$$R_v w_a = \frac{1 + \frac{1}{R_E} \left(\frac{u_e}{sE} \times R_{in} w_e \right)}{1 + \frac{1}{R_E} \left(1 + \frac{u_e}{sE} \right)}$$

In the present study, $R_E = R_S$ is 99.8/0.2, that is, 499, but inlet water is made of natural water (very small R_{in}). Here, $u_e/sE R_E$ is in the range $3.7 \cdot 10^{-4} \text{ mol s}^{-1} / (10^{-2} \text{ m}^2 \times 10^{-4} \text{ mol m}^{-2} \text{ s}^{-1} \times 499) = 0.76$, so that the right term in the numerator can be neglected and $R_v w_a \sim 1/1.76 = 0.57$. With a value of $w_a = 0.01 \text{ mol mol}^{-1}$, we find $R_v \sim 57$. Using Eqn A1 we then have $R_e = 166$, which is equivalent to 99.4% D₂O. In other words, the dilution of heavy water in the leaf by inlet H₂O is very small. This is consistent with the very small effect of heavy v. light inlet water on ¹²C/¹³C isotopic data in the present paper (Figs 1, 2).

Other steps possibly slowed down in heavy water

As already stated in the main text, heavy water has an effect on two main steps, namely, the Rubisco-catalysed reaction, and the CO₂-HCO₃⁻ equilibrium, catalysed by carbonic anhydrase (Table 1). Other slowing effects caused by heavy water are negligible in the present study:

- (i) there is no effect of heavy water on the ¹²C/¹³C isotope effect associated with diffusion in water (Table 2), and
- (ii) the effect of heavy water on the ¹²C/¹³C isotope effect associated with dissolution in water is unknown, but one may assume it is small, as deuteration of water acts on dissolution through (a), the partition function of CO₂ in the liquid phase and (b), the binding energy between solvent water molecules and CO₂. The effect on binding is thought to be a negligible component (Vogel *et al.* 1970). Because the degrees of freedom and the motions of CO₂ are similar, the partition function of CO₂ in the liquid phase is likely not to be much influenced by deuteration of water, so that the change in the ratio of ¹³CO₂ and ¹²CO₂ solubility remains unchanged. This

would be in agreement with the very small effect of atomic substitution changes on solubility in water: for example, the H₂O/D₂O isotope effect on the solubility of CH₄ is 1.005 but that of (the much larger molecule) CF₄ is 1.017 (Cosgrove and Walkley 1981). If an effect of heavy water on the ¹²C/¹³C isotope effect of dissolution were to occur, the overall impact would be very small, because of its order of magnitude (<1%).

Thus, heavy water has roughly no effect on the carbon isotope fractionation by diffusion and dissolution (Table 2), and a change in the photosynthetic discrimination, if any, would have to be related to enzymatic effects.

Appendix II. Calculation of the conductance when inlet water is H₂O

The outlet D₂O vapour mole fraction, denoted as d_a , follows the mass-balance equation for heavy water, that is (as R_{in} is small compared with 1):

$$u_e w_e R_{in} + \Phi s \frac{R_s}{1 + R_s} = d_a (u_e + \Phi s)$$

where u_e is the entering air flow through the chamber, Φ is the water flux taken up by the leaf through the petiole, s is the leaf surface area, and R_s the D/H isotope ratio of source water. R_{in} and w_e are the D/H isotope ratio and the water mole fraction of inlet water, respectively. Because the ratio R_{in} is very small when natural water is used for inlet air, we have:

$$d_a = \frac{R_s/(1 + R_s)}{1 + u_e/\Phi s}, \text{ that is close to } \frac{1}{1 + u_e/\Phi s} \text{ because } R_s/(1 + R_s) = 0.998$$

Although the leaf transpired heavy water, the natural vapour pressure (H₂O of inlet air) had to be taken into account because it may have entered the leaf in the steady-state (see also above). At ambient temperatures, the combination of both natural and heavy waters behave linearly with respect to their relative quantities (Abdulkadirova *et al.* 2002) so that the saturation vapour pressure of the mixture at the evaporating sites is $(1 - \epsilon)d_i + \epsilon w_i$, where ϵ is the proportion of natural water at the evaporative sites, and w_i and d_i are the saturation mole fractions of H₂O and D₂O at leaf temperature [in other words, the actual H₂O vapour mole fraction in the leaf is ϵw_i and that of D₂O is $(1 - \epsilon)d_i$]. Then the water exchange between the atmosphere and the leaf is made up of D₂O loss, that is, $g_{D_2O}((1 - \epsilon)d_i - d_a)$, and H₂O uptake, $g_{H_2O}(w_a - \epsilon w_i)$, where w_a is the outlet mole fraction of H₂O. As the leaf compensated for the small diffusive influx of H₂O by consuming less D₂O through the petiole we have (assuming a mole-to-mole compensation):

$$g_{D_2O}((1 - \epsilon)d_i - d_a) - \alpha_k g_{D_2O}(w_a - \epsilon w_i) = \Phi$$

where the diffusion ratio (H₂O/D₂O isotope effect) is denoted as $g_{H_2O}/g_{D_2O} = \alpha_k$ (~1.040) and the saturation mole fraction pressure w_i/d_i denoted as α^+ . The net heavy water uptake by the leaf is denoted as Φ . Rearranging, this gives:

$$g_{D_2O} = \frac{\Phi}{d_i(1 - \epsilon + \epsilon\alpha^+\alpha_k) - (d_a + \alpha_k w_a)}$$

If ϵ is small enough (see Appendix I), we define the 'efficient' heavy water mole fraction as:

$$d_{eff} = d_a + \alpha_k w_a$$

The D₂O saturation vapour pressure values (at leaf temperature), denoted as d_{sat} , were taken from data compiled for the NIST chemistry webbook (<http://webbook.nist.gov/chemistry>) and from Besley and Bottomley (1973). Stomatal conductance to D₂O is then given by:

$$g_s^{D_2O} = \frac{\Phi(1 - \frac{d_{sat} + d_{eff}}{2})}{d_{sat} - d_{eff}}$$

The CO₂ conductance was then calculated with the ordinary equations (von Caemmerer and Farquhar 1981), with the ratio of diffusivity between CO₂ and D₂O of 1.5 (instead of 1.6 for H₂O) (Marrero and Mason 1973). It is noted that the diffusion coefficients of CO₂ in (heavy) water vapour and in air are not equal (the H₂O-to-D₂O ratio of CO₂ diffusivity is of around ~1.07), in contrast with the usual case (von Caemmerer and Farquhar 1981). However, the effect on the internal CO₂ concentration (c_i) is very small, typically of around 0.2–0.5 $\mu\text{mol mol}^{-1}$, and it was neglected in the present study.