Grass blades as tree rings: environmentally induced changes in the oxygen isotope ratio of cellulose along the length of grass blades

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Summary

• In this study, we tested the hypothesis that environmentally induced changes in the oxygen isotope ratio of leaf water are recorded in grass blade cellulose during leaf-blade expansion.

• Grasses were grown hydroponically in chambers that allowed for control of relative humidity while keeping isotopic inputs (namely source water) constant.

• In experiments where relative humidity was changed from 35% to 93% during grass blade expansion, a 10‰ shift in cellulose $\delta^{18}O$ was observed along single grass blades of *Lolium multiflorum*. However, statistically significant changes were not detectable with relative humidity of 93% to 70%.

• It is shown that grass blades, analogously to tree rings, record environmental change on an interseasonal basis. In light of this, care must be taken to compare leaves of the same developmental stage to avoid confusion of environmental effects with physiological effects in interpretations of leaf organic material $\delta^{18}O$. The results presented here confirm the ability of the current empirical models to predict the oxygen isotope ratio of cellulose in both grass blades that expanded in constant and variable growth conditions.

Key words: oxygen isotopes, cellulose, *Lolium multiflorum*, stable isotopes, tree rings.

Introduction

The evaporative enrichment of ^{18}O in plant leaf water is a function of known fractionation factors, the $\delta^{18}O$ of the environment (source water and atmospheric water vapor), and the evaporative gradient for water loss (Craig & Gordon, 1965; Flanagan *et al.*, 1991). Assuming the isotopic inputs remain constant, the $\delta^{18}O$ of plant leaf water will change predictably with changes in relative humidity and temperature (Farris & Strain, 1978; Yakir *et al.*, 1990; Flanagan *et al.*, 1991; Roden & Ehleringer, 1999a). Hence, any integrative record of leaf water $\delta^{18}O$ is also an integrated record of the growth environment.

Barbour *et al.* (2000) demonstrated that changes in leaf water $\delta^{18}O$ ($\delta^{18}O_{wl}$) are directly recorded in $\delta^{18}O$ values of sucrose translocated from the leaf. Thus, it is expected that cellulose synthesized from sucrose should provide some integrated measure of $\delta^{18}O_{wl}$ during the period of cellulose synthesis. Several recent studies support this hypothesis. Changes in $\delta^{18}O_{wl}$ forced by changes in relative humidity during growth were recorded in the tree-ring cellulose of three tree species in both greenhouse and field conditions (Roden & Ehleringer, 1999a,b). In addition, Barbour & Farquhar (2000) showed that cotton leaf cellulose $\delta^{18}O$ ($\delta^{18}O_{cl}$) recorded relative humidity and abscisic acid induced changes in $\delta^{18}O_{wl}$. Lastly, Helliker & Ehleringer (2002) found that grass blade $\delta^{18}O_{cl}$ changed in concert with $\delta^{18}O_{wl}$ in growth relative humidity regimes ranging from 35% to 96%.

While there is support that $\delta^{18}O_{wl}$ is a primary control of $\delta^{18}O_{cl}$, there is a need to both quantify this effect and to determine how variability in $\delta^{18}O_{wl}$ within a leaf affects the final $\delta^{18}O_{cl}$ signature. Grass blades present a particularly interesting scenario in which leaf water at the tip of the leaf can be

significantly more enriched in ^{18}O than the base of the blade (often exceeding 30‰; Helliker & Ehleringer, 2000, 2002). Furthermore, the leaf development pattern is unique in that the tip of the blade is older than the base. Hence, grass leaves may present both a developmental and isotopic analogy to tree rings.

The first set of cell divisions in the grass apical meristem results in the birth of cells that will form the grass blade tip and the intercalary meristem (IM), such that both the rest of the developing blade and sheath are a result of divisions of these IM daughter cells (Kaufman, 1959; Fahn, 1990). The most mature portion of the grass blade is the tip and continued maturation occurs in a basipetal direction, with the base of the blade next to the ligule being the final section to mature. As leaf cells differentiate and expand, the cellulose precursors used for this expansion are initially imported from other, fully mature leaves, and eventually from photosynthate derived from the mature portions of the expanding grass leaf blade. Anatomical and radio-labeled $^{14}CO_2$-label studies provide support for this general pattern (Crafts & Yamaguchi, 1958; Forde, 1965; Fahn, 1990).

Based on the developmental pattern above, the $\delta^{18}O$ signature of cellulose in recently expanded cells should be reflective of the isotopic signature of photosynthates from mature leaves on the same plant, or the mature portion of the expanding leaf blade. The primary hypothesis of this study is that any sequential change in the $\delta^{18}O$ of cellulose precursors in an expanding grass blade will be recorded sequentially in that same blade. For example, if a step change in relative humidity was imposed on a grass with several mature leaf blades and one blade which was partially expanded, then sucrose translocated from those mature leaves should carry a $\delta^{18}O$ signature representative of the relative humidity change. Consequently, the $\delta^{18}O$ of cellulose in the expanding leaf would record that change, such that the cellulose $\delta^{18}O$ of the tip of the expanding blade would represent the initial relative humidity, and the cellulose $\delta^{18}O$ of the base would represent the altered relative humidity.

Helliker & Ehleringer (2002) showed that the bulk leaf cellulose ($\delta^{18}O_{cl}$) of grass blades was explained by Barbour *et al.* (2000) model,

$$\delta^{18}O_{cl} = \delta^{18}O_{wx} + (\delta^{18}O_{wl} - \delta^{18}O_{wx})(1 - p_{ex} \cdot p_x) + \varepsilon_O \quad \text{Eqn 1}$$

where $\delta^{18}O_{wx}$, and $\delta^{18}O_{wl}$ represent the oxygen isotope ratio of source and leaf-water, ε_O is the oxygen fractionation factor from water to organic material (27‰; Yakir & DeNiro, 1990; Sternberg, 1989), p_{ex} is the proportion of exchangeable oxygen atoms during cellulose synthesis and p_x is the proportion of xylem and/or phloem water within the cells where cellulose is being synthesized. In a best-fit analysis, $p_{ex} \cdot p_x$ was found to be 0.25 for 10 grass species (Helliker & Ehleringer, 2002). However, individual values for p_{ex} and p_x could not be determined.

To assess the applicability of eqn 1 to a variety of plants, it is imperative to determine whether $p_{ex} \cdot p_x$, or either individual variable, is constant across species or even functional types. Furthermore, the question of whether the pattern of cellulose synthesis is similar both between organs of the same plant, and across plant species, can be more easily answered by separating $p_{ex} \cdot p_x$. The growth pattern of grasses, as with tree rings where $p_x = 1$, allows for the partitioning of $p_{ex} \cdot p_x$ into individual components. If all grass blade cell expansion occurs near the IM, then the value of p_{ex} can be determined by sampling for water at the IM and by using the following proportional mixing model used to predict the $\delta^{18}O$ of tree-ring cellulose (Roden *et al.*, 2000),

$$\delta^{18}O_{cl} = (p_{ex} \cdot \delta^{18}O_{wm}) + ((1 - p_{ex}) \cdot \delta^{18}O_{wl}) + \varepsilon_O \quad \text{Eqn 2}$$

where $\delta^{18}O_{wm}$ represents the oxygen isotope ratio of IM water; ε_O and p_{ex} are as defined above ($p_{ex} = f_O$ in the notation of Roden *et al.*, 2000).

There were four primary objectives in this study: First to test the primary hypothesis that changes in growth relative humidity during leaf expansion would be seen as changes in cellulose $\delta^{18}O$ along the leaf blade; second to determine if all leaf expansion occurs at the base of a grass blade in the IM region; third to assess the ability of the Barbour and Farquhar model (Eqn 4.1) to predict change in $\delta^{18}O_{cl}$ along the grass leaf blade, using both observed and predicted values of $\delta^{18}O_{wl}$ and $\delta^{18}O_{wm}$; and fourth to use eqn 2 with observed values of $\delta^{18}O_{wl}$, $\delta^{18}O_{wm}$, and $\delta^{18}O_{cl}$ to determine p_{ex} in a range of growth conditions in the C_3 grass *Lolium multiflorum.*

Materials and Methods

Plant material and growth conditions

The C_3 grass *Lolium multiflorum* was germinated from seed in 1-l pots within open-top plastic chambers under hydroponic conditions and an isotopically constant source water, as described by Helliker & Ehleringer (2000) and Helliker & Ehleringer (2002). To obtain relative humidity environments from 50 to 93%, Plexiglas tops were sealed to the upper lips of the chambers. Relative humidity differences between these treatments were obtained by varying the tightness of the seal between Plexiglas and the upper lip of the chambers and/or the removal of clear plastic sampling ports that were inserted into the Plexiglas tops. The chamber tops were left open in the lowest relative humidity treatment; thus the chamber experienced Salt Lake City (UT, USA) ambient relative humidity. Thermocouples (wet and dry bulb) were placed at plant height to measure air temperature and relative humidity throughout the experiments. Photon flux density at plant height was approximately 1000 μmol m^{-2} s^{-1} (Licor light sensor, Lincoln, NE, USA) throughout a 16-h photoperiod.

Thermocouples and light sensor were averaged at 15-min intervals with a datalogger (Campbell Scientific, model 21X, Logan, UT, USA).

Experimental plan

The growth conditions are referred to in the following manner: grasses that were grown in one environment will be referred to as CC (constant condition) plants, grasses that were switched to different environments during growth will be referred to as VC (variable condition) plants. For both the VC and CC plants, at least two fully mature leaves were allowed to expand. Plants in which only the first photosynthetic leaf was allowed to expand will be referred to as seedlings and are discussed below. In all of the experiments, approximately 100 seeds of the annual ryegrass *Lolium multiflorum* were germinated in each pot, and all the plants within each pot were considered as one replicate. Five replicates of CC plants were grown for 4 wk in an environment where mean daytime relative humidity (RH) was 53 ± 6% and daytime temperature was 27.7 ± 2.5°C; vapor pressure deficit (VPD) was 1.75 kpa. Source water $\delta^{18}O$ was –15.7‰, atmospheric water vapor $\delta^{18}O$ was –23‰.

The VC experiment was conducted as follows: 10 replicates were germinated in daytime conditions of 76 ± 4% RH and 28.8 ± 2.4°C; VPD was 0.95 kpa. The first photosynthetic leaf was allowed to expand in these conditions and then the plants were moved to a chamber with conditions of 35 ± 6% RH and 28.2 ± 3.4°C (VPD = 2.49 kpa) for 5 days to allow the second leaf to partially expand. The 10 replicates were then moved to day time conditions of 93 ± 2% RH and 32.2 ± 2.7°C (VPD = 0.34 kpa) for 5 d in which time the second photosynthetic leaf completed expansion and the third photosynthetic leaf partially expanded. The 10 replicates were then placed in chambers with daytime conditions of 70 ± 4% RH and 28.4 ± 2.0°C (VPD = 1.16 kpa), for 5 d where the third leaf fully expanded. In all of these conditions, five additional replicates of plants were germinated and the first photosynthetic leaf was allowed to expand for the duration that the 10 replicates were in a given growth chamber. These plants will be referred to as seedlings. Source water $\delta^{18}O$ was –16.3‰, atmospheric water vapor $\delta^{18}O$ was –22‰, –24‰, –20‰, –23‰ for the 76%, 35%, 93% and 70% RH growth conditions, respectively.

Leaf water models

The $\delta^{18}O$ of leaf water was predicted by the Craig-Gordon model (Craig & Gordon, 1965; Flanagan *et al.*, 1991):

$$R_l = \alpha^*\left[\alpha_k R_s\left(\frac{e_i - e_s}{e_i}\right) + \alpha_{kb} R_s\left(\frac{e_s - e_a}{e_i}\right) + R_a\left(\frac{e_a}{e_i}\right)\right] \quad \text{Eqn 3}$$

where R_l, R_s and R_a represent the molar isotope ratios of $^{18}O : {}^{16}O$ of leaf water, plant source water and atmospheric water vapor, respectively. α^* is the temperature dependent equilibrium fractionation factor as described by Majoube (1971). α_k and α_{kb} are the kinetic fractionation factors for molecular diffusion out of the stomata and turbulent diffusion in leaf boundary layer, respectively. The water vapor pressure of the leaf, boundary layer and atmosphere is represented by e_i, e_b and e_a, respectively. This model has been shown to be robust in a variety of dicot species (Flanagan *et al.*, 1991a,b; Roden & Ehleringer, 1999a) and to work well for a variety of C_3 grass species (Helliker & Ehleringer, 2002).

The pattern of leaf water enrichment in grasses was shown to be analogous to the string-of-lakes model developed by Gat & Bowser (1991; in Helliker & Ehleringer, 2000). In this study, meristem water $\delta^{18}O$ observations were compared with predictions of the Gat-Bowser model.

$$\delta_n = \delta_{n-1} + \frac{\left(\delta_a + \frac{\varepsilon}{h}\right) - \delta_{n-1}}{1 + \frac{F + (1-h)}{Eh}} \quad \text{Eqn 4}$$

where the subscript n represents the nth element in a series and δ_1 was considered to be equal to $\delta^{18}O_{wm}$. δ_a and δ_{n-1} are the $\delta^{18}O$ of atmospheric water vapor and water entering a leaf (for the first element, δ_1), respectively. F_+ is the flux into a segment and E represents the evaporative flux out of a segment. Relative humidity is represented by h, and $\varepsilon = \varepsilon_{eq} + (1 - h)\varepsilon_k$, where ε_{eq} and ε_k represent the equilibrium and kinetic fractionation factors. ε_{eq} and ε_k are related to α^* and α_k above by $\varepsilon = (1 - \alpha) \cdot 1000$. In this study steady state conditions, well-mixed pools of water and constant E in all elements were assumed. Under these assumptions, the mean $\delta^{18}O$ value of water from all segments has the same value as predicted by the single-water-source Craig-Gordon model. The absolute number of elements ($|n|$) in the series was varied from 3 to 10 to obtain predictions for $\delta^{18}O_{wm}$ at all growth conditions. These predictions were plotted against observed values of $\delta^{18}O_{wm}$ to obtain the value of n that most accurately predicted observed values (Fig. 1).

Sample collection and isotope analysis

All isotope values were presented in the standard delta notation where:

$$\delta^{18}O = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \cdot 1000 \quad \text{Eqn 5}$$

R is the molar ratio of $^{18}O : {}^{16}O$ of the sample or of the internationally accepted standard SMOW (Standard Mean Ocean Water).

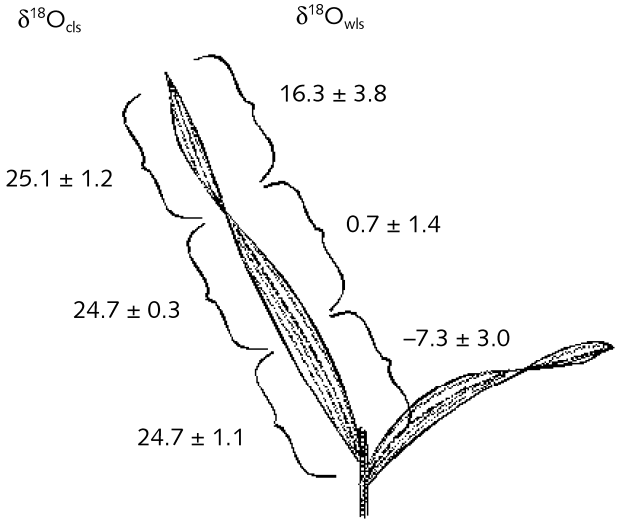

Fig. 1 The oxygen isotope ratio of leaf sections for cellulose ($\delta^{18}O_{cls}$) and leaf water ($\delta^{18}O_{wls}$) for the CC experiment. Samples for $\delta^{18}O_{cls}$ were taken after the full expansion of the second photosynthetic leaf. $\delta^{18}O_{wls}$ values are averages of samples taken once a week for 3 wk. Plus and minus values are standard deviations, $n = 3$ for all values.

After excision, leaf samples were immediately sealed in glass vials and frozen, for eventual extraction by cryogenic distillation (Ehleringer & Osmond, 1989). Bulk leaf water samples and meristem water samples were taken once ($n = 3$) at each growth condition from replicates not used for cellulose analysis. This was done to avoid the effect that any overlap in leaf cutting and regrowth would have on cellulose ^{18}O patterns. Meristem water samples ($n = 3$) were taken by sampling the most basal portion of *c.* 20 leaf blades per replicate. The samples were *c.* 5 mm in length. Leaf water samples of leaf segments were taken in the CC experiments. Each segment represented a third of the leaf blade lengths of *c.* 20 leaves per replicate ($n = 3$). Leaf cellulose samples were taken after the second leaf had fully matured in the CC experiments, after the third leaf had matured in the VC experiments, and as specified above for the first photosynthetic leaf in the seedlings.

All water samples were analyzed by the $CO_2 : H_2O$ equilibration technique of Socki *et al.* (1992) and the isotope ratio determined by on-column injection into a gas chromatograph (GC) – isotope ratio mass spectrometer (IRMS) system in continuous flow mode (GC- Varian, Model 3300, Walnut Creek, CA; MS-Model 252, Finnigan MAT). The equilibration technique and GC-IRMS set up are described in full in Helliker & Ehleringer (2000). Leaf materials for cellulose extraction were placed in coin envelopes and dried at 70°C. α-cellulose was extracted from whole leaf material using the methods of Leavitt & Danzer (1992) as modified by Roden & Ehleringer (1999a). The $\delta^{18}O$ and $\delta^{13}C$ of leaf α-cellulose was determined on a Delta S isotope ratio mass spectrometer (Finnigan MAT) by the pyrolysis method (for $\delta^{18}O$ values) described by Saurer *et al.* (1998) and Roden & Ehleringer (1999a).

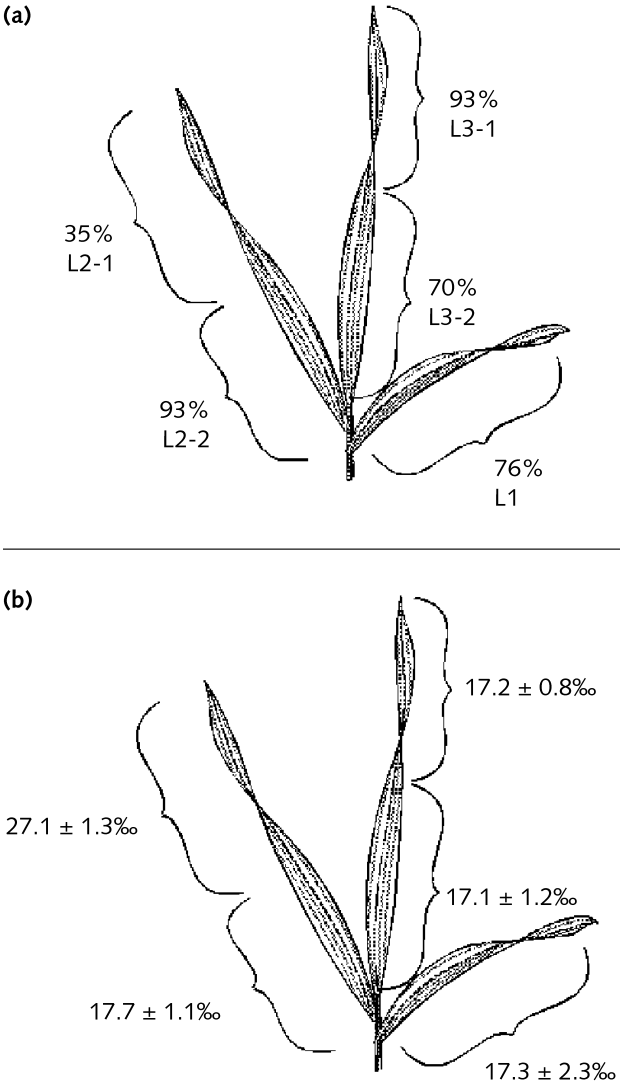

Fig. 2 Relative humidity and $\delta^{18}O_{cls}$ for the VC experiment. Panel a represents the mean day time relative humidity in which a particular grass blade section expanded. Panel b represents the observed $\delta^{18}O_{cls}$. Plus and minus values are standard deviations, $n = 3$ for all values.

Results

The results from the CC (constant growth conditions) experiment revealed a large base-to-tip gradient in leaf water $\delta^{18}O_{wl}$ that exceeded 20‰ (Fig. 2). The $\delta^{18}O_{wl}$ values represent means of samples taken once a week throughout the experiment. Samples of bulk leaf water $\delta^{18}O$ ($n = 3$ for each week of growth) did not vary significantly throughout the growth period (values of $\delta^{18}O_{wl}$ are in Table 1). The $\delta^{18}O$ values of cellulose for a particular grass blade section ($\delta^{18}O_{cls}$) did not change significantly along the grass blade. From base to tip these values were, 24.7, 24.7, and 25.1, respectively.

The growth conditions implemented in the variable conditions (VC) experiment during the period of grass blade

Table 1 Observed $\delta^{18}O$ of bulk leaf water ($\delta^{18}O_{wl}$) and meristem water. ($\delta^{18}O_{wm}$) in the VC and CC experiments. Plus and minus values represent standard deviations, $n = 3$ for each value

Daytime RH (%); experiment	Observed $\delta^{18}O_{wl}$ (‰)	Observed $\delta^{18}O_{wm}$ (‰)
76; VC	–7.0 ± 1.2	–10.7 ± 1.1
35; VC	5.6 ± 1.6	–9.7 ± 1.1
93; VC	–10.5 ± 0.2	–13.2 ± 0.2
70; VC	–8.1 ± 0.5	–13.0 ± 0.1
53; CC	0.5 ± 1.8	–10.3 ± 1.8

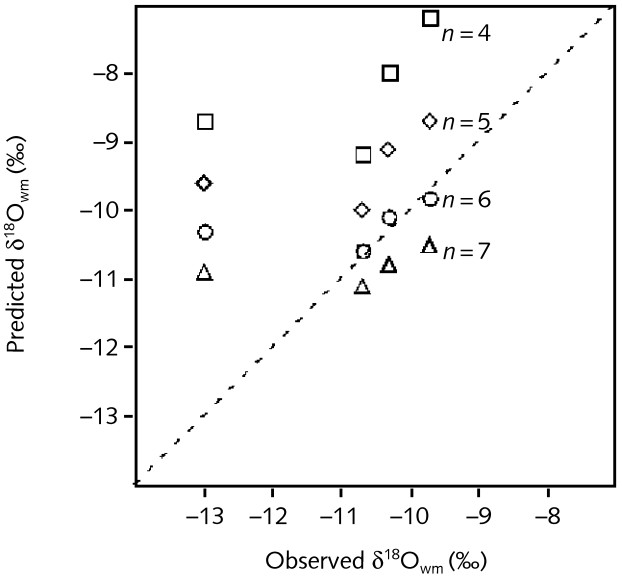

Fig. 3 Predicted vs observed values of $\delta^{18}O_{wm}$. Predicted values are from the Gat-Bowser model (Eqn 4). The first element in the string of evaporatively enriched pools (δ_1) was taken to be equal to $\delta^{18}O_{wm}$. The different values of n represent the varying numbers of elements in the string of pools. The grey dashed line represents the 1 : 1 line.

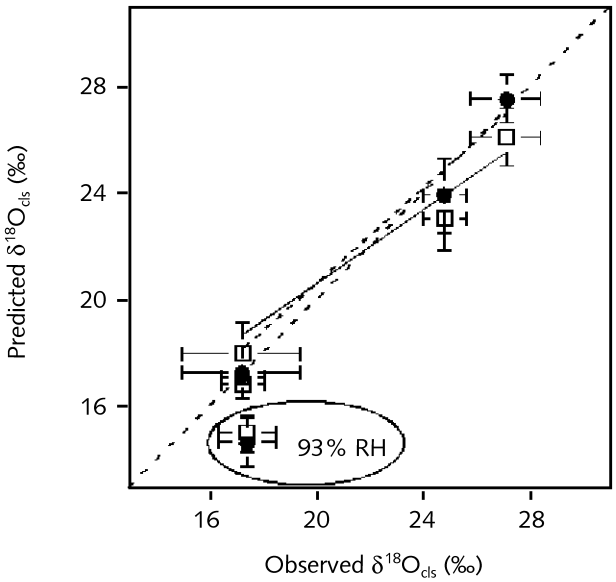

Fig. 4 Predicted vs observed values of $\delta^{18}O_{cls}$. Predicted values are from the Barbour and Farquhar model (2000; Eqn 1; filled circle) and the tree-ring model (Eqn 2; open square), using observed values of $\delta^{18}O_{wl}$ for Eqn 1 and observed values of $\delta^{18}O_{wm}$ and $\delta^{18}O_{wl}$ for Eqn 2. $p_{ex} \cdot p_x$ was found to be 0.22 and p_{ex} was found to be 0.35, both values were obtained by best-fit. The black solid line represents predictions made from using Craig-Gordon predictions for $\delta^{18}O_{wl}$ and Gat-Bowser predictions for $\delta^{18}O_{wm}$ and the tree-ring model for $\delta^{18}O_{cls}$. The black dashed line represents predictions of $\delta^{18}O_{cls}$ made with the Barbour and Farquhar model using the Craig-Gordon model to predict $\delta^{18}O_{wl}$ and $p_{ex} \cdot p_x = 0.22$. The dashed grey line equals a 1 : 1 correlation. Error bars represent standard deviation, $n = 3$ for each point.

expansion are detailed in Fig. 3(a). The $\delta^{18}O_{cls}$ values showed nearly a 10‰ shift along the grass blade of L2, from L2-1 to L2-2 (from Fig. 3b). Nominally, this shift in cellulose $\delta^{18}O$ is in accordance with the large change in relative humidity implemented during the development of L2 from 35% to 93%. Alternatively, there was little change within a grass blade when allowed to expand in environments of 70% to 93% relative humidity (L3-1 to L3-2).

The number of string elements in the Gat-Bowser model (Eqn 4) was varied between 3 and 10 to assess which element number most accurately predicted the observed values of $\delta^{18}O_{wm}$ (Fig. 1). For our predictions of $\delta^{18}O_{cls}$ (Fig. 4, see below) $n = 6$ was used to predict $\delta^{18}O_{wm}$ due to the close numerical association to observed values.

The predictions of $\delta^{18}O_{cls}$ (from both the VC and CC experiments) from eqns 1 and 2 are presented in Fig. 4. Observed values of $\delta^{18}O_{wl}$ were used as input for eqn 1 and $p_{ex} \cdot p_x$ was determined by best-fit ($y = 0.998x - 0.11$) to be 0.22. The mean data points represent predictions obtained using observed values of $\delta^{18}O_{wl}$ and $\delta^{18}O_{wm}$ (listed in Table 1) as inputs for eqn 2. The solid line represents the predictions of $\delta^{18}O_{cls}$ from eqn 2 using predicted values of $\delta^{18}O_{wl}$ and $\delta^{18}O_{wm}$ (eqns 3 and 4), and the dashed line represents predictions from eqn 1 using predicted $\delta^{18}O_{wl}$ and the best-fit value of 0.22 for $p_{ex} \cdot p_x$ from above. The values for 93% RH conditions in the VC experiment were not included in the analysis of eqns 1 or 2 (see Discussion).

In all growth conditions of the VC experiment, the seedlings (grasses with only the first photosynthetic leaf expanded) $\delta^{18}O_{cl}$ were more enriched than the corresponding L2 or L3 $\delta^{18}O_{cls}$ that expanded in the same environment (Fig. 5). As growth relative humidity increased, so did the difference between the observed $\delta^{18}O_{cls}$ of second and third photosynthetic leaves and the $\delta^{18}O_{cl}$ of the first photosynthetic leaf. This relationship was significant with $r = 0.66$ and $P < 0.05$. The $\delta^{18}O$ of whole seeds was 25.8‰ ($n = 3$, data not shown).

The carbon isotope ratio of leaf sections ($\delta^{13}C_{cls}$) was measured for the base and tip sections in L2 of both the CC and

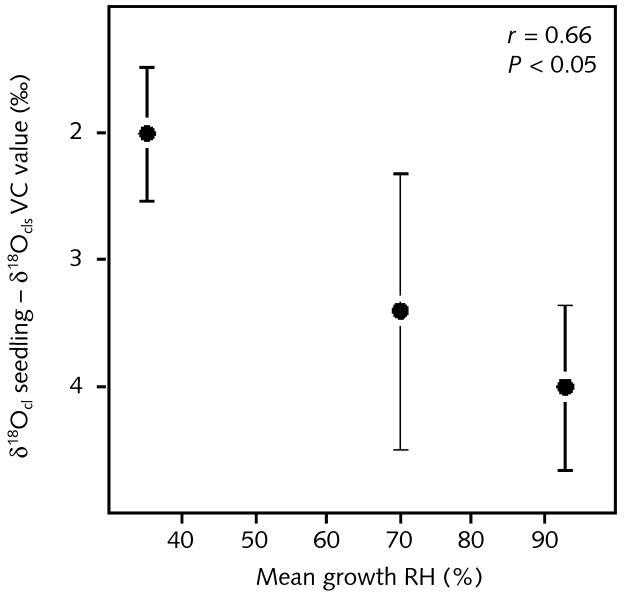

Fig. 5 The $\delta^{18}O_{cl}$ of seedling plants (the first photosynthetic leaf) minus the $\delta^{18}O_{cls}$ of leaf blades L2 and L3 from the VC experiment at a given relative humidity. This relationship was significant with $r = 0.66$ and $P < 0.05$. The $\delta^{18}O$ of whole seeds was 25.8‰ ($n = 3$).

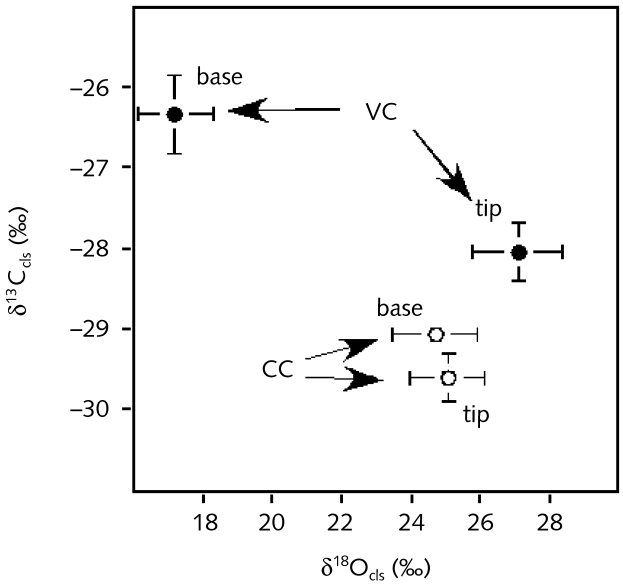

Fig. 6 The cellulose $\delta^{13}C$ and $\delta^{18}O$ of the base and tip leaf sections of L2 leaf blades from the VC and CC experiments. Both $\delta^{13}C_{cls}$ and $\delta^{18}O_{cls}$ are significantly different in the VC experiment ($P < 0.01$ and $P < 0.001$, respectively). $\delta^{13}C_{cls}$ values are significantly different in the CC experiment ($P < 0.05$). $n = 3$ for all samples, error bars are representative of standard deviations. Statistical significance was determined by an unpaired Student's *t*-test.

VC experiments and plotted against the corresponding $\delta^{18}O_{cls}$ values (Fig. 6). $\delta^{13}C_{cls}$ values were significantly different between the base and tip for both the CC and VC experiments ($P < 0.05$ and $P < 0.01$, respectively). There was also a trend in both experiments for $\delta^{13}C_{cls}$ to decrease as $\delta^{18}O_{cls}$ increased, although the trend was only significant in the VC experiment ($r^2 = 0.86$, $P < 0.01$).

Discussion

In a $^{14}CO_2$ labeling study, ^{14}C that was fixed by an expanding grass blade was not translocated to the mature portions of the grass (Crafts & Yamaguchi, 1958). Furthermore, in that study when mature leaves were supplied $^{14}CO_2$, the radiolabeled carbon was found in recently expanded material of the growing leaf blade. These studies show that recently assimilated carbon was used for new growth at the IM region of an expanding grass blade; and provide support for the primary hypothesis that any change in the stable oxygen isotope signature of the photosynthate translocated to the expanding grass blade will be retained in the cellulose $\delta^{18}O$ signature of that portion of the blade.

A corollary to this primary hypothesis, it would be expected that no variation in $\delta^{18}O_{cl}$ would be observed along the length of a grass blade grown in a constant environment, because there would be no change in leaf water $\delta^{18}O$ during growth and consequently photosynthate $\delta^{18}O$ used for cellulose synthesis. Furthermore, grass blades show large progressive ^{18}O enrichment of leaf water from base-to-tip, which, if the primary hypothesis was supported, should not be manifested in $\delta^{18}O_{cl}$, because all new growth occurs at one location near the IM. These assumptions were tested in the CC experiment where the second photosynthetic leaf was allowed to expand in a relatively constant environment. While base-to-tip differences in leaf water $\delta^{18}O$ were in excess of 20‰, the $\delta^{18}O$ of leaf cellulose was not significantly different along the blade (Fig. 2). In a constant environment, and with large gradients of leaf water along the leaf blade, there was no significant change in $\delta^{18}O_{cls}$ from base to tip. It can then be assumed that if any change in $\delta^{18}O_{cl}$ along a grass blade is observed, the change must be a result of variability in environmental conditions during leaf expansion.

The VC experiment was developed to test the primary hypothesis that changes in environment would force changes in leaf water, and the consequent change in the $\delta^{18}O$ of photosynthate would be recorded in expanding grass blades. There was a large change in cellulose $\delta^{18}O$ of nearly 10‰ in L2 when the first portion of the leaf expanded in 35% relative humidity and the second portion of the leaf expanded in 93% relative humidity (Fig. 3). Hence, the hypothesis was supported by this data set. Alternatively, there was no change in L3 where growth environments were 93% and 70% relative humidity for the tip and basal portions of the leaf, respectively.

Both the Barbour and Farquhar model (Eqn 1) and the tree-ring model (Eqn 2) worked well in predicting the cellulose $\delta^{18}O$ of grass leaf blade sections (Fig. 4). In this study, estimates of p_{ex} were constrained by observed values of $\delta^{18}O_{wl}$

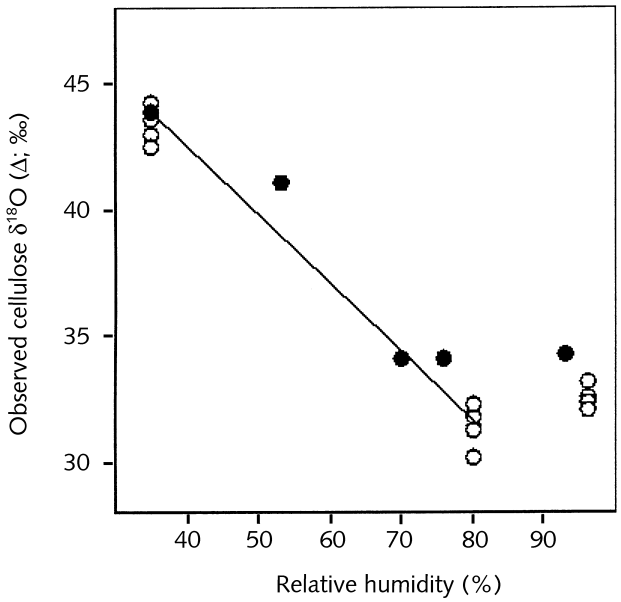

Fig. 7 Cellulose Δ_{18O} vs relative humidity. Open circles represent Δ_{18O} whole leaf blade values from five C_3 grass species in Helliker & Ehleringer (2002). Closed circles represent Δ_{18O} leaf section values from this study. $\Delta = \delta^{18}O_{cl} - \delta^{18}O_{wx}$, where $\delta^{18}O_{wx}$ represents the molar isotope ratios of the source water of a respective experimental system. $n = 3$ for each data point. The least squares equation for RH ranging from 35% to 81% = $-0.27x + 53.1$, $r^2 = 0.97$.

and $\delta^{18}O_{wm}$, the two possible sources of oxygen isotope signatures during cellulose synthesis, as inputs for the tree-ring model. The similar performance of the tree-ring model and the Barbour and Farquhar model, and the different inputs for the two models, allow for partitioning of $p_{ex} \cdot p_x$ (0.22 in this study) and assignment of a numerical value to both p_{ex} and p_x. The results allow the assumption that p_{ex} was constant in conditions ranging from 35% to 76% RH, so that we can simply solve for p_x, and arrive at $p_x = 0.62$, which was within the p_x range of 0.50–0.62 estimated in Helliker & Ehleringer (2002) in 10 species of grass.

To determine the best-fit value of $p_{ex} \cdot p_x$, the results for the leaf sections expanded at 93% RH were left out of the analysis. The basic pattern of cellulose $\delta^{18}O$ enrichment changed above 90% RH in this study, and in all grass species examined in a previous study (Helliker & Ehleringer, 2002). Including $\delta^{18}O$ data from this previous study, there was a negative correlation of $\delta^{18}O$ and growth RH up to 80% RH, after which the relationship deteriorated (Fig. 7; $\delta^{18}O$ values presented as Δ to normalize). In fact, for all data points in Fig. 4 above 90% RH, leaf-water $\delta^{18}O + \varepsilon_O$ was as good a predictor of cellulose $\delta^{18}O$ as either eqns 1 or 2 (data not shown). It is possible that above 90% RH that p_{ex} changed significantly (Barbour & Farquhar, 2000). However, it seems unlikely that p_{ex} suddenly changed above 90% RH, rather it is more probable that the characteristics of evaporative enrichment of leaf and meristem water were different under high relative humidity such that the $\delta^{18}O$ of water at the site of cellulose synthesis was not much different from bulk leaf water. When data from Helliker & Ehleringer (2002) were reanalyzed without the 96% RH data, a $p_{ex} \cdot p_x$ value of 0.21 was obtained, which was very similar to the value of 0.22 in this study.

Obtaining an accurate value for $\delta^{18}O_{wm}$ is crucial to obtain an independent estimation of p_{ex}. We have shown here that it is possible to obtain direct observations of $\delta^{18}O_{wm}$, however, the number of leaves required for such sampling was large (5 mm sections of about 20 leaves per replicates for this study) and as such, is not always feasible. Hence, one of the goals of this study was to compare Gat-Bowser estimates of $\delta^{18}O_{wm}$ with observed $\delta^{18}O_{wm}$ so that future studies will not have to rely on destructive sampling measures. There was a fairly close fit between predicted and observed $\delta^{18}O_{wm}$ using an $n = 6$ and this value was used to predict $\delta^{18}O_{wm}$ for the input to eqn 2. Importantly, by varying $n = 4 - 6$ there was only a slight modification of predicted values for $\delta^{18}O_{cls}$ from eqn 2 (data not shown) so that using a value of n within this range should be a close, nondestructive approximation of $\delta^{18}O_{wm}$.

In all of the VC growth conditions, five replicates of plants were germinated and the first photosynthetic leaf was allowed to expand for the duration that the VC replicates were in a given growth chamber. This was done to qualitatively determine if the $\delta^{18}O_{cl}$ of seedling leaves, the first photosynthetic leaf blade, was primarily affected by cellulose precursors imported from the seed or by the growth environment. In all growth conditions of the VC experiment, the seedling $\delta^{18}O_{cl}$ was more enriched than the corresponding L2 or L3 $\delta^{18}O_{cls}$ that expanded in the same environment (Fig. 5). As growth relative humidity increased, so did the difference between the observed $\delta^{18}O_{cls}$ of second and third photosynthetic leaves and the $\delta^{18}O_{cl}$ of the first photosynthetic leaf ($r = 0.66$, $P < 0.05$). Interestingly, it appeared that seedling $\delta^{18}O$ (25.8‰) was a larger proportion of the seedling leaf as relative humidity increased. It was possible that more seed store carbon was used for root construction instead of leaf construction as relative humidity decreased, but this is only conjecture at this time.

If the primary hypothesis holds for $\delta^{18}O$ of photosynthate translocated to the IM, then it should also hold for $\delta^{13}C$ of translocated material. The $\delta^{13}C$ of leaf sections ($\delta^{13}C_{cls}$) was sampled to assess the covariation of $\delta^{13}C$ and $\delta^{18}O$ as growth environment changed with leaf blade expansion (Fig. 6). While $\delta^{18}O_{cls}$ values did not differ significantly in the CC experiment, $\delta^{13}C_{cls}$ values were significantly different between the base and tip for both the CC and VC experiments, although the absolute difference between base and tip $\delta^{13}C_{cls}$ was much greater in the VC experiment (nearly 2‰). There was a general trend of $\delta^{13}C_{cls}$ decreasing as $\delta^{18}O_{cls}$ increased in both experiments, although the trend was significant in the VC experiment only. This trend is indicative of the intercellular concentration of CO_2 decreasing as humidity increased and there are two possible mechanisms to support such a

trend: first that stomatal conductance decreased as relative humidity increased, or second that photosynthetic capacity decreased as relative humidity increased (Farquhar *et al.*, 1989; Scheidegger *et al.*, 2000). It was not possible to quantitatively determine which scenario was most likely to explain the $\delta^{13}C$ vs $\delta^{18}O$ results presented here, as gas exchange measurements were not made during this experiment.

The $\delta^{18}O$ signature in plant organic matter has many potential applications, which include assessing integrated stomatal conductance differences between plants grown in the same environment, augmentation of $\delta^{13}C$ values, reconstructing both temperature and relative humidity in contemporary and ancient plant specimens and geo-location of illicit, plant-derived substances (Epstein *et al.*, 1977; Farquhar *et al.*, 1998; Switsur & Waterhouse, 1998; Roden & Ehleringer, 1999a; Barbour *et al.*, 2000; Roden *et al.*, 2000). Cellulose $\delta^{18}O$ signatures in tree rings offer insight to environmental change on an interannual basis, analogously, grass blades may offer the same on an interseasonal basis. Additionally, this work shows that care must be taken to compare leaves of the same developmental stage to avoid confusion of environmental effects with physiological effects in interpretations of leaf organic material $\delta^{18}O$. The results presented here confirm the ability of the Barbour and Farquhar model and the tree-ring model to predict cellulose $\delta^{18}O$ signatures in grasses, and therefore contribute to recent studies that are bringing the potential uses of plant organic $\delta^{18}O$ into fruition.

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