Oxygen isotope fractionations across individual leaf carbohydrates in grass and tree species

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Summary statement

Almost no $\delta^{18}O$ data are available for individual leaf carbohydrates, limiting our understanding of oxygen isotope fractionation processes within plants. Here we show that the leaf water signal can be traced via individual carbohydrates into leaf cellulose, with sucrose generally being ^{18}O-enriched compared to hexoses and cellulose across grass and tree species. Interestingly, damping factor $p_{ex}p_x$ responded to rH conditions if modeled from $\delta^{18}O$ of bulk leaf water, but not if modeled directly from $\delta^{18}O$ of individual carbohydrates. Thus, our results are useful for better understanding (post-)photosynthetic isotope fractionation processes and for improving models of oxygen isotope fractionation.

Abstract

Almost no $\delta^{18}O$ data are available for leaf carbohydrates, leaving a gap in the understanding of the $\delta^{18}O$ relationship between leaf water and cellulose. We measured $\delta^{18}O$ values of bulk leaf water ($\delta^{18}O_{LW}$) and individual leaf carbohydrates (e.g. fructose, glucose, sucrose) in grass and tree species, and $\delta^{18}O$ of leaf cellulose in grasses. The grasses were grown under two relative humidity (rH) conditions. Sucrose was generally ^{18}O-enriched compared to hexoses across all species with an apparent biosynthetic fractionation factor (ε_{bio}) of more than 27‰ relative to $\delta^{18}O_{LW}$, which might be explained by isotopic leaf water and sucrose synthesis gradients. $\delta^{18}O_{LW}$ and $\delta^{18}O$ values of carbohydrates and cellulose in grasses were strongly related, indicating that the leaf water signal in carbohydrates was transferred to cellulose (ε_{bio} = 25.1‰). Interestingly, damping factor $p_{ex}p_x$, which reflects oxygen isotope exchange with less enriched water during cellulose synthesis, responded to rH conditions if modeled from $\delta^{18}O_{LW}$, but not if modeled directly from $\delta^{18}O$ of individual carbohydrates. We conclude that due to isotopic leaf water gradients, $\delta^{18}O_{LW}$ is not always a good substitute for $\delta^{18}O$ of synthesis water. Thus, compound-specific $\delta^{18}O$ analyses of individual carbohydrates are helpful to better constrain (post-)photosynthetic isotope fractionation processes in plants.

Keywords: invertase, isomerization, non-structural carbohydrates (NSC), phosphoglucose isomerase, sucrose synthase, sugars, trioses

Introduction

Carbohydrates, as the most abundant biological molecules, are involved in various biochemical processes and are thus an essential component for all living organisms. In autotrophic plant tissues, photosynthetically assimilated CO_2 is used for the production of small carbohydrates such as trioses, which are the main precursors of sucrose and starch (Buchanan *et al.*, 2015). Sucrose is translocated between plant tissues and leaf sections, functioning as precursor for other carbohydrates (e.g. fructose and glucose) in target cells, as well as for much more complex carbohydrate polymers such as starch or cellulose (Buchanan *et al.*, 2015).

Previous studies have shown that isotope fractionation processes in plants during the synthesis of cellulose from individual carbohydrate molecules can be well studied with stable carbon isotope measurements (Rinne *et al.*, 2015a, Rinne *et al.*, 2015b). However, little is known on the oxygen isotope ratio ($\delta^{18}O$) of individual carbohydrates and their relationships to $\delta^{18}O$ values of water (Schmidt *et al.*, 2001, Werner, 2003) and cellulose (Barbour, 2007, Sternberg, 2009).

The oxygen isotopic composition of plant bulk material and carbohydrates is mainly driven by $\delta^{18}O$ of the plants' source water, which is taken up via roots and transported to leaves via the xylem stream without measurable isotope fractionation (Dawson & Ehleringer, 1991). However, water in the leaves becomes ^{18}O-enriched due to preferential loss of lighter water isotopologues (Cernusak *et al.*, 2016). The leaf water enrichment is mainly driven by changes in relative humidity (rH), air temperature, and by the isotopic composition of the water vapour (Kahmen *et al.*, 2011), but also by plant physiological parameters such as leaf temperature, stomatal conductance, and transpiration (Farquhar *et al.*, 2007, Saurer *et al.*, 2016). $\delta^{18}O$ values of bulk leaf water ($\delta^{18}O_{LW}$) can be modulated by the Péclet-effect

(Farquhar & Lloyd, 1993, Barbour *et al.*, 2000a), where the advection of xylem water towards evaporative sites causes mixing of less ^{18}O-enriched xylem water with back-diffused ^{18}O-enriched evaporative site water. The degree of the Péclet-effect is dependent on interveinal distances, pathway tortuosity, water flow velocity, and on the transpiration rate (Cernusak & Kahmen, 2013). As a result, this can cause strong isotopic leaf water gradients in broadleaf (Wang & Yakir, 1995, Gan *et al.*, 2002, Cernusak *et al.*, 2016) and grass species (Helliker & Ehleringer, 2000, Helliker & Ehleringer, 2002, Gan *et al.*, 2003). Models of various complexities have been developed that are generally well able to characterize leaf water enrichment and its spatial distribution in the leaf (Farquhar & Gan, 2003, Cuntz *et al.*, 2007).

Since atmospheric CO_2 entering the stomatal cavity rapidly equilibrates with leaf water (Uchikawa & Zeebe, 2012), it is expected that the isotopic signal of leaf water is imprinted on $\delta^{18}O$ of organic compounds ($\delta^{18}O_{Organics}$) during photosynthetic CO_2 assimilation. However, a biosynthetic fractionation factor (ε_{bio}) of ~27‰ causes the organic compounds to be ^{18}O-enriched compared to leaf water (Sternberg & DeNiro, 1983). ε_{bio} is assumed to represent an oxygen isotope exchange between leaf water and carbonyl groups in simple individual carbohydrates shortly after assimilation (Sternberg & DeNiro, 1983). It was observed to be especially rapid for trioses (Reynolds *et al.*, 1971, Sternberg & DeNiro, 1983), the first photosynthetic product, but to be slower for hexoses (Model *et al.*, 1968, Mega *et al.*, 1990). Previous studies assumed ε_{bio} to be constant across environmental conditions, but a more recent study suggested that ε_{bio} is temperature dependent, showing an exponential decrease with increasing temperature (Sternberg & Ellsworth, 2011). An ε_{bio} of 27‰ was particularly observed for tree-ring cellulose (Roden *et al.*, 2000, Sternberg, 2009), which have been used in many studies to extract past environmental signals (Saurer *et al.*, 1997, Saurer *et al.*, 2016), however, this has been met with mixed success (Treydte *et al.*,

2014). Part of the limitation is our incomplete understanding of oxygen isotopic fractionation processes occurring between leaf water, primary assimilates (carbohydrates), and cellulose (Brandes *et al.*, 2007, Offermann *et al.*, 2011, Gessler *et al.*, 2013). Hence, there is need to fill this knowledge gap by directly assessing the $\delta^{18}O$ values of carbohydrates, in order to better interpreting the physiological and environmental signals that are recorded within leaf or stem cellulose.

Barbour and Farquhar (2000) pointed out that the leaf water signal in carbohydrates is partly exchanged with local water before incorporation into the cellulose polymer (p_{ex}), potentially at the triose level in the futile cycle (Hill *et al.*, 1995). Generally, p_{ex} is assumed to be on average 0.42 across plant species (Barbour & Farquhar, 2000, Roden *et al.*, 2000). In addition to p_{ex}, also the water in which cellulose is synthesized might be diluted by xylem water, where p_x describes the fraction of unenriched water in this water pool. Especially in leaf tissues, p_x is most likely < 1. In combination, $p_{ex}p_x$ reflects a damping factor that causes cellulose to be less ^{18}O-enriched than carbohydrates. It has been observed that $p_{ex}p_x$ varies between 0.25 in grass (Helliker & Ehleringer, 2002) and ~0.38 in cotton leaves (Barbour & Farquhar, 2000). To date, $p_{ex}p_x$ was calculated using $\delta^{18}O$ values of carbohydrates modeled from $\delta^{18}O_{LW}$:

$$\Delta^{18}O_{Cellulose} = \Delta^{18}O_{LW}\ (1- p_{ex}p_x) + \varepsilon_{bio} \qquad \text{Eqn. 1}$$

where $\Delta^{18}O_{Cellulose}$ is the enrichment of cellulose above source water. However, when direct $\delta^{18}O$ measurements of carbohydrates are available, $p_{ex}p_x$ can be calculated as:

$$\Delta^{18}O_{Product} = \Delta^{18}O_{Substrate}\ (1- p_{ex}p_x) \qquad \text{Eqn. 2}$$

where $\Delta^{18}O_{Product}$ and $\Delta^{18}O_{Substrate}$ are the enrichment of a product (i.e. cellulose and hexoses) and a substrate (i.e. sucrose or hexoses) relative to source water, respectively. Thus, $p_{ex}p_x$

values derived from Equation 2 reflect the change in $\Delta^{18}O_{substrate}$ towards $\Delta^{18}O_{Product}$, that is caused by oxygen isotope exchange with water or by other isotope fractionation processes.

Until recently, $\delta^{18}O$ measurements of organic matter were limited to bulk carbohydrates with thermal conversion/elemental analysis isotope ratio mass spectrometry (TC/EA-IRMS; Kornexl *et al.*, 1999a, Kornexl *et al.*, 1999b). However, a new method was recently developed to precisely and accurately measure $\delta^{18}O$ values of individual carbohydrates such as fructose, glucose, and sucrose by gas chromatography/pyrolysis - isotope ratio mass spectrometry (GC/Pyr-IRMS) (Lehmann *et al.*, 2016). In the current study, we have applied this method to expand our knowledge on the relationship between $\delta^{18}O$ values of water, individual carbohydrates, and cellulose in leaves and how the $\delta^{18}O$ values in the reaction chain from leaf water to cellulose are influenced by environmental and biochemical processes.

We measured $\delta^{18}O_{LW}$, $\delta^{18}O$ of different bulk fractions (e.g. bulk leaf material, water soluble compounds), and $\delta^{18}O$ of different individual organic compounds (cellulose, sucrose, fructose, glucose, and alditols) in two C_3 grass species, as well as in needles and leaves from larch and oak trees. The grasses were grown under two different rH treatments in climate controlled growth chambers. Our objectives were (1) to test if our carbohydrate extraction and purification method is significantly adulterated by the $\delta^{18}O$ values of lab water, (2) to determine $\delta^{18}O$ values of individual carbohydrates in grass and tree species and their ε_{bio} (3) to identify possible oxygen isotope fractionations (i.e. $p_{ex}p_x$ values) along the reaction and transport chain from leaf water to cellulose in grasses, and (4) to develop an isotope fractionation scheme.

Materials and methods

Plant material

We grew two C_3 grass species, *Lolium perenne* and *Dactilys glomerata*, under controlled conditions in two different climate chambers with rH of 50% and 75% (Gamarra *et al.*, 2016). Air temperature of about 28.5 °C and light intensity of 250 µmol m^{-2} s^{-1} were similar in both chambers during the 16 h light period. For each rH condition, we grew four replicates of each species on soil in 20x20 cm pots of 5 liters volume. To minimize evaporation, aluminum foil and an additional layer of gravel (~1cm) was used to cover the soil surface. The $\delta^{18}O$ value of irrigation water was –10.9‰, with a maximum variation of 0.1‰ (SD) throughout the experiment. The atmospheric water vapour was collected as described previously (Gamarra *et al.*, 2016) and showed an identical $\delta^{18}O$ value of -15.1‰ in both chambers at the sampling day. The average $\delta^{18}O$ values of the source water derived from the root crown (see Barnard *et al.*, 2006) under high (-7.9‰) and low (-7.1‰) rH conditions were similar across all treatments and species ($P > 0.05$, Table 1). Mature leaf samples were harvested at midday, 37 days after planting, and dried at 60 °C in an oven for 48 h for further analyses. For $\delta^{18}O$ analyses of leaf and soil water, samples were transferred and stored in 10 mL gastight exetainers at -20 °C until water extraction.

Furthermore, we measured $\delta^{18}O$ values of individual carbohydrates in two C_3 tree species: (1) Mature larch trees (*Larix gmelinii*) grown in a Siberian boreal forest were sampled on July 17, 2012. Crown needle samples were taken around midday and stored in a cooler. Subsequently, samples were microwaved 2 h later in the lab to stop any metabolic activities and kept frozen until analysis. Temperature and rH conditions were 21.1 ± 2.3 °C and 52.3 ± 7.6% at the sampling day, respectively (Rinne *et al.*, 2015a, Rinne *et al.*, 2015b, Saurer *et al.*,

2016). (2) Two year old oak saplings (*Quercus robur*) were kept in a greenhouse for 8 weeks during summer. Mature leaf lamina samples were taken at 09:00 am in the morning, frozen in liquid nitrogen, and stored at -20 °C until analysis. Average temperature and rH conditions during the week before sampling were 21.7 ± 1.5 °C and 56.0 ± 5.2%, respectively. Thus, both species experienced a similar vapour pressure deficit as the grasses at high rH (1.1 ± 0.1 kPa).

Extraction and isotopic analysis of water samples

Leaf and soil water samples were cryogenically extracted as previously described (West *et al.*, 2006, Saurer *et al.*, 2016). In brief, exetainers with samples were immersed in a hot water bath at 80 °C and water was extracted under vacuum (10^{-2} mbar) for 1.5 h and trapped in U-tube that was constantly cooled with liquid N_2. Subsequently, the U-tubes were closed with rubber stoppers and the trapped water thawed and transferred to 2 ml reaction vials for isotopic analysis.

Oxygen isotope ratios of extracted water samples were determined with a high-temperature elemental analyzer coupled via a ConFlo III referencing interface to a Delta^{Plus}XP isotope ratio mass spectrometer (TC/EA-IRMS, all supplied by Finnigan MAT, Bremen, Germany), using a glassy carbon reduction method as previously described (Gehre *et al.*, 2004). 0.5 - 1 µl of water samples were injected with an autosampler (GC-PAL, Zwingen, Switzerland) equipped with a gas tight syringe. $\delta^{18}O$ values were measured on the resulting gas CO and referenced to the international V-SMOW standard (IAEA, Vienna, Austria). Measurement precision of quality control standards were < 0.2‰ (SD).

Extraction and purification of leaf carbohydrates and cellulose

Cryogenically- or oven-dried leaf material was milled to a fine powder using a steel ball mill (Retsch, Haan, Germany). The water soluble compounds (WSC) were extracted from 100 mg grass or 60 mg tree leaf material in a 2 ml reaction vial (Sarstedt, Nümbrecht, Germany) in a water bath at 85 °C for 30 min using 1.5 ml deionized water (Lehmann *et al.*, 2015). Subsequently, samples were centrifuged (2 min, 10000 *g*) and the supernatant with the WSC transferred to a new reaction vial and stored at -20 °C. Later, WSC samples were purified with ion exchange cartridges (OnGuard II A, H, and P, Dionex, Thermo Fisher Scientific, Bremen, Germany), specifically removing ionic compounds (like amino and organic acids) and polyphenols (Rinne *et al.*, 2012). Cartridges were pre-cleaned with 30 ml deionized H_2O, WSC samples added, and purified carbohydrates eluted with 6 ml deionized H_2O. The purified carbohydrate samples were frozen (-20 °C), freeze-dried, and the remaining pellets dissolved in deionized H_2O. Aliquots of these solutions were used for bulk or individual leaf carbohydrate $\delta^{18}O$ analyses as described below.

Leaf α-cellulose extraction and purification were performed in an ultrasonic bath and specifically described in a recent study (Weigt *et al.*, 2015): In brief, small pieces of plant material (~100mg) were packed in Teflon filter bags (Ankom Technology, Macedon, NY, USA) and transferred to sodium chlorite solutions (pH 4-5) to remove lignin at 70 °C for 48 h (Fisher Scientific, Reinach, Schweiz). After a washing step with deionized water for 2 h, samples were placed in a sodium hydroxide solution without heating for 45 min to remove fats, oils, tannins, and hemicellulose. This solution was mixed with 3% hydrochloric acid for 45 min and samples transferred and washed in hot (90 °C) deionized water until a neutral pH was established. Subsequently, white cellulose pellets were oven-dried at 50 °C overnight, transferred to a reaction vial with 1 ml deionized water, and stored at 4 °C for 2

days for softening. In a final step, cellulose was ultrasonically homogenized (UP200St, Hielscher, Teltow, Germany) and freeze-dried for 48 h.

Methylation derivatization of individual carbohydrates

For isotopic analyses of individual carbohydrates we used a methylation derivatization method, which was previously described in detail (Lehmann *et al.*, 2016). In brief, proton acceptor silver oxide (Ag_2O) was freshly produced from 15 g silver nitrate (Sigma-Aldrich, Buchs, Switzerland) and 3.5 g sodium hydroxide (VWR, Zurich, Switzerland) and kept in a lightproof vial. In general, about 1.5 mg purified leaf carbohydrates were transferred to a 2 ml reaction vial and freeze-dried to ensure a fine pellet for optimal methylation. 20 mg silver oxide, 300 µL acetonitrile (Sigma-Aldrich), 30 µl methyl iodide (Sigma-Aldrich), and two glass beads were added. The methylation derivatization reaction was started by adding 10 µl dimethyl sulfide (Merck, Darmstadt, Germany) and vigorously mixing the samples. Subsequently, samples were placed in a tray, darkened, and shaken on a horizontal shaker (280 turn min^{-1}) for 24 h. Samples were then spiked with 15 µl deionized H_2O, vigorously mixed, and centrifuged (2 min, 10000 *g*) to remove residues of hydrophilic compounds. Finally, the supernatants with the methylated carbohydrates were taken up with syringe and needle, cleaned from particles with 0.45 µm syringe filters (Infochroma, Zug, Switzerland), and transferred to a GC vial (Infochroma) for further GC/Pyr-IRMS analyses. Commercial available carbohydrates were used for standard mixes and methylated the same way as described above. Between the analyses, methylated samples and standards were stored at -20 °C.

Isotopic analyses of individual carbohydrates, cellulose, and bulk material

$\delta^{18}O$ analyses of individual carbohydrates were performed with a Trace GC Ultra gas chromatograph that was coupled via a reactor unit (Isolink) and a reference interface (Conflo IV) to a MAT253 isotope ratio mass spectrometer (GC/Pyr-IRMS, all supplied by Thermo Fisher Scientific, Bremen, Germany). Purified helium 6.0 (Air Liquide, Paris, France) was used as a carrier gas with a flow rate of 1.4 mL min^{-1}. 1 µl of sample was injected by a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) with a 10 µl syringe (Hamilton, Reno, USA). The constantly tempered (250 °C) split-splitless injector was set to splitless mode for 1 min after injection. To prevent GC column pollution, the liner was partially filled with glass wool and beads. Individual carbohydrates were separated on a 60 m x 0.25 mm x 0.25 µm ZB-SemiVolatiles GC column (Zebron, Phenomenex, Torrance, USA). A sample run took 1020 s, with the GC-oven temperature at 90 °C for 1 min, then progressively heated with 17.5 °C min^{-1} to 300 °C, and kept constant for 4 min. Pyrolysis of carbohydrates to CO at 1280 °C was performed in a custom-built reactor, consisting of an outer ceramic tube (length 320 mm, o.d. 1.6 mm, i.d. 0.8 mm, Friatec, Mannheim, Germany) and an inner platinum tube (99.95%, length 320 mm, o.d. 0.7 mm, i.d. 0.4 mm, Goodfellow, Huntingdon, England) partially filled with nickel wires (0.125 mm, Goodfellow). An auxiliary gas (1% H_2 in He 6.0; Messer, Lenzburg, Switzerland) with a flow of ~0.6 ml min^{-1} was added via a T-piece to the helium flow to facilitate a reducing atmosphere in the reactor. The Reactor was periodically conditioned with carbon, using 1 µl toluene (VWR) injections in 20:1 split mode. In addition, a liquid-N_2 trap was used before the Conflo IV interface during the measurement to avoid any remaining impurities from pyrolysis reaching the IRMS. This resulted in a more stable background for mass 30 and better $\delta^{18}O$ reproducibility. In general, up to ten standard analyses were performed before the actual measurement

sequence in order to reach stable conditions. A sequence of five to eight samples, which were measured each two to four times, was interspersed by standards of different concentrations for offset, drift, and amount corrections as described in detail by Lehmann *et al.* (2016). While all samples included glucose, fructose, and sucrose, tree samples additionally contained species-specific alditols, which were pinitol in larch and quercitol in oak. The retention time of quercitol was 10 s before glucose, while pinitol and fructose peaks in larch were not fully baseline-separated and thus potentially influenced by each other (Lehmann *et al.*, 2016). The averaged precision (SD) across all measurements was 0.4‰ for glucose/fructose, 0.5‰ for both alditols, and 0.7‰ for sucrose, however, precision of individual measurements ranged between 0.1 and 1.4‰. In our recent study (Lehmann *et al.*, 2016), we showed that the accuracy, i.e. the $\delta^{18}O$ offset between GC/Pyr-IRMS and TC/EA-IRMS standard analyses, was about 0.2‰ for most of the carbohydrates. However, due the lack of international standards for carbohydrates and cellulose we could not fully test if the accuracy of those compounds might be biased by water residues and unknown matrix effects in the TC/EA pyrolysis reactor, which could slightly change the observed results in this study.

For bulk isotope analyses, 1 mg of solid organic material (e.g. bulk leaf material and cellulose) or aliquots (~0.5 mg) of WSC and purified bulk carbohydrates were transferred to silver capsules and freeze-dried. All samples were measured by TC/EA-IRMS (vario PYRO cube, Elementar, Hanau, Germany) and referenced to the international benzoic acid standards 601 and 602 (IAEA, Vienna, Austria). Laboratory control standard (27.6‰, cellulose, Merck) was typically measured with a precision of < 0.3‰ (SD).

Statistics

General linear models were used to test for treatment and species-specific differences. One-way ANOVA and Tukey-HSD post hoc were applied to show differences among $\delta^{18}O$ values of bulk and individual compounds, $\Delta^{18}O_{XSW}$ values, as well as among $p_{ex}p_x$ values. Linear regression analyses were used to investigate relationships among $\delta^{18}O_{LW}$ and $\delta^{18}O$ values of individual carbohydrates and cellulose. All statistical analyses were performed in R 3.1.1. (R Core Team, 2015).

Results

Oxygen isotope exchange in hexoses, but not in sucrose

To determine the effect of oxygen isotope exchange with water during sample preparation, we extracted and purified leaf carbohydrates from *Lolium perenne* with lab water ($\delta^{18}O$ = -9.2‰) or ^{18}O-depleted water ($\delta^{18}O$ = -367.4‰; derived from residues of water enrichment processes at the Paul Scherrer Institute). While $\delta^{18}O$ values of sucrose were very similar under both water treatments (Fig. 1), we observed an ^{18}O-depletion in both hexoses under ^{18}O-depleted water compared to lab water ($P < 0.001$, t-test). The ^{18}O-depletion of 10.1‰ for glucose was slightly higher, but not significantly different compared to the ^{18}O-depletion of 9‰ for fructose ($P > 0.05$, t-test).

Influence of rH on $\delta^{18}O$ values of water and organics in grass leaves

We analyzed the oxygen isotopic composition of leaf water, bulk materials, and individual compounds (cellulose, fructose, glucose, and sucrose) from two grass species grown under two rH treatments (Figs. 2, 3). The rH treatment significantly affected leaf water and all analyzed compounds in both species (Table 1), while only some compounds showed

species-specific differences. Bulk leaf water was significantly affected by the rH treatment ($P < 0.001$), resulting in a significant ^{18}O-enrichment of 3‰ as average of both species under low rH compared to high rH conditions. Averaged across species, bulk leaf material and water soluble compounds (WSC) were 3.2‰ and 1.8‰ ^{18}O-enriched under low rH compared to high rH conditions, respectively ($P < 0.001$ and $P < 0.05$). Additionally, bulk leaf material and WSC showed species-specific differences of up to 2‰ across rH treatments (both compounds, $P < 0.01$), with generally higher $\delta^{18}O$ values in *Lolium perenne* than in *Dactylis glomerata*. Bulk carbohydrates, which were 4.1‰ to 9.6‰ ^{18}O-enriched compared to bulk leaf material and WSC ($P < 0.001$, ANOVA and Tukey-HSD), showed no clear species-specific difference across rH treatments ($P > 0.05$). Averaged across species, bulk carbohydrates were 4.5‰ ^{18}O-enriched under low rH compared to high rH conditions ($P < 0.001$). $\delta^{18}O$ of the non-carbohydrate fraction (NCF), calculated as the difference between $\delta^{18}O$ of bulk carbohydrates and WSC, reflects ionic and phenolic compounds. The NCF showed a rH treatment effect ($P < 0.001$) and a species-specific difference ($P < 0.01$), both of about 2.8‰ as average of both species or rH treatments.

Compound-specific $\delta^{18}O$ analyses revealed clear differences among individual leaf carbohydrates in both grass species (Fig. 3; Table 1). While no difference was observed among the species, the maximum $\delta^{18}O$ difference among individual carbohydrates was 1.5‰ under high and 3‰ under low rH conditions as average of both species, respectively. Generally, the highest $\delta^{18}O$ values were found for sucrose within species and rH conditions, which were 1.2‰ to 3.2‰ higher than those of hexoses ($P < 0.05$, ANOVA and Tukey-HSD). $\delta^{18}O$ values of fructose and glucose were not significantly different within species and rH conditions ($P > 0.05$, ANOVA and Tukey-HSD). $\delta^{18}O$ values of cellulose were significantly lower compared to sucrose (6.6‰ to 8.5‰; $P < 0.001$, ANOVA and Tukey-HSD) and hexoses

(2.5‰ to 5.6‰; $P < 0.01$) within species and rH conditions, thus showing the lowest $\delta^{18}O$ values among all compounds. Individual carbohydrates showed rH treatment effects of 5‰ for sucrose and 3.5‰ for both hexoses as average of both species, with higher $\delta^{18}O$ values under low rH compared to high rH conditions ($P < 0.001$, Table 1). Only glucose exhibited an averaged species-specific difference of 1‰ across rH treatments ($P < 0.05$). Depending on the species, the rH treatment effect on cellulose was 4.1‰ in *Dactylis glomerata* and 5‰ in *Lolium perenne* ($P < 0.05$).

Moreover, we found strong relationships ($r^2 = 0.84$ to 0.94) between $\delta^{18}O_{LW}$ and $\delta^{18}O$ values of individual carbohydrates and cellulose across different rH treatments and species, which were significant at $P = 0.032$ to 0.086 (Fig. 3, Table 2). The intercept of 33.1‰, reflecting ε_{bio}, was highest for sucrose, followed by 30‰ and 29.6‰ for glucose and fructose, and 25.1‰ for cellulose. The slopes, reflecting the degree to which leaf water affected the $\delta^{18}O$ value of a compound, were similar for sucrose and cellulose, with a ~1.4‰ increase in $\delta^{18}O$ of the sucrose/cellulose per 1‰ change in $\delta^{18}O_{LW}$. In comparison, the slope of the relationship between $\delta^{18}O$ of hexoses and $\delta^{18}O_{LW}$ was similar to 1. Additionally, $\delta^{18}O$ values of individual carbohydrates and cellulose were strongly related among each other (Fig. S1; Table 2, $r^2 =$ 0.83 to 0.98, $P < 0.05$).

Oxygen isotope fractionation across leaf carbohydrates of different tree species

Furthermore, we determined oxygen isotope fractionation pattern of leaf carbohydrates in tree species (Fig. 4). The maximum $\delta^{18}O$ difference among individual carbohydrates was 7.8‰ in larch and 10.6‰ in oak. Sucrose was ^{18}O-enriched compared to fructose and pinitol in larch ($P < 0.01$, ANOVA and Tukey-HSD) and to all hexoses (fructose, glucose, and quercitol) in oak ($P < 0.001$). The $\delta^{18}O$ values of hexoses showed no significant differences among each other in both tree species ($P > 0.05$, ANOVA and Tukey-HSD), yet the $\delta^{18}O$

differences between fructose and glucose tended to be higher in larch compared to oak. The $\delta^{18}O$ values of sucrose clearly exceeded the 27‰ average by about 7‰ in oak, which was not the case in larch; however, the apparent offset in oak might be overestimated since samples were taken in the morning and thus the leaf water enrichment was not at maximum. HPLC data revealed no significant differences between concentrations of fructose, glucose, and sucrose in oak (data not shown), while both hexoses were about 80% lower compared to sucrose in larch (Rinne *et al.*, 2015a). Thus, the oxygen isotope fractionation pattern of individual leaf carbohydrates was more variable in tree than in grass species, but overall similar, with the strongest ^{18}O-enrichment in sucrose.

Calculated $\Delta^{18}O_{SW}$ and $p_{ex}p_x$ across rH treatments and species

Under the assumption of ε_{bio} to be 27‰ for carbohydrates and cellulose across the leaf (Schmidt *et al.*, 2001; see discussion), we modeled the average isotopic difference between $\delta^{18}O$ values of synthesis water and bulk leaf water ($\Delta^{18}O_{XSW}$) for different compounds (Eqn. 4; Table 3). Mean $\Delta^{18}O_{XSW}$ values were clearly different among the compounds, with highest $\Delta^{18}O_{XSW}$ values for sucrose ($\Delta^{18}O_{SSW}$ = 4.9‰), followed by hexoses ($\Delta^{18}O_{HSW}$ = 2.7‰), and cellulose ($\Delta^{18}O_{CSW}$ = -2.7‰). $\Delta^{18}O_{SSW}$ and $\Delta^{18}O_{CSW}$ values were increasing with decreasing rH ($P < 0.05$, Table 5), while the rH effect for $\Delta^{18}O_{HSW}$ was dependent on the species ($P < 0.05$).

We calculated the damping factor $p_{ex}p_x$ by a best-fit approach for various compounds (Table 4). Equation 1 was used to calculate $p_{ex}p_x$ values modeled from $\delta^{18}O_{LW}$ (p_{mod}). Mean p_{mod} values of 0.6 were higher compared to all other $p_{ex}p_x$ values, showing a significant response to rH treatment ($P < 0.001$, Table 5), with higher values under high compared to low rH conditions. In contrast, $p_{ex}p_x$ values derived from measured $\delta^{18}O$ values of carbohydrates (Eqn. 2) showed generally no response to rH treatments and grass species (Table 5). Mean p_{sc} (0.21) and p_{hc} values (0.15) tended to be higher compared to p_{sh} values (0.06). In

addition, p_{sh} values were lower in grass than in tree species ($P < 0.01$, ANOVA and Tukey-HSD).

Discussion

Low oxygen isotope exchange during extraction and purification

The oxygen isotopic composition of individual carbohydrates holds valuable information about physiological and biochemical processes. As a first step, however, it is necessary to verify that the measured $\delta^{18}O$ values of individual leaf carbohydrates are not significantly biased by potential oxygen isotope fractionation effects during extraction or sample preparation. We tested this by preparing the leaf samples, using strongly ^{18}O-depleted water ($\delta^{18}O$ = -367.4‰). We observed no changes in sucrose, but a clear ^{18}O-depletion of about 9-10 ‰ for hexoses in *Lolium perenne* (Fig. 1). This is expected for hexoses, since one out of six oxygen atoms is in a carbonyl group, which may exchange oxygen with the surrounding water (Model *et al.*, 1968, Mega *et al.*, 1990, Waterhouse *et al.*, 2013). An exchange is, however, only possible when the molecule is in the unlikely straight form during mutarotations (not in the usual ring). Glucose tended to have more ^{18}O-depleted values than fructose. This agrees with the theory that the oxygen isotope exchange of aldehyde groups with the local water is faster than that of ketone groups (Byrn & Calvin, 1966, Schmidt *et al.*, 2001). The degree of oxygen isotope exchange can be estimated by the following simple calculation:

$$\delta_{obs} = \delta_c(1-x) + x(\delta_{ew} + 27) \quad \text{Eqn. 3}$$

where δ_c is the original $\delta^{18}O$ value of the carbohydrate (extracted in normal lab water), δ_{obs} is the observed $\delta^{18}O$ value of the carbohydrate (extracted in ^{18}O-depleted water), δ_{ew} is the $\delta^{18}O$ value of the ^{18}O-depleted extraction water (-367.4‰), and x is the proportion of

exchanged O in %. Solving equation 3 for x, we find that 3% of the oxygen atoms have exchanged with extraction water in whole hexose molecules. If we assume that this affects only carbonyl groups in hexoses, which is 1 out of 6 oxygen atoms, only 18% of this position have exchanged. For our carbohydrate extraction and purification, where we used lab water, which was ca. 10‰ less enriched compared to leaf water, this would cause a maximum ^{18}O-depletion of about 0.3‰ in hexoses. Hence, we could show that isotope fractionation caused by O-exchange with water is nearly negligible for our method. This allows conclusions to be drawn on biochemical and physiological processes encoded in the oxygen isotopic composition of individual carbohydrate.

The rH treatment effect on $\delta^{18}O_{LW}$ is imprinted on carbohydrates and transferred to cellulose in grasses

Relative humidity (i.e. the partial pressure ratio of the ambient (e_a) versus the leaf internal (e_i) vapor pressure) is the most important environmental driver for leaf water enrichment (Cernusak *et al.*, 2016). Accordingly, to produce a clearly detectable signal in $\delta^{18}O_{LW}$, we grew two grass species under two different rH conditions for 37 days. We found that the effect of rH on δ^{18}O of leaf water was imprinted on the δ^{18}O values of bulk organic matter (Fig. 2, Table 1). Bulk carbohydrates were clearly enriched in ^{18}O in both species compared to bulk leaf material and WSC and showed higher responses to changes in rH conditions. Interestingly, the non-carbohydrates fraction (NCF) showed both a rH treatment effect and a species-specific difference. This shows (1) that the responses to the different rH conditions in the δ^{18}O values of less purified bulk fractions are dampened by other compounds (like ionic, phenolic, and other compounds) compared to δ^{18}O values of purified carbohydrates, (2) that the δ^{18}O values of the compounds in the damping fraction (i.e. NCF) can be different

between species, which may complicate the interpretation of WSC as a proxy for plant carbohydrates (Gessler *et al.*, 2013), and (3) that non-carbohydrate compounds in the WSC fraction are mainly ^{18}O-depleted compared to carbohydrates (Schmidt *et al.*, 2001).

The rH treatment effects were also observed in bulk leaf water and individual compounds in both grass species, with the strongest rH effects for sucrose and cellulose (Fig. 3, Table 1). Since $\delta^{18}O$ values of source water were similar across treatments and species (Table 1), and other environmental factors were constant (temperature and light), we conclude that the variation in $\delta^{18}O$ values of the individual compounds are mainly driven by the rH treatment effects in $\delta^{18}O_{LW}$, integrating effects of leaf physiology (Péclet effect, stomatal regulation, leaf temperature). It is thus evident that the environmental and physiological changes affecting the grass leaf water signal are imprinted on the individual carbohydrates during photosynthesis, which is in line with studies measuring $\delta^{18}O$ of leaf phloem organic matter in *Ricinus communis* (Barbour *et al.*, 2000b, Cernusak *et al.*, 2003).

Moreover, the leaf water signal in $\delta^{18}O$ of carbohydrates was propagated along the reaction chain into cellulose. A slope of 1 for the relationship between $\delta^{18}O$ of cellulose and sucrose indicates a strong channeling of the sucrose breakdown by sucrose synthase (SuSy) with cellulose biosynthesis in the leaf growth and differentiation zone at the base position of the growing leaf (Table 2; Fujii *et al.*, 2010, Nelissen *et al.*, 2016). This is in line with studies showing that the sucrose production in grasses can be linked to cellulose synthesis in the intercalary meristem at the leaf base (Fig. 5; Helliker & Ehleringer, 2002, Cernusak *et al.*, 2016).

Isotopic leaf water and sucrose synthesis gradients may explain ε_{bio} > 27 ‰ for sucrose

The maximum $\delta^{18}O$ difference among individual carbohydrates was clearly different for grasses (3‰ in both grass species, rH = 50%) and trees (7.8‰ in larch, and 10.6‰ in oak). However, the $\delta^{18}O$ pattern among the carbohydrates was overall consistent across species (Figs. 3, 4), with sucrose being ^{18}O-enriched compared to hexoses (including fructose, glucose, and alditols), which showed no significant differences among each other. Especially in grasses and oak, this resulted in ε_{bio} values of sucrose deviating up to 5‰ to 7‰ from the often cited average of 27‰.

The unexpectedly high ^{18}O-enrichment in sucrose could be related to isotopic leaf water gradients (Fig. 5). Leaf water is known to vary strongly along the blades of grass leaves, resulting in longitudinal isotope gradients from the base to the tip (Helliker & Ehleringer, 2000, Helliker & Ehleringer, 2002, Gan *et al.*, 2003), but there is also growing evidence for radial isotope gradients from the vein out to the leaf margins in broadleaves (Wang & Yakir, 1995, Gan *et al.*, 2002, Farquhar & Gan, 2003). Furthermore, it is known that the isotopic heterogeneity in leaf water is more pronounced with decreasing rH conditions in both longitudinal (Helliker & Ehleringer, 2002, Gan *et al.*, 2003) and radial direction (Gan *et al.*, 2002, Cheesman & Cernusak, 2016). Also fine-scale spatial gradients in leaf water have been suggested to affect $\delta^{18}O$ values of carbohydrates (Farquhar *et al.*, 1998), e.g. higher enrichment in the mesophyll compared to xylem water or higher enrichment in chloroplast water compared to cytosolic water in mesophyll cells (Yakir *et al.*, 1994, Wang *et al.*, 1998). Thus, measured $\delta^{18}O_{LW}$ integrates isotopic leaf water gradients and thus might deviate from the actual $\delta^{18}O$ value of synthesis water ($\delta^{18}O_{SW}$) of different compounds due to synthesis occurring in particular leaf tissues and cellular compartments.

Schmidt *et al.* (2001) showed that $\delta^{18}O$ values of carbohydrates derived from different plant tissues can strongly vary and hypothesized that the ε_{bio} values of carbohydrate are also post-photosynthetically modified. Nevertheless, the authors assumed an average ε_{bio} value of 27‰ for carbohydrates and cellulose. If true, we can actually model the average isotopic difference between $\delta^{18}O_{SW}$ of a specific compound X and $\delta^{18}O_{LW}$ ($\Delta^{18}O_{XSW}$) as follows:

$$\Delta^{18}O_{XSW} = \delta^{18}O_X - (\delta^{18}O_{LW} + 27) \qquad \text{Eqn. 4}$$

where $\delta^{18}O_X$ is the $\delta^{18}O$ value of a specific compound X. Although $\Delta^{18}O_{XSW}$ values of cellulose might be slightly biased by the influence of the leaf water signal derived from carbohydrate precusors, we can show that $\delta^{18}O_{LW}$ does only roughly reflect $\delta^{18}O_{SW}$ and associated rH responses for carbohydrates and cellulose under the assumption of ε_{bio} = 27‰ (Tables 3, 5). This might be an explanation for the relationships between $\delta^{18}O_{LW}$ and $\delta^{18}O$ of sucrose or cellulose, showing slopes > 1 and ε_{bio} values deviating from 27‰ (Fig. 3, Table 2). Thus, the observed ε_{bio} values for carbohydrates and cellulose are most likely apparent values deviating from the actual ε_{bio} value at the site of synthesis.

However, since isotopic leaf water gradient would have been integrated when compound synthesis rates occur similarly across the leaf, we assume that the compounds have been produced in spatially disproportional rates, e.g. higher sucrose synthesis rates at the leaf tip/margin compared to leaf base/veins (Helliker & Ehleringer, 2002). In grasses, higher sucrose concentrations in the tips than in the base have been observed (Williams *et al.*, 1993). Such gradients are regulated by the plant internal carbon transport, e.g. carbohydrate export rates into the phloem (Williams *et al.*, 1993), but might also be influenced by gradients in CO_2 assimilation (Meinzer & Saliendra, 1997, Ocheltree *et al.*, 2012). In broadleaf species, sucrose synthesis might also be influenced by spatial variability in leaf gas-exchange (Nardini *et al.*, 2008, Li *et al.*, 2013), including patchy stomatal

conductance (Mott & Buckley, 2000). This might be different for larch needles, where the isotopic leaf water and sucrose synthesis gradients were potentially not as strongly expressed or sucrose synthesis occurred closer to the needle base, causing the lowest ε_{bio} for carbohydrates among species (Fig. 4). Species-specific difference in ε_{bio} might be also explained by differences in carbohydrate turn-over times and by mixing with "older" carbohydrates in storage (Gessler *et al.*, 2013). It remains to be determined if compound synthesis water is influenced by vein water via a Péclet effect or if it is a distinct pool (Song *et al.*, 2015, Holloway-Phillips *et al.*, 2016), and to be investigated how compound synthesis rates change across leaves.

$p_{ex}p_x$ differs if derived from $\delta^{18}O_{LW}$ or from $\delta^{18}O$ of carbohydrates

The oxygen isotope fractionation between leaf water and cellulose deviating from 27‰ is often explained by the damping factor $p_{ex}p_x$, which is based on biochemical considerations about oxygen isotope exchange of carbohydrates with local water during cellulose synthesis (Eqn. 1; Barbour & Farquhar, 2000). $p_{ex}p_x$ values derived from $\delta^{18}O_{LW}$ clearly responded to rH conditions (p_{mod}, Tables 4, 5), which is in agreement with a recent study showing also increasing $p_{ex}p_x$ values with increasing relative humidity (i.e. decreasing vapour pressure deficite, Liu *et al.*, 2016). However, extraordinarily high p_{mod} values of ~0.9 at high rH conditions would indicate an almost complete loss of the leaf water signal, which can hardly be true. If we assume p_{mod} to be ~0.2 under high rH conditions (i.e. p_{ex} = 0.4, p_x = 0.5; similar to p_{sc} values in Table 4) and use $\Delta^{18}O$ values of cellulose, we can solve Equation 1 for $\Delta^{18}O_{LW}$. The resulting $\Delta^{18}O_{LW}$ values were 3.3‰ lower compared to the measured $\Delta^{18}O_{LW}$ across both species, which would be almost similar to $\delta^{18}O$ of source water. This result is supported by studies showing that the intercalary meristem water in grasses in which cellulose is produced can be isotopically less enriched compared to bulk leaf water (Helliker

& Ehleringer, 2002, Gan *et al.*, 2003). Similarly, also isotope ratios of n-alkanes indicate a low enrichment above source water for grass meristem water (Kahmen *et al.*, 2013, Gamarra *et al.*, 2016). This suggests that p_{mod} is not solely driven by p_{ex} and p_x variations, but also by the isotopic heterogeneity in leaf water, causing that $\delta^{18}O_{LW}$ does not correctly reflect $\delta^{18}O_{SW}$ values (Table 3). In summary, our results show that $p_{ex}p_x$ values derived from $\delta^{18}O_{LW}$ can be unreliable and that new approaches are necessary to overcome this.

This can be done by $\delta^{18}O$ measurements of individual carbohydrates using Eqn. 2, allowing the determination of more reliable $p_{ex}p_x$ values by excluding the uncertainties in $\delta^{18}O_{LW}$. In contrast to p_{mod} values, the different types of $p_{ex}p_x$ values derived from $\delta^{18}O$ of carbohydrates (p_{sc}, p_{hc}, and p_{sh}) were not affected by rH treatments (Tables 4, 5). Accordingly, this suggests that p_{ex} and p_x do not vary with rH conditions, which would be in line with the study by Helliker and Ehleringer (2002). If p_{ex} is assumed to be 0.42 (Roden *et al.*, 2000), p_x would vary between 0.15 - 0.5 for the different types of $p_{ex}p_x$ values, suggesting $\delta^{18}O$ differences in different synthesis water pools along the leaf. Thus, $\delta^{18}O$ measurements of individual carbohydrates can be helpful to constrain oxygen isotope fractionation processes in plants.

Oxygen Isotope fractionations between individual carbohydrates

Finally, differences in p_{sh} values between grass and tree species demonstrate that oxygen isotope fractionation processes between sucrose and hexoses in leaf tissues might be different across species of different functional groups (Table 3). Sucrose and hexose synthesis gradients might be differently shaped within and among species, with spatially different rates causing differences in sucrose and hexose concentrations along a leaf (Williams *et al.*, 1993). Besides, explanations for differences in $\delta^{18}O$ of sucrose and hexoses need to consider biochemical oxygen isotope fractionations as schematically outlined in

Figure 5. Sucrose is probably translocated within the leaf phloem from the tip/margin to the base/veins. On the way, a smaller proportion of sucrose might be partly unloaded from the phloem into sink cells in mid leaf sections. There, sucrose can be broken down by invertase to free fructose and glucose and/or by sucrose synthase (SuSy) to glucose-UDP and free fructose, although the relative contribution of the enzymes to the sucrose breakdown in leaf tissues is not fully understood (Buchanan *et al.*, 2015). In both cases, hydrolysis of sucrose adds local water and thus an additional oxygen atom to the C-1 position of free glucose or glucose-UDP. In sink cells, the local water that is used during the breakdown of sucrose may be less ^{18}O-enriched compared to the sucrose synthesis water in source cells, causing a position-specific ^{18}O-depletion in glucose. The ^{18}O-depletion might be additionally strengthened if isotope effects occur under non-quantitative hydrolysis of sucrose molecules (Schmidt *et al.*, 2001). Via isomerization and enzymatic reactions, the ^{18}O-depletion in free glucose and glucose-UDP is further transferred to fructose molecules. Potentially, the phosphoglucose isomerase reaction may also include so far unknown equilibrium or kinetic isotope effects (Fig. 5), as observed for carbon and hydrogen stable isotopes (Schleucher *et al.*, 1999, Gilbert *et al.*, 2012), causing differences between δ^{18}O values of glucose and fructose (Figs. 3, 4). δ^{18}O values of hexoses across the leaf might be additional modified by the futile cycle (Hill *et al.*, 1995; Fig. 5), causing heterogeneous intramolecular oxygen isotope pattern in hexoses (Schmidt *et al.*, 2001, Werner, 2003, Schmidt *et al.*, 2015). The latter is in agreement with previous observed intramolecular carbon and hydrogen isotope patterns in carbohydrates or cellulose (Rossmann *et al.*, 1991, Augusti *et al.*, 2006, Waterhouse *et al.*, 2013). In addition, we found no clear relationship between the relative amounts of individual carbohydrates and the δ^{18}O values of larch and oak (data not shown). Thus, the oxygen isotope fractionations across the individual

carbohydrates and cellulose, observed here across different grass and tree species, may be a result of isotopic leaf water and sucrose synthesis gradients in concert with various oxygen isotope effects and exchange processes (Fig. 5).

Conclusion

i) We investigated the oxygen isotope ratio of individual leaf carbohydrates in comparison to leaf water in different grass and tree species. Isotope fractionation during sample preparation is a potential concern. Yet we could show that this is of minor importance. We recommend, however, testing any new preparation protocol with isotopically labelled water before its application on real samples to avoid misinterpretations.

ii) We also recognized that ionic and phenolic compounds in the water soluble compound fraction causes a species-specific damping of $\delta^{18}O$ values and thus purification of carbohydrates is advisable.

iii) While we could show that the rH signal in leaf water was clearly incorporated into carbohydrates and transferred to cellulose, $p_{ex}p_x$ values derived from measured $\delta^{18}O$ values of individual carbohydrates were clearly different from those derived from $\delta^{18}O_{LW}$. This strongly suggests that $\delta^{18}O$ measurements of individual carbohydrates are necessary to better constrain oxygen isotope fractionation processes and to avoid potential misjudgments (i.e. rH effect on $p_{ex}p_x$ values). Although we could advance our understanding about oxygen isotope fractionation processes among carbohydrates, it is not clear where exactly those occur across the leaf, e.g. during tip-to-base translocation of carbohydrates. Additionally, $p_{ex}p_x$ values might be temporally influenced during leaf development, e.g. cellulose synthesis might occur during night when leaf water is less ^{18}O-enriched, and might also differ among species. It is therefore necessary to thoroughly investigate these

processes by measuring the temporal and spatial variability in $\delta^{18}O$ of individual carbohydrates, cellulose, and leaf water along leaves of different species.

iv) We also showed that due to isotopic leaf water gradients, the $\delta^{18}O$ of bulk leaf water is not always a good substitute for $\delta^{18}O$ of synthesis water, in particular in grasses. This is a problem, when $\delta^{18}O_{LW}$ is used to determine ε_{bio} or $p_{ex}p_x$ values. Thus, the apparent ε_{bio} value of sucrose exceeding the common average of 27‰ by up to 7‰ may not be fully correct and reflect an upper bound. In addition, bulk leaf lamina water (main vein removed) is often more ^{18}O-enriched than bulk leaf water and thus might be an alternative indicator for synthesis water of carbohydrates, as seen by $\delta^{18}O$ analysis of leaf phloem organic matter (Cernusak *et al.*, 2003).

v) Finally, our results strongly suggest that the high ^{18}O-enrichment in sucrose might be driven by sucrose synthesis gradients, with higher proportions in leaf tips/margins than in base/veins. We conclude that studying $\delta^{18}O$ values of sucrose could be a useful tool to investigate how plant physiological and ecohydrological information in $\delta^{18}O$ values of leaf water is translocated across different leaf sections, loaded into phloem, and ultimately stored in the cellulose of the tree-ring archives. Thus, it is very useful to determine $\delta^{18}O$ values of individual carbohydrates for a better understanding of (post-)photosynthetic isotope fractionation processes and for improving models of oxygen isotope fractionation.

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Table 1: Results of general linear models for $\delta^{18}O$ values of different bulk fractions, individual compounds, and water samples. P-values are given for individual effects of grass species and relative humidity treatments (rH), and their interactions. Significant values are marked in bold. WSC and NCF denote the water soluble compounds and non-carbohydrate fraction. Please refer to Figures 2 and 3 for corresponding data.

Compound	Species	rH	Species*rH
Bulk leaf material	**0.002**	**< 0.001**	0.475
WSC	**0.008**	**0.013**	0.617
Bulk carbohydrates	0.134	**< 0.001**	0.835
NCF	**0.003**	**0.001**	0.621
Sucrose	0.429	**< 0.001**	0.426
Fructose	0.416	**< 0.001**	0.825
Glucose	**0.044**	**< 0.001**	0.798
Cellulose	**< 0.001**	**< 0.001**	**0.025**
Leaf water	0.385	**< 0.001**	0.125
Source water	0.119	0.058	0.800

Table 2: Linear regression parameters among $\delta^{18}O$ values of bulk leaf water ($\delta^{18}O_{LW}$), individual carbohydrates, and cellulose. Intercept (IC), slope, correlation coefficient (r^2), and P-value (P) are given. Standard errors are shown for IC and slope. For better comparison, $\delta^{18}O$ values of fructose and glucose were merged and denoted as $\delta^{18}O_{Hexoses}$. n denotes the number of data points used for a regression line, with each point reflecting average data of up to four individuals. Please refer to Figures 3 and S1 for linear regressions.

X-Axis	$\delta^{18}O_{LW}$	$\delta^{18}O_{LW}$	$\delta^{18}O_{LW}$	$\delta^{18}O_{LW}$	$\delta^{18}O_{Fructose}$	$\delta^{18}O_{Sucrose}$	$\delta^{18}O_{Cellulose}$	$\delta^{18}O_{Cellulose}$
Y-Axis	$\delta^{18}O_{Sucrose}$	$\delta^{18}O_{Fructose}$	$\delta^{18}O_{Glucose}$	$\delta^{18}O_{Cellulose}$	$\delta^{18}O_{Glucose}$	$\delta^{18}O_{Hexoses}$	$\delta^{18}O_{Sucrose}$	$\delta^{18}O_{Hexoses}$
IC	33.1±0.8	29.6±0.9	30.0±0.7	25.1±1.3	0.0±2.5	6.7±1.3	6.9±5.3	11.9±2.8
Slope	1.5±0.3	1.0±0.3	1.1±0.2	1.3±0.4	1.0±0.1	0.7±0.0	1.0±0.2	0.7±0.1
r^2	0.94	0.87	0.91	0.84	0.98	0.98	0.90	0.83
P	0.032	0.068	0.047	0.086	0.008	<0.001	0.052	0.002
n	4	4	4	4	4	8	4	8

Table 3: Modeled $\Delta^{18}O_{XSW}$ values for different compounds across rH treatments and species under the assumption of ε_{bio} = 27‰. Equation 4 was used to calculate $\Delta^{18}O_{XSW}$ values for sucrose ($\Delta^{18}O_{SSW}$), hexoses ($\Delta^{18}O_{HSW}$), and cellulose ($\Delta^{18}O_{CSW}$). For $\Delta^{18}O_{HSW}$, $\delta^{18}O$ values of alditols (only tree species), fructose, and glucose of each species were averaged. Uppercase letters denote differences among different types of $\delta^{18}O_{sw}$ values (One-way ANOVA and Tukey-HSD). Dg = *D. glomerata*, Lp= *L. perenne*, Lg = *L. gmelinii*, Qr = *Q. robur*. Means and standard errors are given (n = 3-5).

Species	rH	$\Delta^{18}O_{SSW}$	$\Delta^{18}O_{HSW}$	$\Delta^{18}O_{CSW}$
Dg	Low	6.2 ± 0.5	3.5 ± 0.4	-2.3 ± 0.8
Dg	High	3.6 ± 0.6	2.0 ± 0.3	-4.4 ± 0.7
Lp	Low	5.4 ± 0.7	2.3 ± 0.4	-1.5 ± 0.5
Lp	High	4.3 ± 1.3	2.9 ± 0.6	-2.5 ± 0.6
Mean	-	**4.9 ± 0.4A**	**2.7 ± 0.43B**	**-2.7 ± 0.4C**
Lg	-	-1.4 ± 1.9	-7.0 ± 0.6	-
Qr	-	7.4 ± 0.8	-2.0 ± 1.3	-

Table 4: Damping factor $p_{ex}p_x$ for different compounds across rH treatments and species. p_{mod} denotes $p_{ex}p_x$ values derived from $\Delta^{18}O_{LW}$ (Eqn. 1). p_{sc} or p_{sh} indicate $p_{ex}p_x$ values derived from $\Delta^{18}O$ of carbohydrates (Eqn. 2), where sucrose is the substrate ($\Delta^{18}O_{Substrate}$) and cellulose or hexoses the product ($\Delta^{18}O_{Product}$). p_{hc} denotes $p_{ex}p_x$ values (Eqn. 2), where hexoses reflects the substrate and cellulose the product. For $p_{ex}p_x$ values derived from hexoses (p_{sh}, p_{hc}), $\delta^{18}O$ values of alditols (only tree species), fructose, and glucose of each species were averaged. Uppercase letters denote differences among different types of $p_{ex}p_x$ values (One-way ANOVA and Tukey-HSD). Dg = *D. glomerata*, Lp= *L. perenne*, Lg = *L. gmelinii*, Qr = *Q. robur*. Means and standard errors are given (n = 3-4).

Species	rH	p_{mod}	p_{sc}	p_{hc}	p_{sh}
Dg	Low	0.37 ± 0.10	0.22 ± 0.01	0.16 ± 0.01	0.07 ± 0.00
Dg	High	0.87 ± 0.06	0.23 ± 0.01	0.19 ± 0.01	0.05 ± 0.01
Lp	Low	0.25 ± 0.09	0.18 ± 0.01	0.11 ± 0.01	0.08 ± 0.01
Lp	High	0.93 ± 0.07	0.19 ± 0.02	0.15 ± 0.02	0.05 ± 0.02
Mean	**-**	**0.60±0.09A**	**0.21±0.01B**	**0.15±0.01B**	**0.06±0.01B**
Lg	-	-	-	-	0.13 ± 0.04
Qr	-	-	-	-	0.15 ± 0.01

Table 5: Results of general linear models for different types of $\Delta^{18}O_{XSW}$ and $p_{ex}p_x$ values. P-values are given for individual effects of grass species and relative humidity treatments (rH), and their interactions. Significant values are marked in bold. Please refer to Tables 3 and 4 for corresponding data.

Parameters	Species	rH	Species*rH
$\Delta^{18}O_{SSW}$	0.424	**0.028**	0.325
$\Delta^{18}O_{HSW}$	0.071	**0.038**	**0.036**
$\Delta^{18}O_{CSW}$	0.410	**0.038**	0.372
p_{mod}	0.345	**0.001**	0.332
p_{sc}	0.069	0.602	0.905
p_{hc}	**0.021**	0.109	0.812
p_{sh}	0.549	0.151	0.945

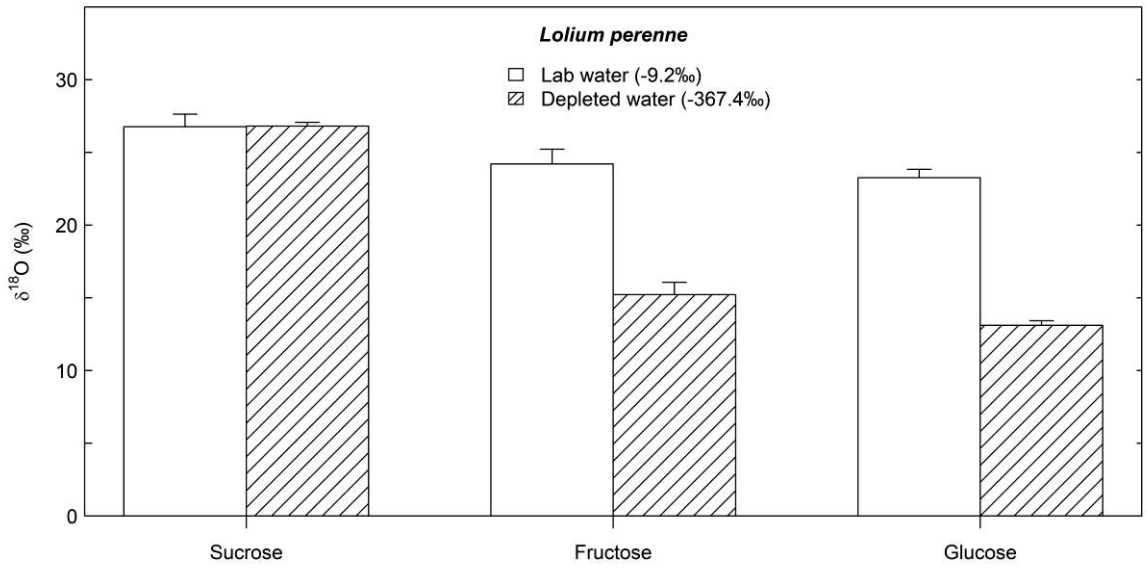

Figure 1: δ18O values of leaf carbohydrates in Lolium perenne (rH = 75%) extracted and purified with different 18O-labelled water. Mean δ18O values and standard errors are given (n = 3-4).

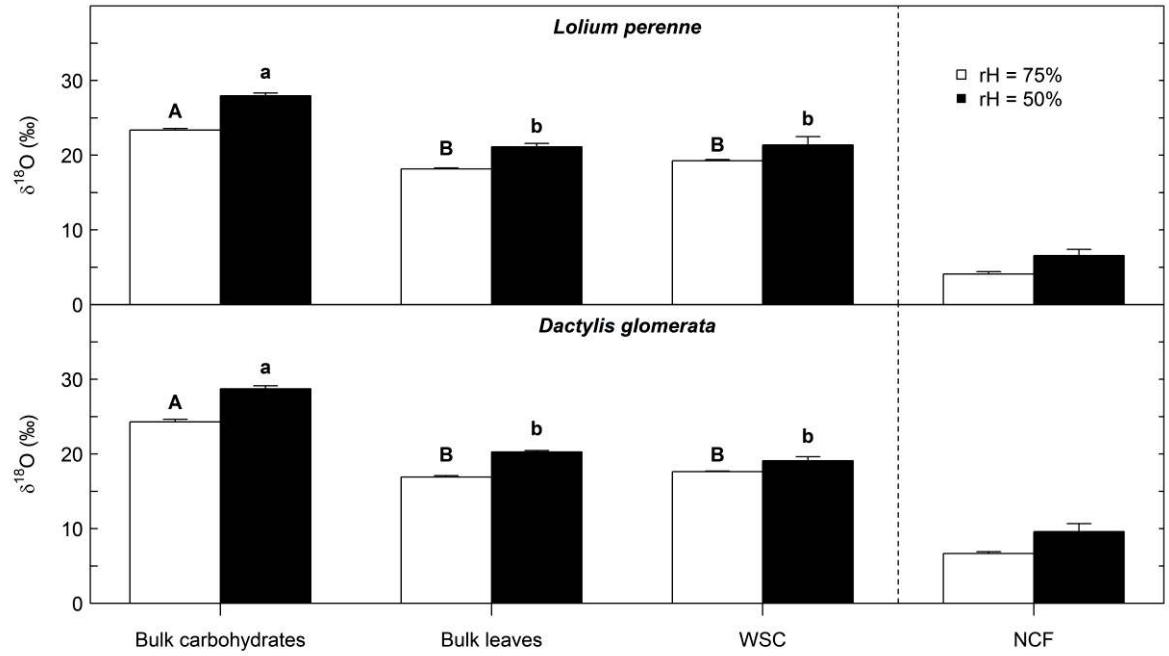

Figure 2: δ18O values of different leaf bulk fractions under two different relative humidity conditions in two grass species. All extraction and purification steps were performed with normal lab water. Significant differences among the different bulk fractions for each treatment in both species are indicated by capital (high rH) and lowercase (low rH) letters (One-way ANOVA and Tukey-HSD). WSC = water soluble compounds. NFC = Non-carbohydrate fraction, denoting the difference between δ18O values of bulk carbohydrates and WSC. Mean δ18O values and standard errors are given (n = 3-4). Please refer to Table 1 for more statistics.

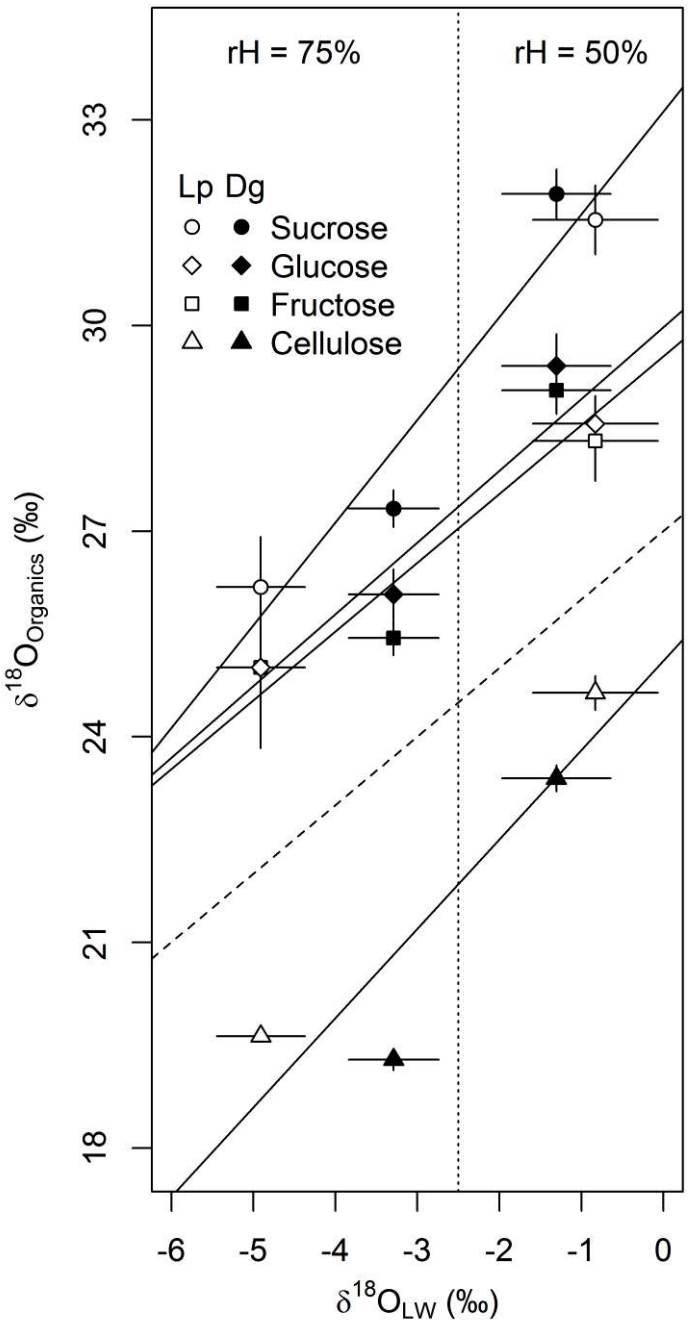

Figure 3: Relationships between δ18O values of bulk leaf water (δ18OLW) and δ18O values of individual carbohydrates and cellulose (δ18Oorganics) under two different relative humidity conditions in two grass species. All extraction and purification steps were performed with normal lab water. Open and closed symbols reflect L. perenne (Lp) and D.

glomerata (Dg), respectively. For better comparison, the biosynthetic fractionation factor of 27‰ is indicated by a dashed line using a generic regression equation y = x + 27. The vertical dotted line separates low and high rH conditions for better visualization. Please refer to Tables 1 and 2 for statistics. Mean δ18O values and bi-dimensional standard errors are given (n = 3-4).

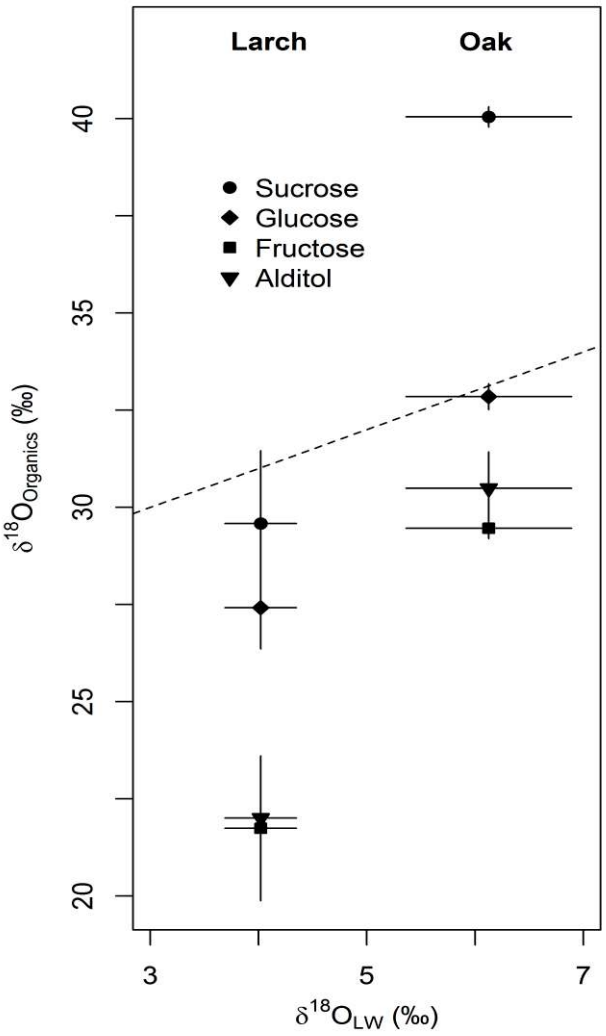

Figure 4: Relationships between δ18O values of bulk leaf water (δ18OLW) and δ18O values of individual carbohydrates (δ18Oorganics) in two tree species. All extraction and purification steps were performed with normal lab water. Alditol denotes quercitol in oak and pinitol in larch. For better comparison, the biosynthetic fractionation factor of 27‰ is indicated by a dashed line using a generic regression equation y= x + 27. Mean δ18O values and bidimensional standard errors are given (n = 2-5).

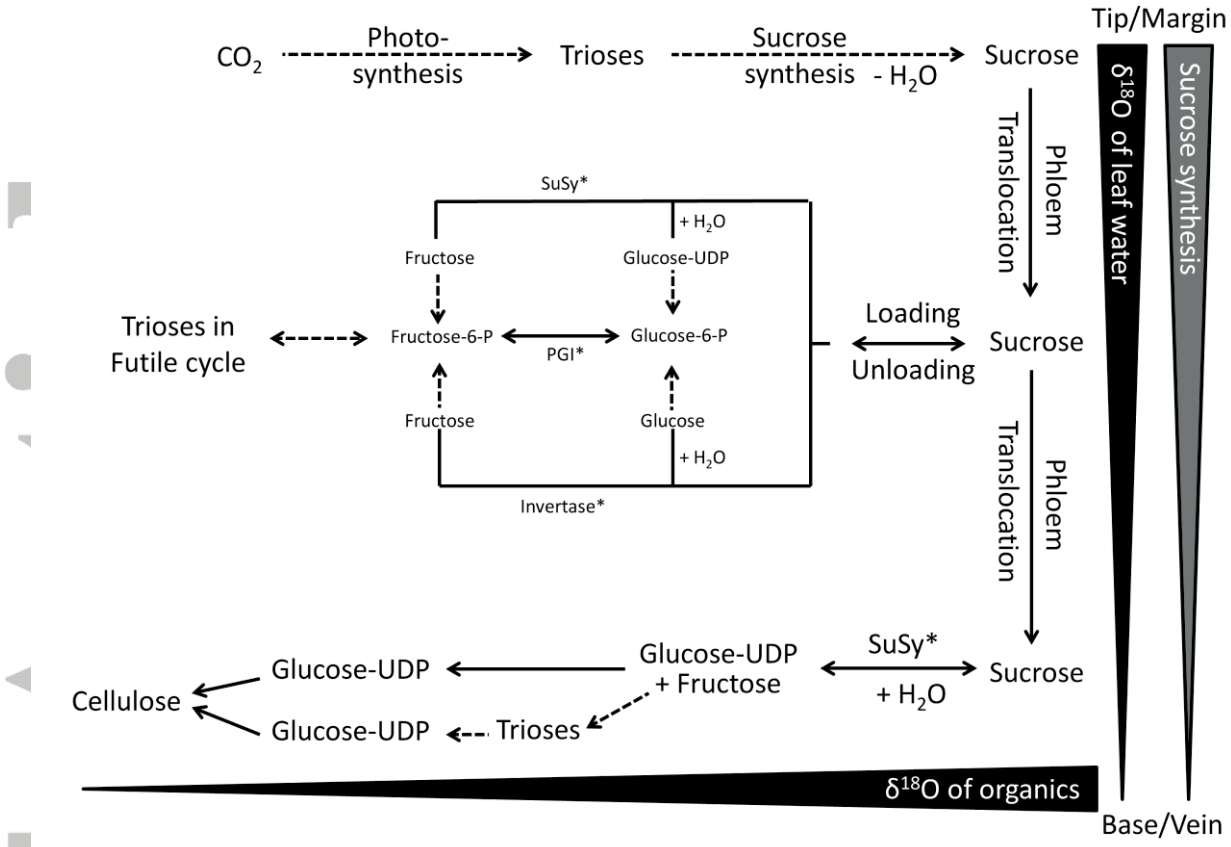

Figure 5: Oxygen isotope fractionation scheme for organic compounds in leaves from monocots (tip/base) and dicots (margin/vein). Sucrose and hexose synthesis occur along the leaf, however, potentially in spatially different rates. For instance, higher sucrose synthesis rates in ^{18}O-enriched leaf tip/margin water than in ^{18}O-depleted leaf base/vein water may explain the ^{18}O-enrichment in sucrose compared to other carbohydrates in grass and tree species, as well as the biosynthetic fractionation factor for sucrose exceeding the common average of 27‰. During phloem translocation from leaf tip/margin to base/vein, sucrose might be partly unloaded from the phloem in mid leaf sections and hydrolyzed to hexoses via invertase and sucrose synthase (SuSy), using relatively less ^{18}O-enriched leaf water. The hexoses undergo isomerization by the phosphoglucose isomerase (PGI) and breakdown to trioses, causing exchange of oxygen isotopes with water via carbonyl groups in the futile cycle and thus additional ^{18}O-depletion in hexoses compared to sucrose. Sucrose re-

synthesis via trioses and SuSy and loading into phloem may be possible. Sucrose that is translocated to the leaf base/vein is unloaded from the phloem into sink cells and hydrolyzed to hexoses by SuSy, using less ^{18}O-enriched leaf water. A certain proportion of this hexoses undergoes the trioses futile cycle before the final incorporation into cellulose, which showed clearly lower $\delta^{18}O$ values compared to carbohydrates in both grass species.

The black bold arrow marking the x-axis denotes a gradient in $\delta^{18}O$ of organics such as sucrose, hexoses, or cellulose. The black bold arrow marking the y-axis indicate a gradient in $\delta^{18}O$ of leaf water, while the grey bold arrow denotes a gradient in sucrose synthesis. Dashed arrows denote reactions with intermediate steps. Stars (*) indicate potential equilibrium and kinetic isotope fractionation during sucrose hydrolysis and hexose isomerization, causing additional oxygen isotope fractionation.