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Hydrogen exchange during cellulose synthesis distinguishes climatic and biochemical isotope fractionations in tree rings

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Summary

• The abundance of the hydrogen isotope deuterium (D) in tree rings is an attractive record of climate; however, use of this record has proved difficult so far, presumably because climatic and physiological influences on D abundance are difficult to distinguish.
• Using D labelling, we created a D gradient in trees. Leaf soluble sugars of relatively low D abundance entered cellulose synthesis in stems containing strongly D-labelled water. We used nuclear magnetic resonance (NMR) spectroscopy to quantify D in the C-H groups of leaf glucose and of tree-ring cellulose.
• Ratios of D abundances of individual C-H groups of leaf glucose depended only weakly on leaf D labelling, indicating that the D abundance pattern was determined by physiological influences. The D abundance pattern of tree-ring cellulose revealed C-H groups that exchanged strongly (C(2)-H) or weakly (C(6)-H_2) with water during cellulose synthesis.
• We propose that strongly exchanging C-H groups of tree-ring cellulose adopt a climate signal stemming from the D abundance of source water. C-H groups that exchange weakly retain their D abundance established in leaf glucose, which reflects physiological influences. Combining both types of groups may allow simultaneous reconstruction of climate and physiology from tree rings.

Key words: climate reconstruction, deuterium (D), hydrogen exchange, isotopomer, nuclear magnetic resonance (NMR) spectroscopy, tree-ring cellulose.

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Introduction

Forecasts of climate and of the effect of climate on the biosphere are hampered by two uncertainties. One concerns long-term interactions between climate and the biosphere that are incompletely understood. Climate–biosphere interactions have been studied in manipulative experiments (Long *et al.*, 2004; Nowak *et al.*, 2004), but these experiments can only cover timescales of a few years. The results of such experiments may therefore reflect transient responses (Knorr *et al.*, 2005), which raises the questions as to whether plants adapt to environmental changes on timescales of centuries, and how such adaptations can be detected. A second uncertainty concerns climate records with better geographic coverage and with high time resolution, which are required to calibrate climate models and detect trends in climate extremes.

Tree rings are an attractive tool to address both uncertainties, because their composition is strongly influenced by environmental conditions. Moreover, tree-ring series covering centuries or millennia can be sampled at annual or even seasonal resolution; thus, they can give high-resolution information on past climate variability with good geographic coverage (Schweingruber, 1996). Several properties of tree rings can be measured; properties with robust correlations to environmental conditions can serve as climate proxies. Tree-ring width and density have been used extensively for climate reconstruction (Briffa *et al.*,

2004) but these parameters reflect many different environmental signals, and great care must be taken in order to distinguish climate signals.

Ratios of stable isotopes of hydrogen (D : H), carbon (^{13}C : ^{12}C), and oxygen (^{18}O : ^{16}O) have also been used to extract climate and physiological signals from tree-ring series (McCarroll & Loader, 2004). In these studies, known environmental variables are correlated with isotope ratios of plant metabolites, commonly tree-ring cellulose. In a second step, these correlations are used to reconstruct environmental parameters from tree-ring series. However, several environmental variables influence each isotope ratio and it is therefore difficult to reconstruct individual variables from isotope ratios. This problem may be particularly severe for the hydrogen isotopes (Pendall, 2000; Waterhouse *et al.*, 2002), because the D : H ratio is influenced by several D fractionation mechanisms. Firstly, the D abundance of precipitation water contains an air temperature signal (Dansgaard, 1964), which is transferred to plant material, because water is taken up by tree stems without isotope fractionation (White *et al.*, 1985; Ehleringer & Dawson, 1992). Secondly, leaf transpiration enriches leaf water in D (Wershaw *et al.*, 1966; Roden & Ehleringer, 2000). This enrichment depends on the difference in vapour pressure between leaves and the surrounding air, and therefore confers a humidity signal to leaf water (Craig & Gordon, 1965). During photosynthesis, both signals are transferred to primary photosynthate. Thirdly, enzymes have kinetic D isotope effects, which discriminate against D in particular C-H groups of metabolites. Fourthly, when cellulose is synthesized in the tree trunk, enzymes catalyse hydrogen exchange of carbon-bound hydrogen of translocated sugars with xylem water. This exchange has been studied in an aquatic plant (Yakir & DeNiro, 1990), in seedlings (Terwilliger & DeNiro, 1995), and in tree rings (Hill *et al.*, 1995; Roden & Ehleringer, 1999; Waterhouse *et al.*, 2002), and it was found that about 40% of all carbon-bound hydrogen exchanges during cellulose synthesis. Studying the related exchange of oxygen isotopes, Sternberg *et al.* (2003) found that the oxygen at carbon 2 of glucose exchanges 100% with water. By analogy, we expect that hydrogen exchange affects specific C-H groups, determined by the enzyme activities involved in cellulose synthesis.

Biochemical D discriminations are central to understanding D abundance in plant matter, and to reconstruct paleo-signals from tree rings. These discriminations have previously been summarized as an autotrophic (photosynthetic) fractionation factor of approx. −150‰ (Estep & Hoering, 1981; Yakir & DeNiro, 1990), and a fractionation factor for heterotrophic (postphotosynthetic) carbohydrate metabolism of approx. +150‰ (Yakir & DeNiro, 1990; Luo & Sternberg, 1992). Underlying these composite fractionation factors of metabolic pathways are isotope effects of several enzymes. Because each enzyme acts on a specific chemical bond, enzymes imprint a specific D abundance on each C-H group of a metabolite (Martin *et al.*, 1992; Schmidt, 2003). A molecule carrying D in a specific C-H group is called a D isotopomer; thus, the abundance pattern of D in the C-H groups of a metabolite can be described as deuterium isotopomer distribution (DID). Isotope effects can deplete metabolites several-fold in particular D isotopomers. Because of this, D isotopomer abundances can easily show 50% variation, but isotopomer variation cannot be pinpointed from the D abundance of the whole molecule (Schleucher *et al.*, 1999). An enzyme's D isotope effect describes the D depletion it causes *in vitro*. The extent to which it causes isotope depletion *in vivo* depends on the regulation of the biosynthetic pathway; thus, DIDs of metabolites carry signals on metabolic regulation (Schleucher *et al.*, 1999; Schmidt, 2003).

Based on the four discrimination mechanisms outlined above, we propose the following framework for the D abundance of tree-ring cellulose. The D abundance of leaf water is determined by the D abundance of source water and D enrichment resulting from leaf transpiration. When hydrogen originating from leaf water enters photosynthesis, kinetic isotope effects of photosynthetic enzymes reduce the abundance of each D isotopomer of glucose by a factor specific for this isotopomer. Thus, the abundance of each D isotopomer of glucose is given by the leaf water D abundance, scaled down by a factor that reflects isotopomer-specific enzyme isotope effects. We can therefore state hypothesis 1 of this work: because the D abundance of leaf water is a constant influence for all isotopomers, ratios of isotopomer abundances are independent of leaf water. In other words, the DID of photosynthetic glucose should only reflect enzyme isotope effects. When translocated photosynthate is used to synthesize tree rings, hydrogen exchange can reintroduce the D abundance of source water, irrespective of all D discriminations at the leaf level. Hypothesis 2 of this work states that isotopomers of tree-ring cellulose can be identified which exchange strongly or weakly during cellulose synthesis. Identification of such isotopomers would allow differentiation of the four mechanisms of D discrimination. The objective is to isolate physical D discrimination mechanisms (source water, evaporative enrichment) to improve paleoclimate reconstructions from tree-ring records. Biochemical fractionations, which used to represent noise for climate reconstruction, instead constitute signals of metabolic fluxes, which may be used to study long-term physiological changes. Parallel reconstruction of climate signals and physiological changes by isotopomer analysis opens a way to study long-term adaptations of trees to environmental changes.

Concept of the experiment

The aim of this experiment was to study hydrogen exchange during cellulose synthesis, using a D gradient between leaves and xylem water. During the growing season of the experiment, trees were watered with D-labelled water and were exposed to high humidity. Conceptually, the D label and high humidity play the role of evaporative D enrichment in previous studies.

However, in our experiment the labelled water was enriched by five times the natural D abundance (i.e. δD was of the order of 5000‰). Therefore the behaviour of the label was the overriding influence on D abundance in the trees. Water vapour exchange between leaves and the atmosphere diluted the D label in leaf water; evaporative enrichment was negligible in comparison. This dilution of the D label in leaf water created a D gradient between leaf water and xylem water in tree stems. Consequently, carbohydrates of relatively low D abundance produced in the leaves were used to synthesize tree-ring cellulose in the presence of xylem water of high D abundance. The large D gradient between leaf metabolites and xylem water allowed us to distinguish effects of hydrogen exchange from possible D fractionations. The abundances of individual D isotopomers were measured using deuterium nuclear magnetic resonance spectroscopy (D NMR). The D content of a sample, measured by isotope ratio mass spectrometry (IRMS), is normally described as the D : H ratio and expressed as the δD value. This nomenclature is not well suited for our experiment, because our D enrichment was so high that the δ scale does not offer an advantage, and because NMR does not measure D : H ratios, but D isotopomer abundances. We use the term D abundance to refer to the D content of a whole molecule, and the term D isotopomer abundance to refer to the D content of specific C-H groups of glucose. The D label used in the experiment was nevertheless a trace amount, below 0.1%. It is highly unlikely that this labelling affected metabolism, and therefore conclusions from the experiment apply to tree-ring series of unlabelled trees. We used oak and spruce trees because these species represent broadleaved and coniferous trees, and they are widespread in Europe, and often used in tree-ring studies.

Materials and Methods

Plant material

Oak trees (*Quercus petraea* Agder, 1.5 years old) and spruce trees (*Picea abies* Minsk, 5 years old) from a local nursery were potted (pots of 15 and 10 l, respectively) in normal potting soil in May 2000. They were grown in a glasshouse until March 2001, when six plants of each species were transferred to a growth chamber while six plants of each species remained in the glasshouse. Growth chamber conditions were initially 15°C : 10°C air temperature (light : dark), 10 h light. After bud break, the temperature was switched to 25°C : 23°C and 14 h of light (approx. 400 μmol photons m^{-2} s^{-1}), to simulate the growing season. The air humidity was set to 80%. The plants were kept in an optimal water regime by watering them with nutrient solution (RIKA-S, NPK 7-1-B, Weibulls, Hammenhög, Sweden) every other day. For growth chamber plants (D-labelled plants), 0.75 ml D_2O (99.8 atom% D) was added per litre of nutrient solution, resulting in a D abundance of approximately sixfold natural abundance. The water D abundance was quantified as described below. Control plants, kept in a glasshouse (25°C : 18°C air temperature, 80 : 70% relative air humidity, natural day light) were watered with nutrient solution without D_2O addition. Soils and leaves of D-labelled plants were sampled in May, June and July, to measure the D abundance in soil and leaf water. For each sampling, soil from three plants was pooled, and similarly for leaves. Water from leaves and soil was extracted cryogenically according to Ehleringer & Osmond (1989). At the end of August 2001, fully expanded leaves of control and D-labelled trees were harvested from representative parts of each plant. At the end of October, stems of control and D-labelled trees were harvested. All plant material was stored at −20°C until analysis.

Sugar isolation and NMR sample preparation

Leaf soluble sugars were extracted according to Hurry *et al.* (2000), with the following modifications. Fresh leaf material (10–15 g) ground in liquid nitrogen was extracted four times with 80% ethanol at 80°C for 15 min. The combined extracts were evaporated until the chlorophyll precipitated, which was removed by vacuum filtration through diatomaceous earth. The filtrate was evaporated on a rotary evaporator and dried under vacuum. Late wood of the most recent tree ring was carefully collected by grinding with a file, to obtain a fine powder. Whole wood was hydrolysed to glucose according to Saeman *et al.* (1945), with the following modification. The glucose solution was cooled on ice, neutralized with calcium carbonate, filtered, evaporated and dried. We did not purify sucrose or cellulose, but isolated a glucose derivative from leaf soluble sugars and tree rings. For sucrose, this is justified because the D abundance of leaf water is passed from photosynthetic glucose to all glucose-containing molecules. For cellulose, we assume that the glucose that is incorporated into different polymers undergoes the same hydrogen exchange. To convert sugars into samples for D NMR, leaf soluble sugars and glucose from tree rings were derivatized according to Schleucher *et al.* (1999). Avoiding D fractionation, the derivatization converts all glucose moieties of leaf soluble sugars and tree rings into a pure glucose derivative (3,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose) that is suitable for NMR (T. R. Betson *et al.*, unpublished). The glucose derivative contains two methyl groups, which originate from acetone during the synthesis. All samples were prepared using the same batch of acetone, so that these methyl groups serve as a reference for natural D abundance. We refer to the samples derived from leaf soluble sugars and tree rings as 'leaf glucose' and 'tree-ring glucose', respectively.

Measurement of deuterium enrichment

To quantify the D abundance of plant glucose samples, the glucose derivative (approx. 100 mg) was dissolved in a solvent mixture (16% (v/v) C_6F_6 and 84% acetonitrile containing

0.1% C_6D_6). The solution was filtered with slight overpressure through a Pasteur pipette (containing a cotton plug followed by layers of diatomaceous earth (0.5 cm), $NaHCO_3$ (0.5 cm), and Na_2SO_4 (2 cm)) into the NMR tube. Solvent was added until the NMR sample reached a suitable height (26–31 mm). Deuterium NMR spectra of the glucose derivative were measured using a DRX600 spectrometer (Bruker, Switzerland) equipped with a 5 mm broadband-observe probe with ^{19}F lock. Integratable D spectra were recorded as described in Schleucher *et al.* (1999). The baselines of the spectra were corrected, and integrals of the glucose and methyl signals of the derivative were obtained using the Lorentzian line-shape fit of the XWINNMR program (Bruker). The ratio of these integrals gave the D abundance of the glucose isotopomers relative to the methyl groups. Thus, the natural D abundance of the methyl groups defined the unit for D abundance used in this work.

Deuterium abundance of leaf and soil water was quantified as follows: 100 µl water was mixed with 400 µl acetonitrile and 50 µl deuterated chloroform in an NMR tube. First, the magnetic field of the spectrometer was adjusted ('locked' and 'shimmed') using the deuterated chloroform. Then the lock was turned off, D NMR spectra were measured and the D signals of water and acetonitrile were integrated. By comparing the integrals to the molar ratios of water and acetonitrile, the D abundance of the water relative to the acetonitrile was calculated. The D abundance of water was converted to the abundance scale of the glucose derivative by comparing the NMR signals of acetonitrile to those of the methyl groups of the derivative.

Statistical analysis

P-values, calculated with the *t*-test in Microsoft Excel, were used to test for statistical differences between D isotopomer distributions, and between 'relative exchange' values of individual isotopomers.

Results

Deuterium-labelled and control oak and spruce trees reached a diameter of about 2 cm and formed tree rings of about 2 mm in thickness. Deuterium NMR spectra were recorded on a glucose derivative (see the Materials and Methods section). The spectra (Fig. 1) contain one signal for each D isotopomer D1–D5, $D6^R$, $D6^S$ (the indices *R* and *S* denote the stereochemistry at a CH_2 group), and the abundance of each isotopomer is given by the integral of its signal. To quantify D enrichment, the signals of the methyl groups of the derivative served as reference for natural D abundance. For tree-ring glucose of control oaks (dashed spectrum in Fig. 1), the glucose signals had roughly one-third of the integral of the methyl groups, consistent with natural D abundance. For D-labelled oaks (solid spectrum), the glucose signals were roughly of the same intensity as the methyl signals, indicating strong D

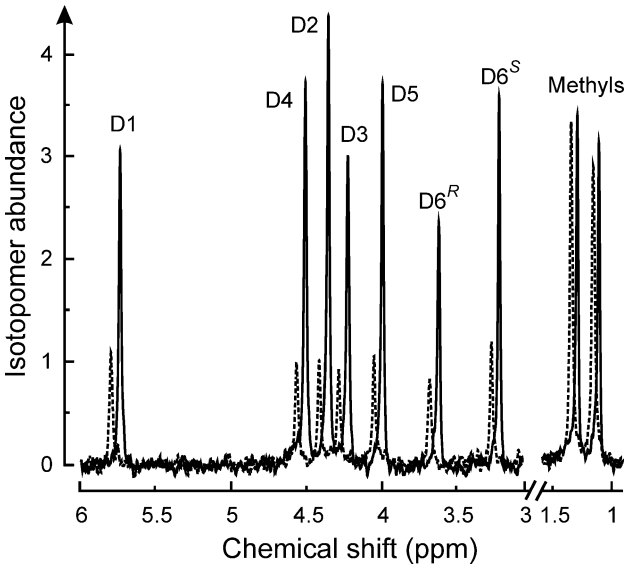

Fig. 1 Deuterium (D) isotopomer abundance of tree-ring glucose of control oaks (*Quercus petraea*; dashed spectrum, shifted for clarity) and D-labelled oaks (solid spectrum). The positions (chemical shift) of the signals in the D nuclear magnetic resonance (NMR) spectra allow the assignment of each isotopomer; signal integrals reflect isotopomer abundance. The seven signals between 6 and 3 ppm originate from the seven D isotopomers of glucose. The two signals between 1.5 and 1 ppm, which were used to scale the spectra, originate from the methyl groups introduced during the derivatization.

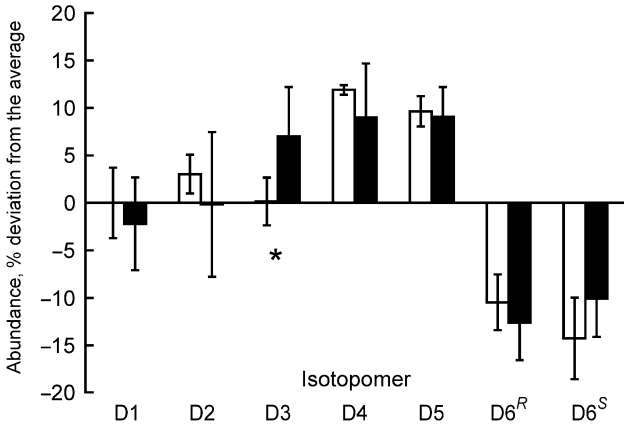

Fig. 2 Isotopomer abundances of leaf glucose of control (open bars) and D-labelled (closed bars) oak (*Quercus petraea*) trees. For each isotopomer, the deviation of its abundance from the average abundance of all isotopomers is given as a percentage. Error bars indicate SD (n = 4–6). *, significantly different isotopomer abundances (t-test, $P < 0.05$).

enrichment as a result of exchange with D-enriched xylem water. Similar spectra were obtained for spruce (data not shown).

Comparing the DIDs of leaf glucose of control trees and D-labelled trees provides a sensitive test for hypothesis 1, which states that DIDs of leaf sugars are determined by enzyme isotope effects, independently of leaf water D abundance. Figure 2 shows DIDs of leaf glucose of control oaks (open

Table 1 Deuterium (D) abundances of water and sugars in D-labelled oak (*Quercus petraea*) and spruce (*Picea abies*) trees

	Deuterium abundance	
	Oak	Spruce
Watering water	5.51 ± 0.39	5.51 ± 0.39
Soil water	4.01 ± 0.56	3.73 ± 0.48
Leaf water	2.37 ± 0.25	1.67 ± 0.16
Leaf glucose	2.27 ± 0.17	1.92 ± 0.20
Tree-ring glucose	3.65 ± 0.16	3.01 ± 0.18

D abundance was measured by D nuclear magnetic resonance in units of natural D abundance, as described in the Materials and Methods section. The D abundances of soil and leaf water represent averages ± SD over three sampling dates and for six plants of each species. For the D abundance of glucose, the abundances of seven D isotopomers were averaged first, then the average ± SD was taken for six plants.

Table 2 Abundances of deuterium (D) isotopomers of tree-ring glucose (a_{ring}) and leaf glucose (a_{leaf}) of D-labelled oak (*Quercus petraea*), and hydrogen exchange parameters

Oak isotopomer	a_{ring}	a_{leaf}	Relative exchange	Percentage exchange
D1	3.19 ± 0.24	2.21 ± 0.13	0.38 ± 0.10 a	30 ± 8
D2	4.85 ± 0.31	2.27 ± 0.30	1.00 b	79 ± 10
D3	3.66 ± 0.22	2.42 ± 0.13	0.48 ± 0.06 a	40 ± 7
D4	4.02 ± 0.13	2.48 ± 0.29	0.60 ± 0.06 c	50 ± 7
D5	4.03 ± 0.18	2.47 ± 0.17	0.61 ± 0.05 c	51 ± 7
$D6^R$	2.79 ± 0.13	1.99 ± 0.20	0.31 ± 0.07 a	23 ± 5
$D6^S$	3.03 ± 0.32	2.04 ± 0.07	0.38 ± 0.10 a	29 ± 10

D isotopomer values are multiples of natural D abundance. Relative exchange values are calculated as $a_{ring} - a_{leaf}$, relative to the highest difference (D2); letters group isotopomers into statistically different classes ($P < 0.05$). Percentage exchange was calculated as $100(a_{ring} - a_{leaf})/(a_{water} - a_{leaf})$. Values are average ± SD ($n = 6$).

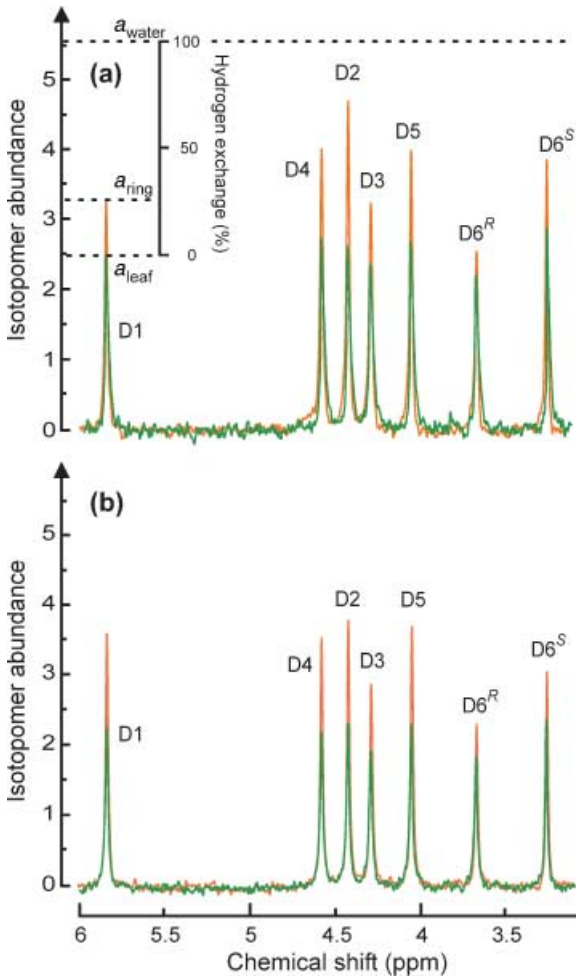

Fig. 3 Increase in deuterium (D) isotopomer abundance between leaf glucose (green) and tree-ring glucose (orange) from D-labelled oak (*Quercus petraea*) (a) and spruce (*Picea abies*) (b). Isotopomer abundance in the D nuclear magnetic resonance (NMR) spectra is expressed in units of natural abundance, relative to the methyl groups of the glucose derivative. For illustration, an exchange scale for the D1 isotopomer is indicated in (a); a_{leaf} and a_{ring}, isotopomer abundance in leaf glucose and tree-ring glucose, respectively; a_{water}, D abundance of watering water.

bars) and D-labelled oaks (closed bars). The DIDs were expressed as percentage abundance deviation relative to the average D abundance of the whole molecule. In oaks, the DID did not differ between control and D-labelled trees, except for a statistically significant abundance difference of 7% of the D3 isotopomer. In spruce, abundance differences up to 11% were observed for the D1 and $D6^S$ isotopomers.

To investigate hydrogen exchange during cellulose synthesis (hypothesis 2), we tracked the D enrichment in the D-labelled trees. The D abundances of watering water, soil water, leaf water, leaf glucose and tree-ring glucose are given in Table 1. The D enrichment decreased from watering water to leaf water; leaf glucose showed approximately the same enrichment as leaf water, while tree-ring glucose was again more D-enriched. This shows that the experiment had created the intended gradient of D abundance in the trees.

To identify the C-H groups that exchanged with xylem water during cellulose synthesis, we compared D NMR spectra of leaf glucose and tree-ring glucose isolated from D-labelled oak (Fig. 3a) and spruce (Fig. 3b). For both oak and spruce, every D isotopomer was more abundant in tree-ring glucose than in leaf glucose.

Tables 2 and 3 show the abundances of the D isotopomers in leaf glucose (a_{leaf}) and tree-ring glucose (a_{ring}) for both species. For leaf glucose, the abundance of all D isotopomers was close to the D abundance of leaf water. In contrast, the D isotopomer abundances of tree-ring glucose were very different, and varied between the D abundances of leaf water and soil water (Table 1). The 'relative exchange' columns give the differences ($a_{ring} - a_{leaf}$), scaled so that the largest difference is equal to 1.

Table 3 Abundances of Deuterium (D) isotopomers of tree-ring glucose (a_{ring}) and leaf glucose (a_{leaf}) of D-labelled spruce (*Picea abies*), and hydrogen exchange parameters

Spruce isotopomer	a_{ring}	a_{leaf}	Relative exchange	Percentage exchange
D1	3.44 ± 0.23	2.01 ± 0.19	0.97 ± 0.13 a	41 ± 7
D2	3.54 ± 0.22	2.06 ± 0.20	1.00 a	43 ± 6
D3	2.81 ± 0.20	1.82 ± 0.23	0.66 ± 0.09 c	27 ± 5
D4	3.27 ± 0.22	1.99 ± 0.21	0.87 ± 0.12 a	36 ± 6
D5	3.40 ± 0.22	2.09 ± 0.22	0.89 ± 0.11 a	38 ± 6
$D6^R$	2.21 ± 0.17	1.69 ± 0.17	0.35 ± 0.04 b	14 ± 3
$D6^S$	2.40 ± 0.16	1.76 ± 0.18	0.43 ± 0.05 b	17 ± 3

D isotopomer values are multiples of natural D abundance. Relative exchange values are calculated as $a_{ring} - a_{leaf}$, relative to the highest difference (D2); letters group isotopomers into statistically different classes ($P < 0.05$). Percentage exchange was calculated as $100(a_{ring} - a_{leaf})/(a_{water} - a_{leaf})$. Values are average ± SD ($n = 6$).

Relative exchange is a quantitative measure of exchange of the C-H groups. In oak, exchange of C(2)-H was more than twofold stronger compared with C(3,4,5)-H, and threefold stronger compared with C(1)-H, C(6)-H^R and C(6)-H^S. In spruce, exchange of C(1,2,4,5)-H was more than twofold stronger compared with C(6)-H^R and C(6)-H^S, while C(3)-H was intermediate.

To quantify percentage exchange, we related a_{leaf} and a_{ring} to the D abundance of xylem water. Xylem water could not be sampled during the whole growing season; therefore, the xylem water D abundance had to be approximated by the D abundance of soil water or watering water. However, the soil water D abundance measured was an underestimate, as evident from the higher abundance of the D2 isotopomer of oak tree-ring glucose compared with soil water (4.85 vs 4.01; Tables 1 and 2). This underestimate was probably caused by H : D exchange between soil and air moisture during sampling. Thus, we used the D abundance of watering water to calculate percentage exchange. For each D isotopomer, the increase in abundance in tree rings compared with leaf glucose is given by $a_{ring} - a_{leaf}$. The maximum possible increase is given by the difference in abundance between watering water and leaf glucose ($a_{water} - a_{leaf}$) (Fig. 3). The percentage hydrogen exchange is given by 100 ($a_{ring} - a_{leaf}$)/($a_{water} - a_{leaf}$). The percentage exchange for the seven C-H groups of tree-ring glucose in oak and spruce are given in the last column of Tables 2 and 3. The averages of these values, the exchange of the whole molecule, were 43 and 31% for oak and spruce, respectively.

Discussion

To use the D abundance of tree rings as a paleo-proxy, the mechanisms of H isotope transfer in plants must be understood. Deuterium labelling combined with D isotopomer measurements allowed us to test two hypotheses: (1) that DIDs of leaf metabolites are determined by enzyme isotope effects, and (2) that hydrogen exchange during cellulose synthesis affects specific C-H groups.

To test hypothesis 1, we compared the DIDs of control and D-labelled leaf glucose. Differences between DIDs of control and D-labelled glucose ranged up to 7% for oak (Fig. 2) and up to 11% for spruce (despite, in addition, the different light intensities in the glasshouse and growth chamber). These differences are small compared with the D enrichments of leaf water and the whole glucose molecule, both of which were of the order of 100% (Table 1). Thus, leaf water D enrichment influenced the D abundance of the whole glucose molecule strongly, reflecting the fact that leaf water is the source of all C-H groups of the photosynthate. In contrast, leaf water D enrichment had only a weak influence on the DID of leaf glucose. We conclude that DIDs of leaf glucose are largely independent of leaf water D abundance, but are determined mainly by enzyme isotope discrimination (mechanism 3). Therefore, changes in DIDs reflect changes in enzyme isotope discrimination, which may be caused by changing metabolic fluxes (Schleucher, 1998) or physiological adaptations to environmental changes.

The gradient in D abundance between watering water, leaf glucose and tree-ring glucose (Table 1) is the signal we used to calculate hydrogen exchange. The relative exchange and the percentage exchange data (Tables 2 and 3) show that there are C-H groups that exchange strongly and weakly, respectively. These data support hypothesis 2, that the C-H groups of tree-ring cellulose have different isotopic origins, and their D abundances should be interpreted individually. There are no previously published data on exchange of individual C-H groups of any species. Thus, we can only compare average exchange of the whole glucose moiety, which was 43% for oak and 31% for spruce. Previous studies have found values between 31 and 45% (Yakir & DeNiro, 1990; Terwilliger & DeNiro, 1995; Roden & Ehleringer, 2000), which agrees with our average exchange values. Furthermore, the high exchange of C(2)-H (79%) agrees with Sternberg *et al.* (2003), who observed that C(2)-O exchanges completely during cellulose synthesis, via a related mechanism. Thus, we are confident that our calculation gave realistic percentage exchange values, which can be interpreted in terms of biochemistry of cellulose synthesis, and can be used to derive paleo-proxies from D isotopomer abundances in tree rings.

Biochemical implications

During synthesis of cellulose, hexoses from translocated sucrose must be converted to sugar phosphates (Fig. 4). It is well known that several enzymes of sugar phosphate metabolism catalyse hydrogen isotope exchange, but not every substrate turnover event proceeds with exchange (Hanson & Rose, 1975). Thus, an observed percentage exchange of a C-H group

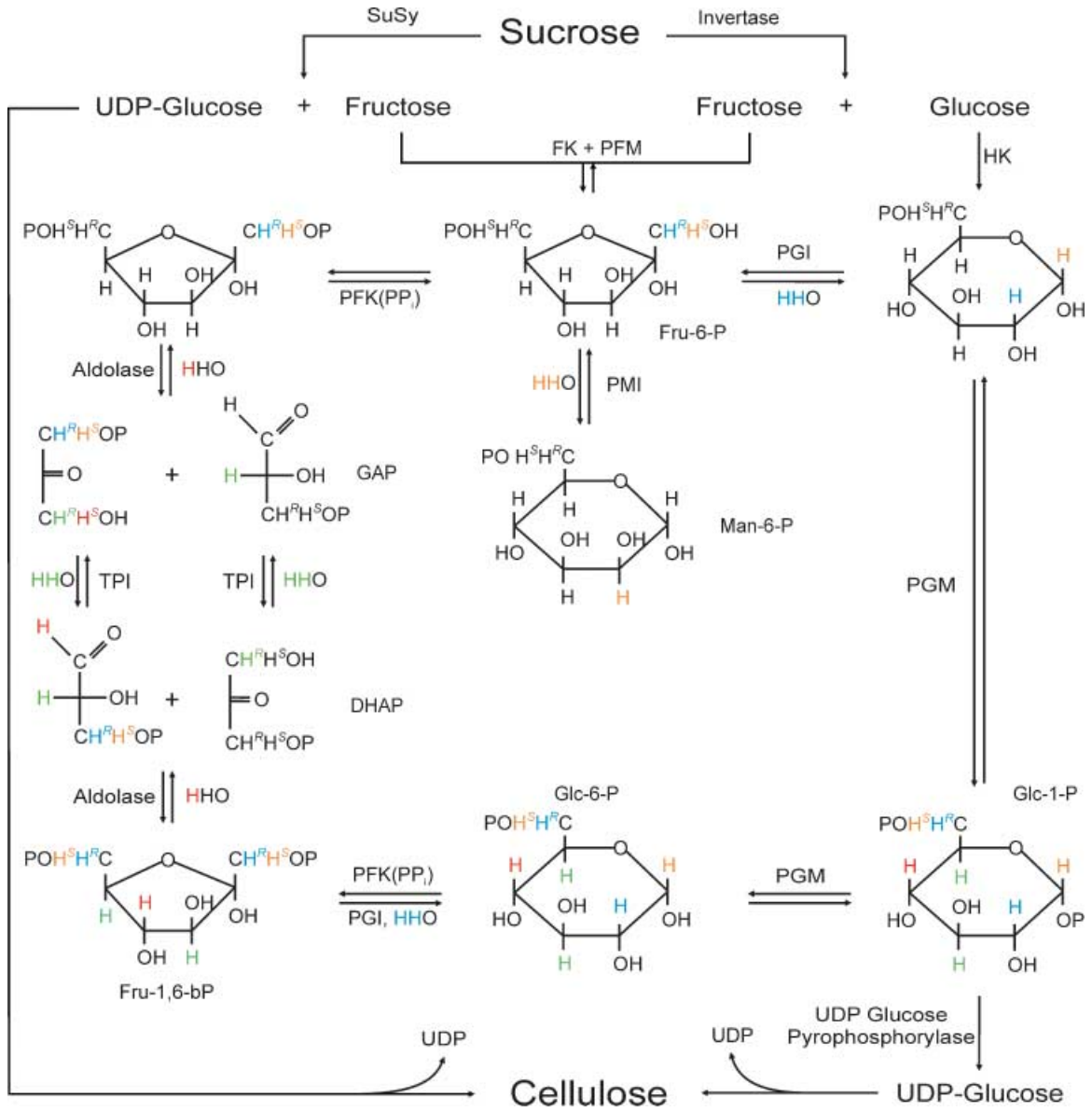

Fig. 4 Enzyme reactions of cellulose synthesis exchange specific C-H groups of glucose. Hydrogens exchanged by different enzymes are colour-coded. FK, fructokinase; HK, hexokinase; Man-6-P, mannose-6-phosphate; PFK(PPi), fructose-6-phosphate 1-phosphotransferase; PFM, phosphofructomutase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; PMI, phosphomannose isomerase; SuSy, sucrose synthase; TPI, triose phosphate isomerase.

indicates that at least this percentage of a metabolite went through a particular enzyme reaction. The exchange pattern in oak can be explained by the following metabolic fluxes. Exchange of C(2)-H of glucose is catalysed by phosphoglucose isomerase (PGI). If sucrose is split by sucrose synthase (SuSy), the UDP-glucose is protected from exchange. The 79% exchange of C(2)-H in oaks (Table 3) shows that, at most, 21% of hexoses did not undergo the PGI reaction. This implies that, at most, 42% of sucrose was split by SuSy, while at least 58% of sucrose must have been split by invertase. Studies of activities of extracted enzymes have deduced predominant SuSy activity or comparable activities of SuSy and invertase (Hauch & Magel, 1998; Magel *et al.*, 2001; Uggla *et al.*, 2001). While it is difficult to deduce *in vivo* metabolic fluxes from the activities of extracted enzymes, isotope exchange can estimate metabolic fluxes with negligible perturbance of the organism. The lower exchange of C(2)-H in spruce (43%, Table 3) suggests that invertase plays a less important role in spruce, compared with oak. C(3)-H, C(4)-H and C(5)-H of glucose are exchanged by triose phosphate isomerase (TPI) and aldolase if hexoses are broken down to triose phosphates and resynthesized (Fig. 4, cf. Schleucher *et al.*, 1998). This

cycling has been invoked before to explain oxygen isotope exchange during cellulose synthesis (Hill *et al.*, 1995; Farquhar *et al.*, 1998). The approx. 50% exchange of these hydrogens in oaks requires that nearly 50% of all hexoses cycled through triose phosphates. This agrees well with the triose phosphate cycling value of 43% found by Roden *et al.* (2000). The weaker exchange of C(3)-H, C(4)-H and C(5)-H in spruce suggests that triose cycling is less active in this species. Phosphomannose isomerase (PMI) and PGI exchange C(1)-H^S and C(1)-H^R of fructose-6-phosphate, which can be transferred to C(6)-H_2 by triose phosphate cycling. This explains the exchange of the C(6)-H_2 group. The hydrogen exchanged by PMI corresponds to C(1)-H of glucose; therefore the stronger exchange of C(1)-H in spruce, compared with oak, is consistent with higher PMI activity in this species, in agreement with the higher abundance of mannose in hemicelluloses of softwood species (Sjöström, 1993).

It is possible that enzyme isotope effects discriminate against D during hydrogen exchange. In this case, the observed percentage exchanges would be apparent values. For example, the 79% exchange of C(2)-H in oak might result from a 100% exchange scaled down by an isotope effect of 1.27. Such an isotope effect would also have reduced the abundance of the D2 isotopomer in the cellulose of unlabelled control plants. However, in control plants, there was no significant difference in the abundance of the D2 isotopomer between soluble sugar and cellulose for oak (3%, not significant) or spruce (1%, not significant). Therefore any isotope effect during hydrogen exchange of C(2)-H must be small.

Relevance for climate reconstruction from tree rings

The differences in D isotopomer abundances in oaks were large (30%, Fig. 2), which is in agreement with previous observations on annual plants (Schleucher *et al.*, 1999). In other words, DIDs of tree-ring cellulose are nonrandom. It follows that the whole-molecule D abundance of tree-ring cellulose (usually expressed as δD) must be treated as an average of the abundances of the seven D isotopomers of glucose. The highly unequal exchange of individual C-H groups (Tables 2 and 3) implies that the four mechanisms of D fractionation affect each D isotopomer individually. The abundance of D isotopomers of strongly exchanging C-H groups will be mostly influenced by δD of source water, while transpirative enrichment will strongly influence weakly exchanging C-H groups. Furthermore, it has been observed that DIDs of annual plants depend on environmental conditions (Schleucher, 1998). In other words, the DID of tree-ring cellulose must be expected to be nonconstant, and variation in tree ring DID is a potential source of information. This has important consequences for the interpretation of δD, and for reconstruction of paleo-proxies from tree-ring deuterium in general.

DID variation influences δD, but the underlying fractionations cannot be identified from δD. We argue that this explains why studies designed to model tree-ring δD based on environmental parameters (temperature, relative humidity) have reported conflicting results (Pendall, 2000; Roden *et al.*, 2000; Waterhouse *et al.*, 2002; McCarroll & Loader, 2004). In contrast, $\delta^{18}O$ of leaf sucrose (Barbour *et al.*, 2000) and tree-ring cellulose (Waterhouse *et al.*, 2002) could be modelled based on source water $\delta^{18}O$ and leaf water ^{18}O enrichment. A likely explanation of this is that D isotope effects of chemical reactions are much larger than physical D fractionations, whereas chemical and physical fractionations are of the same order of magnitude for other isotopes (Melander & Saunders, 1980).

While variation in DID hampers interpretation of δD, it means that individual D isotopomers can be used as separate information channels. Abundances of individual D isotopomers can be calculated from combined measurements of δD (by IRMS) and DIDs (by NMR). In an isotope balance, the D abundance of the whole molecule, derived from IRMS, is allocated to individual D isotopomers in proportion to the DID (T. R. Betson *et al.*, unpublished). Based on the identification of strongly and weakly exchanging C-H groups of tree-ring cellulose, we propose four strategies to reconstruct paleo-signals from abundances of individual D isotopomers.

(i) The D isotopomer *abundance* of strongly exchanging C-H groups will be essentially independent of all leaf-level processes, but will be set by source water D abundance during cellulose synthesis. These D isotopomer abundances should therefore carry signals – primarily temperature – which are present in source water. Concerning the temperature signal, leaf-level D fractionations represent noise; thus, we expect the temperature signal to be clearer in these D isotopomer abundances, as compared with the δD of tree-ring cellulose. Because the pattern of exchange (Tables 2 and 3) is more asymmetrical in oak than in spruce, oak should be better suited for this approach, in particular using the D2 isotopomer. Experiments are in progress to analyse 30- year tree-ring chronologies, to test how well the abundance of the D2 isotopomer correlates with source water δD. Applied to tree-ring series covering centuries, this may open a way for high-resolution temperature reconstructions, which are needed to gauge past climate variability.

(ii) C-H groups that exchange weakly, such as C(6)-H^R, C(6)-H^S, and C(1)-H in oaks, retain in tree rings the D abundances that were imprinted on them by leaf-level D fractionations. The corresponding isotopomer abundances can therefore store leaf-level signals in tree rings. For example, transpirative enrichment of leaf water depends on vapour pressure deficit, which can represent a humidity signal. This enrichment may be conserved in weakly exchanging isotopomers of tree rings, while it should be absent in strongly exchanging isotopomers. *Abundance differences* between nonexchanging and exchanging isotopomers may therefore allow reconstructing changes in growing season humidity.

(iii) *Abundance ratios* of weakly exchanging isotopomers are determined by isotope effects during leaf biochemistry. This follows from the observation that the dependence of the DID

of leaf glucose on the D abundance of leaf water is weak (Fig. 2), as predicted by hypothesis 1. Changes in *abundance ratios* of weakly exchanging isotopomers reflect changes in biochemical D discriminations, and carry signals on metabolic regulation. Changes in metabolism that may be detected include ratios between enzyme activity and metabolic flux, or ratios of metabolic fluxes through alternative pathways. For example, we observed that the ratio of the $D6^R$ and $D6^S$ isotopomers depends on the CO_2 concentration during growth (Schleucher, 1998, A. Augusti *et al.*, unpublished). This ratio may be used to study adaptations of trees to increasing atmospheric CO_2 concentration over the last centuries, or in response to the Pleistocene–Holocene transition.

(iv) DID and δD of cellulose can be interpreted simultaneously, using methods such as multivariate data analysis (principal components analysis and partial least-squares analysis). This approach does not require purely exchanging and non-exchanging isotopomers, but exchange percentages would be used as parameters. This approach should therefore be equally applicable to species which show asymmetrical (oak) or more symmetrical (spruce) exchange patterns. This approach appears to be very promising for the simultaneous reconstruction of several signals, such as temperature, humidity and metabolic flux ratios.

In conclusion, exchange patterns contain information on metabolism, which can be obtained in a noninvasive way (using only D labelling). Isotope exchange experiments can be useful for monitoring changes in metabolism, for example seasonal changes or changes induced by plant breeding. The nonrandom DIDs observed for oak and spruce explain why the δD of tree rings could not be interpreted reliably to date. Taking exchange patterns into account and interpreting abundances of D isotopomers individually will allow isolation of several different signals from tree rings. Recent advances in NMR technology reduce analysis times, enabling analysis of hundreds of samples (Kovacs *et al.*, 2005), and therefore of tree-ring series covering centuries. How best to extract correlated signals from DIDs must be established in ecophysiological experiments on trees, and in tree-ring studies. One particularly interesting prospect is to reconstruct climate and physiological signals in parallel, and to study interactions between climate and plant physiology on timescales of centuries or millennia.

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References

Barbour MM, Schurr U, Henry BK, Wong SC, Farquhar GD. 2000. Variation in the oxygen isotope ratio of phloem sap sucrose from castor bean. Evidence in support of the Péclet effect. *Plant Physiology* **123**: 671–679.

Briffa KR, Osborn TJ, Schweingruber FH. 2004. Large-scale temperature inferences from tree rings: a review. *Global and Planetary Change* **40**: 11–26.

Craig H, Gordon LI. 1965. Deuterium and oxygen-18 variations in the ocean and the marine atmosphere. In: Tongiorgi E, ed. *Proceedings of a Conference on Stable Isotopes in Oceanographic Studies and Paleotemperatures*. Spoleto, Italy: Lischi & Figli Publishers, 9–130.

Dansgaard W. 1964. Stable isotopes in precipitation. *Tellus* **16**: 436–468.

Ehleringer JR, Dawson TE. 1992. Water-uptake by plants – perspectives from stable isotope composition. *Plant, Cell & Environment* **15**: 1073–1082.

Ehleringer JR, Osmond CB. 1989. Stable isotopes. In: Pearcy RW, Ehleringer JR, Mooney HA, Rundel PW, eds. *Plant physiological ecology. Field methods and instrumentation*. London, UK: Chapman & Hall, 281–300.

Estep MF, Hoering TC. 1981. Stable hydrogen fractionations during autotrophic and mixotrophic growth of microalgae. *Plant Physiology* **67**: 474–477.

Farquhar GD, Barbour MM, Henry BK. 1998. Interpretation of oxygen isotope composition of leaf material. In: Griffiths H, ed. *Stable isotopes – integration of biology, ecological and geochemical processes*. Oxford, UK: Bios Scientific Publishers, 27–62.

Hanson KR, Rose IA. 1975. Interpretations of enzyme reaction stereospecificity. *Accounts of Chemical Research* **8**: 1–10.

Hauch S, Magel E. 1998. Extractable activities and protein content of sucrose-phosphate synthase, sucrose synthase and neutral invertase in trunk tissues of *Robinia pseudoacacia* L. are related to cambial wood production and heartwood formation. *Planta* **207**: 266–274.

Hill SA, Waterhouse JS, Field EM, Switsur VR, ap Rees T. 1995. Rapid recycling of triose phosphates in oak stem tissue. *Plant, Cell & Environment* **18**: 931–936.

Hurry V, Strand Å, Furbank R, Stitt M. 2000. The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the *pho* mutants of *Arabidopsis thaliana*. *Plant Journal* **24**: 383–396.

Knorr W, Prentice IC, House JI, Holland EA. 2005. Long-term sensitivity of soil carbon turnover to warming. *Nature* **433**: 298–301.

Kovacs H, Moskaua D, Spraul M. 2005. Cryogenically cooled probes – a leap in NMR technology. *Progress in Nuclear Magnetic Resonance Spectroscopy* **46**: 131–155.

Long SP, Ainsworth EA, Rogers A, Ort DR. 2004. Rising atmospheric carbon dioxide: Plants FACE the Future. *Annual Review of Plant Biology* **55**: 591–628.

Luo Y-H, Sternberg L. 1992. Hydrogen and oxygen isotopic fractionation during heterotrophic cellulose synthesis. *Journal of Experimental Botany* **43**: 47–50.

Magel E, Abel-Latif A, Hampp R. 2001. Non-structural carbohydrates and catalytic activities of sucrose metabolizing enzymes in trunks of two *Juglans* species and their role in heartwood formation. *Holzforschung* **55**: 135–145.

Martin GJ, Martin ML, Zhang B-L. 1992. Site-specific natural isotope fractionation of hydrogen in plant products studied by nuclear magnetic resonance. *Plant, Cell & Environment* **15**: 1037–1050.

McCarroll D, Loader NJ. 2004. Stable isotopes in tree rings. *Quaternary Science Reviews* **23**: 771–801.

Melander L, Saunders WH. 1980. *Reaction Rates of Isotopic Molecules*. New York, USA: Wiley-Interscience, Hoboken.

Nowak RS, Ellsworth DS, Smith SD. 2004. Functional responses of plants to elevated atmospheric CO_2 – Do photosynthetic and productivity data from FACE experiments support early predictions? *New Phytologist* **162**: 253–280.

Pendall E. 2000. Influence of precipitation seasonality on piñon pine cellulose δD values. *Global Change Biology* **6**: 287–301.

Roden JS, Ehleringer JR. 1999. Hydrogen and oxygen isotope ratios of tree-ring cellulose for riparian trees grown long-term under hydroponically controlled environments. *Oecologia* **121**: 467–477.

Roden JS, Ehleringer JR. 2000. Hydrogen and oxygen isotope ratios of tree-ring cellulose for field grown riparian trees. *Oecologia* **123**: 481–489.

Roden JS, Lin G, Ehleringer JR. 2000. A mechanistic model for interpretation of hydrogen and oxygen ratios in tree-ring of cellulose. *Geochimica et Cosmochimica Acta* **64**: 21–35.

Saeman JF, Bubl JL, Harris EE. 1945. Quantitative saccharification of wood and cellulose. *Industrial and Engineering Chemistry* **17**: 35–37.

Schleucher J. 1998. Intramolecular deuterium distributions and plant growth conditions. In: Griffiths H, ed. *Stable isotopes – integration of biology, ecological and geochemical processes*. Oxford, UK: Bios Scientific Publishers, 63–73.

Schleucher J, Vanderveer P, Markley JL, Sharkey TD. 1999. Intramolecular deuterium distributions reveal disequilibrium of chloroplast phosphoglucose isomerase. *Plant, Cell & Environment* **22**: 525–534.

Schleucher J, Vanderveer P, Sharkey TD. 1998. Export of carbon from chloroplasts at night. *Plant Physiology* **118**: 1439–1445.

Schmidt HL. 2003. Fundamentals and systematics of the non-statistical distributions of isotopes in natural compounds. *Naturwissenschaften* **90**: 537–552.

Schweingruber FH. 1996. *Tree Rings and Environmental Dendroecology*. Berne, Switzerland: Haupt Verlag AG.

Sjöström E. 1993. Wood polysaccharides. In: *Wood chemistry, fundamentals and applications*. San Diego, USA: Academic Press, 51–70.

Sternberg LD, Anderson WT, Morrison K. 2003. Separating soil and leaf water O-18 isotopic signals in plant stem cellulose. *Geochimica et Cosmochimica Acta* **67**: 2561–2566.

Terwilliger VJ, DeNiro MJ. 1995. Hydrogen isotope fractionation in wood-producing avocado seedlings: biological constraints to paleoclimatic interpretations of delta D values in tree ring cellulose. *Geochimica et Cosmochimica Acta* **59**: 5199–5207.

Uggla C, Magel E, Moritz T, Sundberg B. 2001. Function and dynamics of auxin and carbohydrates during earlywood/latewood transition in Scots Pine. *Plant Physiology* **125**: 2029–2039.

Waterhouse JS, Switsur VR, Barker AC, Carter AH, Robertson I. 2002. Oxygen and hydrogen isotope ratios in tree rings: how well do models predict observed values? *Earth and Planetary Science Letters* **201**: 421–430.

Wershaw RL, Friedman I, Heller SJ, Frank PA. 1966. Hydrogen isotope fractionation in water passing through trees. In: Hobson GD, ed. *Advances in organic geochemistry. Proceedings of the Third International Congress*. New York, USA: Pergamon, 55–67.

White JWC, Cook ER, Lawrence JR, Broecker WS. 1985. The D/H ratios of sap in trees: Implications for water sources and tree ring D/H ratios. *Geochimica et Cosmochimica Acta* **49**: 237–246.

Yakir D, DeNiro MJ. 1990. Oxygen and hydrogen isotope fractionation during cellulose metabolism in *Lemna gibba* L. *Plant Physiology* **93**: 325–332.