RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 2499–2506
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4068

RCM

Kinetic $^{12}C/^{13}C$ isotope fractionation by invertase: evidence for a small *in vitro* isotope effect and comparison of two techniques for the isotopic analysis of carbohydrates†

Caroline Mauve[1], Jean Bleton[2], Camille Bathellier[3], Caroline Lelarge-Trouverie[1], Florence Guérard[1], Jaleh Ghashghaie[3], Alain Tchapla[2] and Guillaume Tcherkez[1]*

[1]Plateforme Métabolisme Métabolome, IFR87 La Plante et son Environnement, Institut de Biotechnologie des Plantes, Université Paris-Sud XI, 91405 Orsay cedex, France
[2]LETIAM, Institut Universitaire de Technologie, BP127, Université Paris-Sud XI, 91405 Orsay cedex, France
[3]Ecologie, Systematique & Evolution, Université Paris-Sud XI, UMR 8079, 91405 Orsay cedex, France

Received 12 January 2009; Revised 20 March 2009; Accepted 1 April 2009

The natural $^{13}C/^{12}C$ isotope composition ($\delta^{13}C$) of plants and organic compounds within plant organs is a powerful tool to understand carbon allocation patterns and the regulation of photosynthetic or respiratory metabolism. However, many enzymatic fractionations are currently unknown, thus impeding our understanding of carbon trafficking pathways within plant cells. One of them is the $^{12}C/^{13}C$ isotope effect associated with invertases (EC 3.2.1.26) that are cornerstone enzymes for Suc metabolism and translocation in plants. Another conundrum of isotopic plant biology is the need to measure accurately the specific $\delta^{13}C$ of individual carbohydrates. Here, we examined two complementary methods for measuring the $\delta^{13}C$ value of sucrose, glucose and fructose, that is, off-line high-performance liquid chromatography (HPLC) purification followed by elemental analysis and isotope ratio mass spectrometry (EA-IRMS) analysis, and gas chromatography-combustion (GC-C)-IRMS. We also used these methods to determine the *in vitro* $^{12}C/^{13}C$ isotope effect associated with the yeast invertase. Our results show that, although providing more variable values than HPLC∼EA-IRMS, and being sensitive to derivatization conditions, the GC-C-IRMS method gives reliable results. When applied to the invertase reaction, both methods indicate that the $^{12}C/^{13}C$ isotope effect is rather small and it is not affected by the use of heavy water (D_2O). Copyright © 2009 John Wiley & Sons, Ltd.

The carbon isotope composition of plant organic material is a well-recognized tool to elucidate the physiology of photosynthesis and plant cell metabolism. During photosynthetic CO_2 assimilation, there is a $^{12}C/^{13}C$ isotope fractionation, thereby depleting photosynthetic products in ^{13}C compared with atmospheric CO_2 (for a review, see Farquhar *et al.*[1]). Typically, the natural carbon isotope composition ($\delta^{13}C$) of sugars (sucrose or glucose) in C_3 plants falls around −28‰ while that of CO_2 is of around −8‰, so that the average fractionation value is around 20‰. Such a $^{12}C/^{13}C$ fractionation mainly originates from the kinetic isotope effect of the carboxylating enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase[2] (Rubisco, EC 4.1.1.39) and that of CO_2 diffusion from the atmosphere to carboxylation sites. However, other isotopic fractionations that may occur downstream during carbon metabolism can change the isotopic signal of the photosynthetically-fixed carbon. For example, it is known that some isotope effects associated with glycolytic enzymes do occur (for a review, see Tcherkez *et al.*[3]). In addition, it has been shown that the intramolecular isotope distribution in glucose (Glc) is not statistical[4] and this has pervading consequences for metabolites that originate from Glc metabolism.[5] However, the carbon isotope effects of enzymes associated with starch and sucrose (Suc) metabolism are not documented, thus impeding our understanding of carbon-trafficking pathways in plant cells.

Invertases (β-fructofuranosidase, EC 3.2.1.26) catalyze the hydrolytic cleavage of Suc into α-Glc and β-fructose (β-Fru) and so are cornerstone enzymes for Suc metabolism and translocation in plants. They are involved in many metabolic processes within plants, such as carbohydrate export from leaves, storage in tubers and roots, and physiological responses to environmental conditions like water stress.[6] However, the $^{12}C/^{13}C$ isotope effect associated with invertases is currently unknown. If an isotope effect were to occur, a systematic ^{13}C-depletion of Glc and/or Fru

*Correspondence to: G. Tcherkez, Plateforme Métabolisme Métabolome, Batiment 630, Université Paris-Sud 11, 91405 Orsay cedex, France.
E-mail: guillaume.tcherkez@u-psud.fr
†Presented at the 2nd Joint European Stable Isotope User Meeting (JESIUM), Presqu'île de Giens, France, 31 August–5 September, 2008.
Contract/grant sponsor: Institut Fédératif de Recherche 87.

produced from Suc hydrolysis might be expected, and this would typically contribute to the depletion of ^{13}C storage molecules (e.g. starch) or respired CO_2 formed from imported Suc in sink organs.

It is currently accepted that invertase catalysis involves a double-displacement (ping-pong) mechanism[7] in which the Fru moiety of Suc gives an oxonium ion (i.e., with a triple-bonded O^+) after the acidic attack of the Glc moiety. In baker's yeast (*Saccharomyces cerevisiae*), the catalytic residues have been identified as aspartic acid (D23) acting as a nucleophile,[8] and glutamic acid (E204) which serves as the general catalytic acid/base[9] (step 1, Fig. 1). The reaction does not lead to a stereochemical inversion of Fru configuration while it has been recognized that a transitory inversion may occur[8–10] (step 2, Fig. 1). All the steps of the reaction involve C−O bonds and so may possibly cause intrinsic carbon isotope effects. The amplitude of the overall reaction isotope effect depends on the value of such intrinsic isotope effects and the kinetic limitations imposed by the mechanism (limiting steps). Typically, if the isotope-sensitive steps are not limiting at all, the observed isotope effect will be very small compared with the intrinsic isotope effects.[11]

In the study presented in this paper, we investigated both the kinetic $^{12}C/^{13}C$ and the solvent H_2O/D_2O isotope effects associated with Suc consumption by the yeast invertase. The carbon isotope composition ($\delta^{13}C$) of Suc, Glc and Fru was measured by either gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) or off-line liquid chromatography followed by elemental analysis-mass spectrometry (thereafter abbreviated HPLC∼EA-IRMS). Although off-line and time-consuming, the latter technique is commonly used in plant isotopic biology as there remain several uncertainties about the application of (on-line) GC-C-IRMS for carbohydrate analysis. Carbohydrates are not volatile and require chemical derivatizations that may adulterate their back-calculated, natural $\delta^{13}C$ values. For example, trifluoroacetate or alditol acetate derivatizations are not quantitative and are accompanied by a kinetic isotope fractionation, compromising accurate isotopic analyses.[12,13] Although obscuring somewhat the $\delta^{13}C$ value of original compounds because of the large carbon load brought by trimethylsilylation, we used here such a derivatization that allows a quantitative sample conversion through a single step.[13] Our results show that, although providing more variable values, the GC-C-IRMS method gives reliable results for Suc while being sensitive to derivatization conditions (Glc, Fru). When applied to the invertase reaction, both methods indicate that the ^{12}C-Suc/^{13}C-Suc isotope effect is near 1.0011 with respect to total Suc carbon, and is not affected by the use of rate-limiting heavy water (D_2O).

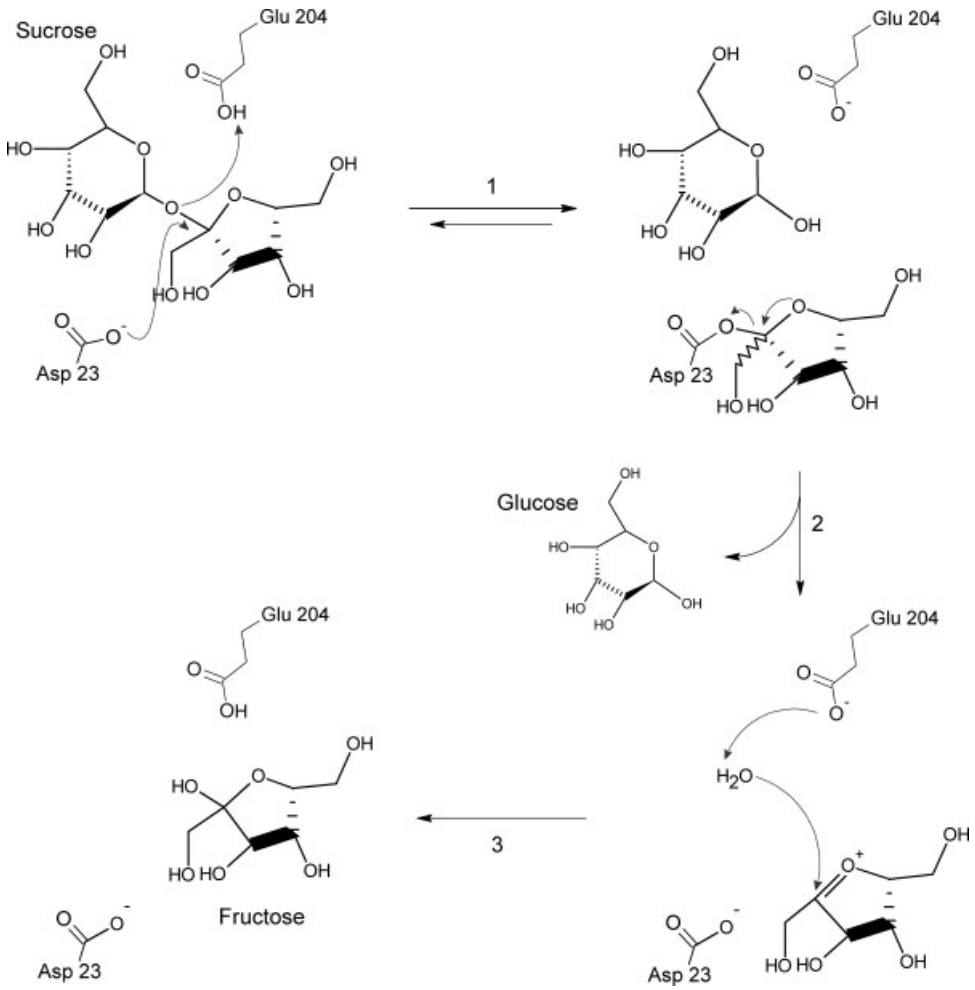

Figure 1. The reaction mechanism of the yeast invertase.

DOI: 10.1002/rcm

EXPERIMENTAL

HPLC analysis of carbohydrates

The HPLC separation was carried out off-line and used to (a) separate and purify sugars from samples of invertase assays or plant extracts (preparative chromatography) for isotopic analysis (with EA-IRMS) and (b) to determine the amount of sucrose during invertase assays (analytical chromatography). The HPLC procedure was that of Duranceau *et al.*,[14] which was shown not to be associated with any carbon isotope fractionation.

Lyophilized samples from invertase assays were dissolved in 1 mL of distilled water. The resulting solution (or aqueous filtered and deproteinated leaf extracts, see below, *Plant material*) was injected on the HPLC system (803C-302, Gilson, Roissy-en-France, France). Suc, Glc and Fru were separated using the procedure described in the literature.[14,15] Briefly, separation was made with a cation-exchange column (SugarPack 6.5×300 mm, Waters, Guyancourt, France) at 90°C, with isocratic degassed water as a mobile phase (flow rate 0.5 mL min^{-1}). Detection was performed using a Gilson-133 refractometer. All samples were injected using the autosampler (analytical quantification) or manually (preparative analyses in which carbohydrates were manually collected). The collected sugars were then dried and weighed in tin capsules for IRMS analyses.

Derivatization for GC-C-IRMS analysis

The trimethylsilyl (TMS) derivatization was carried out by adding hexamethyldisilazane (HMDS) in trimethylchlorosilane (TMCS) and pyridine (Trisil reagent, Pierce-Perbio Science, Brebiere, France) (3:1:9, v/v/v). The lyophilized samples were trimethylsilylated in 200 μL of Trisil in sealed vials. Following vigorous mixing, the vials were incubated for 2 h at 80°C in an oil bath. For the equilibration step, the samples were first dissolved in 100 μL of pyridine for 24 h at room temperature. Before GC injection, 5 μL of eicosane were added as an internal standard (50 mM in hexane).

Isotopic measurements

Isotopic measurements were carried out by the isotopic facilities structure Plateforme Métabolisme-Métabolome of the Institut Fédératif de Recherche IFR87, Orsay, France. Pure crystalline compounds (reference Suc, α-Glc, β-Fru, Fluka Analytical, Saint Quentin Fallavier, France) or compounds purified from samples through HPLC were analyzed using EA-IRMS (Carlo-Erba 1500-Optima, GV Instruments-Elementar, Villeurbanne, France). GC-C-IRMS analyses were carried out with an Agilent 6890N GC system (Agilent Technologies, Massy, France) coupled to a combustion module (Cal 9900, GV Instruments) and an Isoprime isotope ratio mass spectrometer (GV Instruments). A cold water-trap (GV Instruments) was used to remove water generated by the combustion within the furnace tube. GC-C-IRMS analyses were carried out by injecting 1 μL in split mode (split ratio of 1:10) at 280°C (injector temperature). TMS-derivatized sugars were separated on an OV-1 column (25 m, 0.25 mm i.d., 25 μm, Agilent Technologies) with helium as a carrier gas in the constant flow mode. The temperature steps were as follows: 160°C (maintained for 1 min), then 180°C (1°C min^{-1}), then 300°C (10°C min^{-1}) and a plateau at 300°C (2 min). The total run time per injection was 35 min.

Isotopic corrections and calculations

All the $\delta^{13}C$ values are given with respect to V-PDB. Any isotopic offset of the EA-IRMS instrument was corrected with a standard of known $\delta^{13}C$ value (glutamic acid, −28.20‰). The $\delta^{13}C$ values obtained by GC-C-IRMS were corrected against eicosane (C_{20}-alcane, −33.87‰ with EA-IRMS) added to all samples (internal standard, see also above). The $\delta^{13}C$ value of the standard material was, in addition, checked against those of the supplier (standard vg-mix, GV Instruments). The $\delta^{13}C$ of the added carbons (i.e., of the TMS groups, thereafter denoted as δ_{TMS}) was determined with reference samples in which β-Fru, α-Glc or Suc of known $\delta^{13}C$ was derivatized. δ_{TMS} was then calculated using the mass-balance equation:

$$\delta_{dc} = x\delta_{TMS} + (1 - x)\delta_c \quad (1)$$

where δ_{dc} and δ_c are the carbon isotope composition of derivatized and pure (non-derivatized) reference compound, respectively, and x is the proportion of added carbons in the derivatized compound. Such a calibration method (that uses known sugars to infer δ_{TMS} values) allowed us (a) to include any variations in δ_{TMS} between sugar compounds, that is, took into account any possible fractionations during derivatization; nevertheless, such fractionations are not likely as the TMS derivatization is believed to be quantitative (100% yield); and (b) to minimize the effect of possible variations in x from the expected value, simply because the same x value was used with both reference and sample compounds. In other words, the $\delta^{13}C$ of sample sugars depended only weakly on the chosen x value.

For the samples, we used the following equation:

$$\delta_{ds} = x\delta_{TMS} + (1 - x)\delta_s \quad (2)$$

where δ_{ds} and δ_s are the carbon isotope composition of derivatized and natural (non-derivatized) sample sugar. Susbstituting Eqn. (1) into Eqn. (2) gives:

$$\delta_s = \delta_c + \frac{\delta_{ds} - \delta_{dc}}{1 - x} \quad (3)$$

The partial derivative of δ_s with respect to any elemental variation in x is:

$$\frac{\partial\delta_s}{\partial x} = \frac{\delta_{ds} - \delta_{dc}}{(1 - x)^2} \quad (4)$$

On the other hand, if δ_{TMS} were fixed (and, e.g., measured independently by EA-IRMS), we would have:

$$\frac{\partial\delta_s}{\partial x} = \frac{\delta_{ds} - \delta_{TMS}}{(1 - x)^2} \quad (5)$$

In Eqn. (4), the numerator is smaller, typically 3‰, while it is near 11‰ in Eqn. (5). In other words, possible errors in the x value would have a lower effect on the value of δ_s with Eqn. (4).

DOI: 10.1002/rcm

δ_{TMS} obtained with standards was checked to be constant over a range of three Glc, Fru and Suc concentrations: 5, 10 and 25 mmol L^{-1}. For each concentration, three repetitions were carried out so that the δ_{TMS} used for the calculations was the average of nine values.

Enzyme assay and isotope effect

Yeast invertase was purchased from Roche (Meylan, France). It was dissolved in a buffer (5 mg mL^{-1} in 0.2 mol L^{-1} phosphate buffer, pH 4.6) and 53 μL of this solution were added to 2 mL of assay solution (Suc solution in either H_2O or D_2O at 10 mg mL^{-1}, i.e., 30 mmol L^{-1}). Assays were carried out at 30°C, pH (pD) 6.5. At different sampling times (5, 10, 15 min, etc.), 100 μL aliquots were collected; the reaction was stopped with $AgNO_3$ (100 μL at 10 mmol L^{-1}) followed by instant-freezing at −196°C (liquid N_2). The $^{12}C/^{13}C$ isotope effect (denoted as α) was measured with the classical Rayleigh fractionation equation[11] as follows:

$$\alpha = \frac{\ln(1-f)}{\ln(1-f) + \ln(R_t/R_0)} \quad (6)$$

where f is the fraction of consumed Suc and R_t the $^{13}C/^{12}C$ ratio of Suc at sampling time t. R_0 is the initial $^{13}C/^{12}C$ ratio ($t = 0$) of Suc.

Plant material

French bean plants were those used in the work described in Bathellier *et al.*[15] Leaves were collected at different times under continuous darkness. The reader is referred to this paper for further details on growth conditions. Briefly, plants were grown in a greenhouse with a 16 h photoperiod and a minimum photosynthetic photon flux density maintained at approximately 500 μmol m^{-2} s^{-1} by supplementary lighting from a high-pressure sodium light. The temperature and leaf-to-air vapour pressure deficit were maintained at approximately 25.5/18.5°C and 1.4/1.2 kPa day/night, respectively. Watering was carried out with a commercial nutrient solution (Hydrokani C2, Hydro, Neuilly-Sur-Seine, France) twice during the photoperiod. The carbon isotope composition of the CO_2 in the greenhouse was -9.85 ± 0.3‰. Leaves were collected at various times in the dark (continuous darkness) and immediately frozen in liquid nitrogen, lyophilized and ground. Aqueous extracts were obtained with 20 mg leaf powder and 2 mL distilled H_2O by stirring at 0°C, as already described.[15] After centrifugation, the supernatant was filtered and 200 μL aliquots were sampled and injected (HPLC). The remaining extract was freeze-dried under vacuum for derivatization (GC-C-IRMS analysis).

RESULTS

Comparison of analytical methods (GC-C-IRMS and HPLC∼EA-IRMS)

Figure 2 shows the $\delta^{13}C$ values of Suc and Fru during the time course of the invertase reaction (in both H_2O and D_2O), obtained through both analytical methods. Clearly, there is a

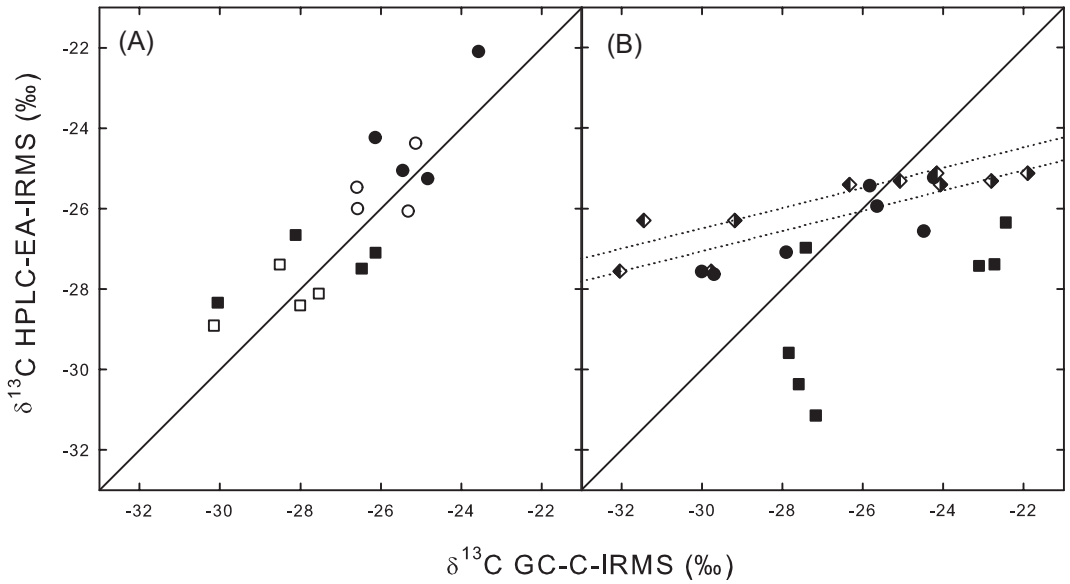

Figure 2. Comparison of GC-C-IRMS and HPLC∼EA-IRMS methods for measuring the isotope composition ($\delta^{13}C$) value of carbohydrates. The continuous line represents the 1:1 relationship. (A) Fru (squares) and Suc (discs) isotope composition during the time course of the invertase reaction (sampling time 5, 10, 15 and 20 min) operating in either H_2O (closed symbols) or D_2O (open symbols). Each individual datum is the mean of two assay replicates. During the derivatization procedure for GC-C-IRMS, an equilibration step (Glc anomerization in pyridine) was included. (B) Glc (diamonds), Fru (squares) and Suc (circles) isotope composition of *Phaseolus* leaf extracts. When making the isotopic correction (due to the derivatization), a $\delta^{13}C$ value of the exogenous carbon of either −46.0‰ (right-filled symbols) or −47.0‰ (left-filled symbols) was used. During the derivatization procedure for GC-C-IRMS, no equilibration step was included. Dotted lines: trends of the relationship for Glc.

DOI: 10.1002/rcm

consistent agreement between both methods, all the data giving values around 1:1. There is, nevertheless, some scatter of the $\delta^{13}C$ values, with the GC-C-IRMS values being slightly ^{13}C-depleted. Such an uncertainty in the GC-C-IRMS-derived values in this δ-range is possibly caused by the low $\delta^{13}C$ values of the exogenous carbons (added by derivatization, typically around −43‰) thereby causing a larger variability when the natural $\delta^{13}C$ values of sugars are back-calculated (the natural $\delta^{13}C$ value of sugars is near −25 to −30‰).

The $\delta^{13}C$ values of Glc are not indicated in Fig. 2(A) as the chemical derivatization process may cause large variations. In fact, the GC of derivatized α-Glc or Fru shows an additional peak, mainly composed of the TMS derivative of the β-stereoisomer of Glc (at equilibrium, its relative abundance is around 60% of the total Glc) and the open form of Fru (relative abundance of around 6% of total Fru) (see Fig. 3). While the open form of Fru may modestly impact on the Fru isotopic signal (small amount; but see below), any slight variation of the α/β anomeric equilibrium during derivatization is likely to change the Glc isotopic signal dramatically. Figure 2(B) shows the $\delta^{13}C$ values of carbohydrates from bean (*Phaseolus vulgaris*) extracts, for which no Glc-anomeric equilibration step was included during sample preparation. It is clear that while the Suc values approach the 1:1 line, there is a subtantial deviation for Glc values obtained through GC-C-IRMS (right-filled diamonds and lower dotted line). Such a poor relationship does not depend on possible errors in the δ-value used for exogenous carbons (TMS groups): shifting such a value by, e.g., −1‰ does not improve the $\delta^{13}C$ of Glc (left-filled diamonds). Figure 4 shows the variations of the $\delta^{13}C$ value in Glc and Fru, obtained with GC-C-IRMS analysis with or without equilibration (during derivatization), compared with those obtained with HPLC∼EA-IRMS. Clearly, the $\delta^{13}C$ values obtained with GC-C-IRMS are very sensitive to preparative conditions: the Glc and Fru values are far from the 1:1 line without the equilibration step (closed symbols). We therefore conclude that reliable results cannot be obtained without rigorous control of the anomeric equilibrium, thereby increasing the uncertainty in the $\delta^{13}C$ values. Because of such difficulties, only the $\delta^{13}C$ value of Suc is used in the following to calculate the isotope effects associated with invertase.

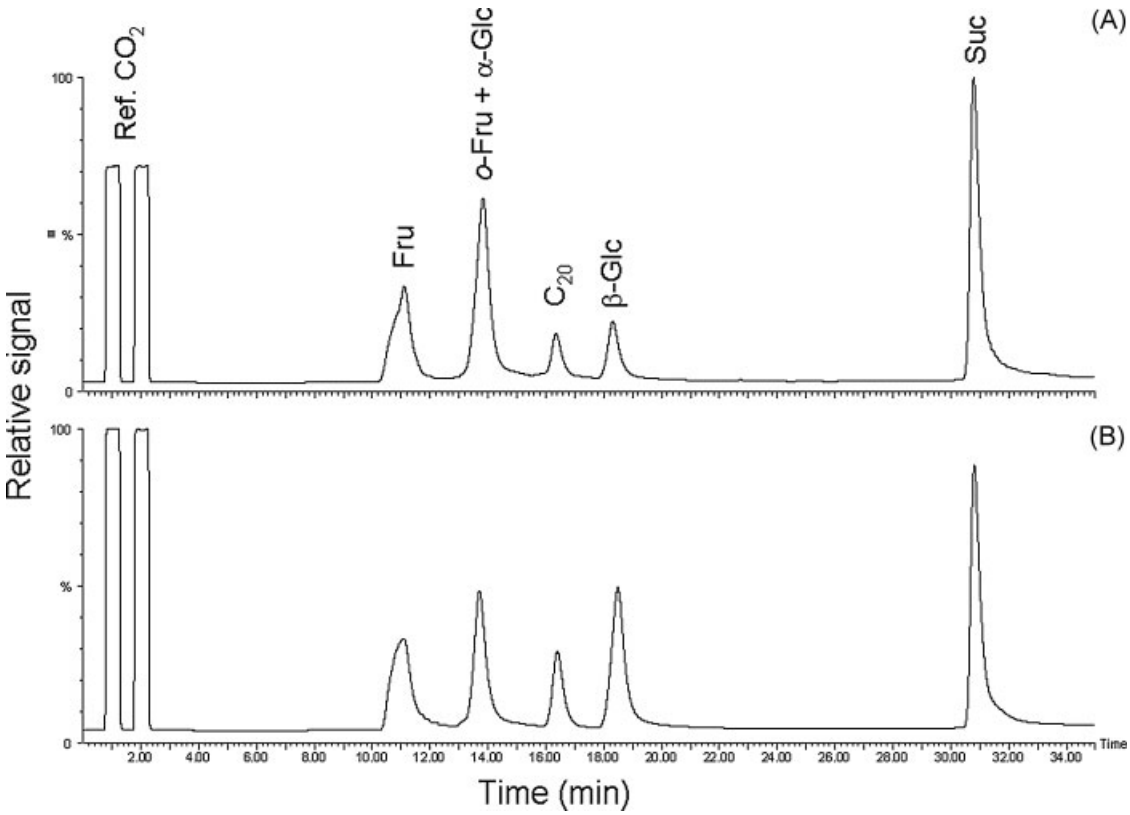

Figure 3. Typical chromatograms associated with GC-C-IRMS analysis of invertase assay samples, without (A) or with (B) the Glc equilibration step (anomeric equilibrium of Glc isomeres with pyridine). Carbohydrates were derivatized by trimethylsilylation (see Experimental section for further details). The relative signal (y-axis) is the mass-44 current (in %). Eicosane (C_{20}) is used as an internal standard. *o*-Fru, open form of Fru. On the left-hand side are the reference CO_2 peaks. The height of sugar peaks (such as Suc) is dissimilar here in (A) and (B) (it is apparent compared with reference CO_2 peaks) because of the different sugars concentrations within the samples. The identification of each peak as shown here was carried out and checked through GC-time-of-flight mass spectrometry before injection into the GC-C-IRMS system (mass data not shown).

DOI: 10.1002/rcm

RCM

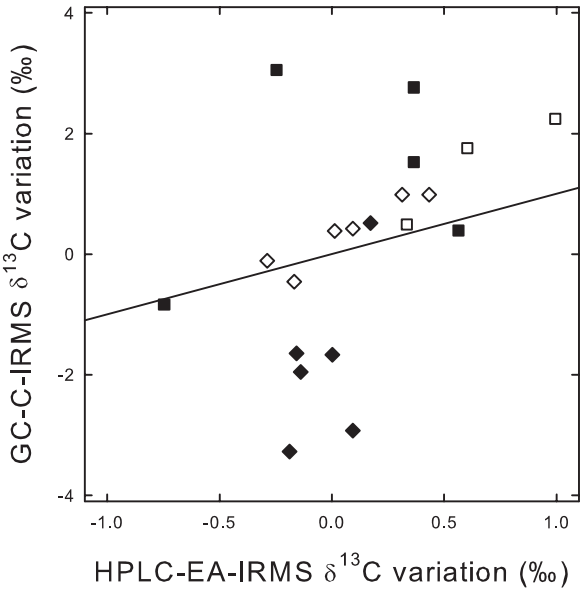

Figure 4. Comparison of the variation (in ‰) in δ^{13}C of Glc (diamonds) and Fru (squares) around a common average value, when samples are simultaneously measured with HPLC∼EA-IRMS and GC-C-IRMS. For the latter technique, an isomeric equilibration is carried out before derivatization (open symbols) or is not (closed symbols). Continuous line, 1:1 relationship.

The $^{12}C/^{13}C$ isotope effect associated with invertase

It is apparent from Fig. 2(A) that Fru produced from Suc hydrolysis by invertase is ^{13}C-depleted compared with Suc. In other words, there is an isotope effect against ^{13}C during invertase catalysis. In order to quantify such an isotope effect, the isotope composition (both analytical methods) and the concentration (obtained through HPLC) of Suc were measured during the reaction and the results are shown in Fig. 5. In both natural and heavy water, the isotope composition of Suc increases as the reaction proceeds, indicating again that the reaction discriminates against ^{13}C. Such a discrimination is nevertheless weak, Suc being enriched by 4‰ only at a consumption fraction as high as 95% (10 min in natural water). The values of the V/K kinetic isotope effect (calculated with Rayleigh's equation, see Experimental section) are indicated in Table 1. On average, the isotope effect is 1.0011. It is worth noting that while absolute δ-values are different between the GC-C-IRMS and HPLC∼EA-IRMS methods (there is an offset of nearly −0.5‰ in the GC-C-IRMS values, Fig. 5), the isotope effect is quite similar whatever the analytical method (Table 1).

The H_2O/D_2O solvent isotope effect

The invertase reaction is slowed down by heavy water (Fig. 5) so that the solvent isotope effect on the initial enzymatic rate is near 1.6. D_2O has no influence on the $^{12}C/^{13}C$ isotope effect: the average value of the V/K isotope effect is 1.0011 in H_2O and 1.0009 in D_2O (Table 1).

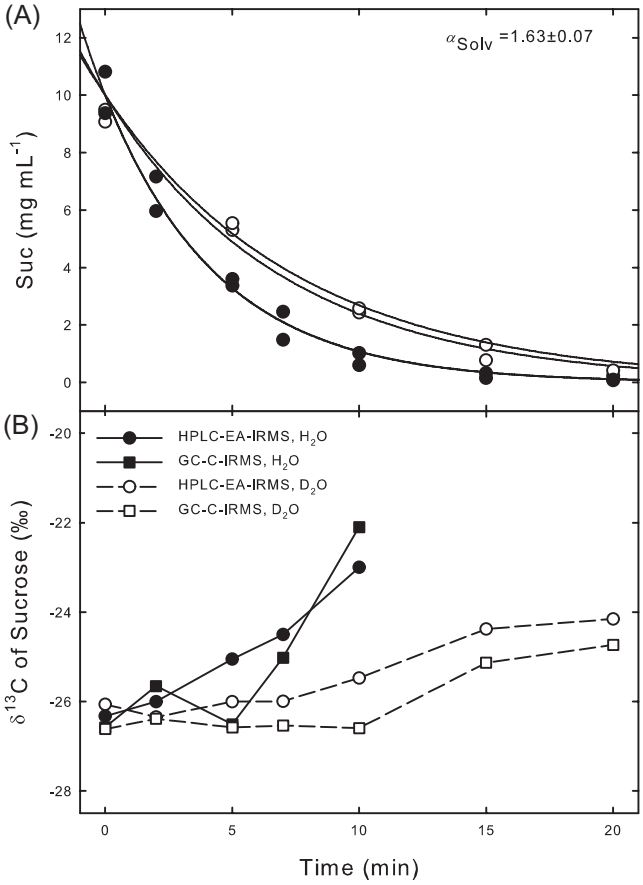

Figure 5. Time course of Suc concentration (A) and isotope composition (B) during the invertase reaction operating in either H_2O (closed symbols) or D_2O (open symbols). (A) Continuous lines: first-order exponential regressions; all of them are significant ($p < 0.001$). The H_2O/D_2O ratio of time constants is 1.63 ± 0.07. (B) The carbon isotope composition of Suc was measured by either GC-C-IRMS (squares) or HPLC∼EA-IRMS (circles). In the assay in H_2O, there is no value at 15 and 20 min because the very low Suc content does not allow reliable isotopic analyses.

DISCUSSION

The kinetic $^{12}C/^{13}C$ and solvent H_2O/D_2O isotope effects associated with Suc consumption by the yeast invertase were investigated. The carbon isotope composition (δ^{13}C)

Table 1. The $^{12}C/^{13}C$ kinetic isotope effect (KIE) of the invertase reaction in either H_2O or D_2O. The δ^{13}C value of substrate Suc was measured with either HPLC∼EA-IRMS or GC-C-IRMS. Each single data is the average over the different sampling times (different fraction of consumed Suc) as indicated in Fig. 5

Technique used for δ^{13}C Suc	Solvent	KIE (mean ± standard error)
HPLC∼EA-IRMS	H_2O	1.0011 ± 0.0001
GC-C-IRMS	H_2O	1.0012 ± 0.0004
HPLC∼EA-IRMS	D_2O	1.0007 ± 0.0007
GC-C-IRMS	D_2O	1.0011 ± 0.0005
Average value	H_2O	1.0011
	D_2O	1.0009

DOI: 10.1002/rcm

of Suc, Glc and Fru was measured by either gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) with TMS derivatives, or high-performance liquid chromatography-elemental analysis-isotope ratio mass spectrometry (HPLC~EA-IRMS).

Application of GC-C-IRMS and TMS derivatives for carbohydrates

In the present study, trimethylsilylation was used to volatilize Glc, Fru and Suc for GC as it is easy and quantitative and so is not accompanied by an isotope effect.[13,16] Nevertheless, it has already been recognized that such a derivatization procedure causes several analytical problems, e.g., the addition of exogenous carbons that adulterate the natural $\delta^{13}C$ value of carbohydrates. As a result, the natural $\delta^{13}C$ value has to be back-calculated with the known $\delta^{13}C$ value of added TMS groups. This may induce very large uncertainties in the final results simply because of the large number of added TMS carbons. We used here a combination of two calibrations: (a) an internal alkane reference (of known $\delta^{13}C$) added to each sample in order to correct for any isotopic offset of the IRMS; (b) the determination of the $\delta^{13}C$ value of TMS groups with standard carbohydrates of known $\delta^{13}C$ values and analyzed using GC-C-IRMS. While such a standard procedure gives consistent results with Suc (similar HPLC and GC-C-IRMS signals, Fig. 2), the Glc values are less reliable due to the possible α/β anomeric (dis)equilibrium, i.e., mutarotation (Fig. 4): in ordinary conditions, α-Glc and β-Glc account for nearly 40 and 60% at equilibrium, respectively.[17] Other authors have used an equilibration step prior to derivatization[18] but the role of such a step is still not well documented. Here we provide evidence that such an anomeric equilibration is necessary (Fig. 4) because a thermodynamic equilibrium isotope effect (EIE) occurs during mutarotation. While the measurement of such a $^{12}C/^{13}C$ EIE has not yet been reported, mutarotation is accompanied by a H/D isotope effect[19] of nearly 3.2. Conversion from α-Glc into β-Glc proceeds through the intermediate open form of Glc (denoted as *o*-Glc hereafter). Such a large H/D isotope effect indicates that the step from *o*-Glc to β-Glc is probably rate-limiting and, presumably, it is also associated with a carbon isotope effect. The isotopic shift between equilibrated and non-equilibrated Glc falls between 3 and 4‰ (Figs. 2(B) and 4) and, thus, we assume that the EIE associated with mutarotation is at least 1.004 (that is, α-Glc is ^{13}C-enriched by 4‰).

We recognize that mutarotation-related difficulties may be overcome with other derivatization techniques such as the use of alditol acetates, in which hexoses are first reduced to aldidol, thus preventing configurational changes.[20,21] Other techniques also show a clear improvement in the number of added carbons, using methylboronic acid.[22] Such alternative techniques are nevertheless more complex (several steps, e.g., with methylboronic techniques) and are accompanied by an isotope effect because they are not quantitative (for a specific discussion, see Docherty *et al*.[20]). The agreement between the GC-C- and HPLC-derived isotopic results provided here (Fig. 2(A)) indicates that TMS derivatives are reasonably suitable for the ^{13}C-analysis of Suc. That said, further improvements will probably be obtained in the near future with recent techniques involving liquid chromatography coupled to IRMS (LC-IRMS).[23]

Kinetic and solvent isotope effects of the yeast invertase

Our results show that invertase is associated with a small $^{12}C/^{13}C$ isotope effect of less than 1‰ when Suc isotope composition is measured during the enzymatic assay (Fig. 5). While such a value is low, it should be emphasized that the positional isotope effect that occurs in Suc atom positions involved in C−O bonds reshuffled by invertase is certainly larger. The Glc moiety of Suc is liberated quite early during invertase catalysis and no C−O bonds are broken (Fig. 1). Thus one may hypothesize that there is no primary isotope effect associated with the Glc moiety. By contrast, the C-2 position of the Fru moiety is first linked to Asp and then attacked by H_2O, thereby causing a primary carbon isotope effect. Under the assumption that secondary isotope effects are negligible, the 1‰ isotope effect measured at the whole Suc level would indicate that the positional kinetic isotope effect in C-2 of the Fru moiety is near 1.012 (as there are 12 carbon atoms in Suc). Such a value is rather small, suggesting that steps 1, 2 or 3 are only partially rate-limiting. Similar values have been obtained in other related catalytic systems. The inorganic hydrolysis of 1-methyl-Glc by acid catalysis ($HClO_4$) is accompanied by a kinetic isotope effect in C-1 of 1.007 (α-methylglycoside) or 1.011 (β-methylglycoside).[24] The enzymatic hydrolysis of 1-methyl-Glc (by α- and β-glucosidases, EC 3.2.1.20 and EC 3.2.1.21, respectively) is accompanied by a kinetic isotope effect in C-1[25] of nearly 1.010 and 1.032 (these experimental values are relative, i.e., arbitrarily assume no isotope effect in C-4). The mechanism of glucosidases is quite similar to invertase, in that the C-1 of the Glc moiety of methyl-Glc binds a $-COO^-$ group of the catalytic site (in invertases, the Fru moiety binds the $-COO^-$ group of Asp, Fig. 1). In glucosidases, it is believed that hexose covalent binding to the catalytic site (see, e.g., step 1, Fig. 1) is partially rate-limiting[25] and, accordingly, it is thought that the lifetime of the oxonium ion is very short, near 10^{-12} s.[26,27] The catalytic efficiency of invertase is not very large, with a K_m(Suc) value as high as 25 mmol L^{-1} [28] and a large specific activity near 2.10^4 s^{-1} $site^{-1}$,[29] giving a V/K_m ratio of ~0.8 L μmol^{-1} s^{-1}; in other words, the moderate catalytic efficiency of invertase might be due to a weak substrate binding and a limited commitment to hydrolysis (k_1/k_{-1}) with respect to sucrose release.

We further found a H_2O/D_2O solvent isotope effect of 1.6 only (Fig. 5). A similar solvent isotope effect (1.5) has been found on the hydrolysis of *p*-nitrophenyl-Glc by α-glucosidase.[30] This suggests that step 1 and/or step 3 may be partially rate-limiting. In fact, step 3 is certainly affected by D_2O as it is water-dependent. Step 1 may also be limiting in D_2O if the Glu proton were exchanged with the solvent, thereby exacerbating the extremely low basicity of Glc moiety (Fig. 1). However, D_2O did not change the $^{12}C/^{13}C$ isotope effect (Table 1), showing that the limiting step responsible for the carbon isotope effect is weakly if at all H/D-dependent. We therefore hypothesize that step 1 is $^{12}C/^{13}C$ isotopically sensitive but it is only partially rate-

DOI: 10.1002/rcm

limiting, i.e., the fructosylation step has an early transition state both in terms of the extent of proton transfer and in the amount of glycosidic bond cleavage. This scenario would also agree with the larger H_2O/D_2O isotope effect associated with the non-enzymatic acidic hydrolysis of Suc of 2.1,[31] indicating that the H^+ attack is less rate-limiting during enzymatic catalysis.

Physiological consequences

Sucrose is the main transport molecule from source organs (leaves) to sink plant organs such as roots, tubers, etc.[32] Unless other processes take place (such as Suc accumulation, e.g., in beet root), it is widely accepted that Suc entering sink organ cells is broken down into Glc and Fru by the interplay of invertase, providing Glc for starch synthesis or other biosyntheses. If the $^{12}C/^{13}C$ kinetic isotope effect found here on the yeast invertase also occurs in the plant enzyme, Fru and Glc molecules produced from hydrolyzed Suc (the positional isotope effect in C-2 of the Fru moiety is assumed to be 1.012, see above) would be slightly depleted in ^{13}C. However, there is substantial evidence that the organic matter of heterotrophic sink organs is consistently ^{13}C-enriched compared with leaves.[33] It is thus likely that the invertase reaction either does not fractionate between isotopes *in vivo* (e.g., it may be quantitative and so cannot discriminate) or is involved in a complex set of metabolic reactions. In the latter case, it is plausible that some fractions of phloem Suc are consumed by invertases along the phloem path within stem cells, thereby enriching in ^{13}C the Suc molecules left behind. If true, this would result in ^{13}C-enriched Suc being available for roots. We nevertheless recognize that other processes contribute to enrich heterotrophic organs, such as the production of ^{13}C-depleted respired CO_2,[15] phospho*enol*pyruvate carboxylase fixation of ^{13}C-enriched HCO_3^-, or the use of starch-derived, ^{13}C-enriched photosynthates, etc. (for a review, see Badeck *et al*.[33] and Cernusak *et al*.[34]).

Water stress is known to trigger the production of ^{13}C-enriched photosynthates, mainly because of a lower stomatal conductance that in turn decreases the photosynthetic fractionation (lower c_i/c_a) (for a review, see Brugnoli and Farquhar[35]). At the leaf level, it is believed that the vacuolar invertase is induced under water deficit, thereby decreasing the Suc content and grain yield in maize (*Zea mays*).[36] Under those circumstances, the Suc export flow from leaves is decreased and made of ^{13}C-enriched Suc because of both the activity of invertase and stomatal closure. In other words, invertase would cause a further ^{13}C-enrichment in grains.

The present work was performed on the yeast invertase and, as such, the value of the isotope effect reported here may not apply to the plant enzyme. Thus, further work is needed to measure the isotope effect of invertase in plants and to elucidate the role of invertase within whole-plant carbon-trafficking pathways, that is, in order to interpret the ^{13}C-signal of carbohydrates in organs other than leaves. The invertase family is multigenic and made up of many isoforms located in several cell compartments, with varying catalytic properties (see, e.g., Tang *et al*.[37] for invertases in *Arabidopsis* and Roitsch and Gonzalez[6] for a review). It thus remains plausible that the kinetic isotope effect differs between them and so further experimental work on isotope effects involved in plant metabolism is required to decipher whether invertase are key components of the ^{13}C-balance within plants.

Acknowledgements

The authors acknowledge the support of the Institut Fédératif de Recherche 87 for this work as well as Profs. Dan Yakir and Gabriel Cornic for valuable discussions on the results. G.T. wishes to give special thanks to Dr Aline Mahé who provided intense support during the time of this work.

REFERENCES

1. Farquhar GD, Ehleringer JR, Hubick KT. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1989; **40**: 503.
2. Roeske CA, O'Leary MH. *Biochemistry* 1984; **23**: 6275.
3. Tcherkez G, Farquhar GD. *Funct. Plant Biol.* 2005; **32**: 277.
4. Rossmann A, Butzenlechner M, Schmidt HL. *Plant Physiol.* 1991; **96**: 609.
5. Tcherkez G, Farquhar GD, Badeck F, Ghashghaie J. *Funct. Plant Biol.* 2004; **31**: 857.
6. Roitsch T, Gonzalez MC. *Trends Plant Sci.* 2004; **9**: 606.
7. Sinnott ML. *Chem. Rev.* 1990; **90**: 1171.
8. Reddy VA, Maley F. *J. Biol. Chem.* 1990; **265**: 10817.
9. Reddy VA, Maley F. *J. Biol. Chem.* 1996; **271**: 13953.
10. Lammens W, Le Roy K, Van Laere A, Rabijns A, Van den Ende W. *J. Mol. Biol.* 2008; **377**: 378.
11. O'Leary MH. *Methods Enzymol.* 1980; **64**: 83.
12. Macko SA, Ryan M, Engel MH. *Chem. Geology* 1998; **152**: 205.
13. Meier-Augenstein W. *J. Chromatogr. A* 1999; **842**: 351.
14. Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. *Plant Cell. Environ.* 1999; **22**: 515.
15. Bathellier C, Tcherkez G, Bligny R, Gout E, Cornic G, Ghashghaie J. *New Phytol.* 2009; **181**: 387.
16. Rieley G. *Analyst* 1994; **119**: 915.
17. Angyals SJ, Pickels VA. *Aust. J. Chem.* 1972; **25**: 1695.
18. Derrien D, Balescent J, Marol C, Santaella C. *Rapid Commun. Mass Spectrom.* 2003; **17**: 2626.
19. Hamill WH, La Mer VK. *J. Chem. Phys.* 1934; **2**: 891.
20. Docherty G, Jones V, Evershed RP. *Rapid Commun. Mass Spectrom.* 2001; **15**: 730.
21. Rumpel C, Dignac MF. *Soil Biol. Biochem.* 2005; **38**: 1478.
22. Jackson SJ, Waterhouse JS, Bluck LJC. *Rapid Commun. Mass Spectrom.* 2007; **21**: 3123.
23. Cabanero AI, Recio JL, Ruperez M. *J. Agric. Food Chem.* 2006; **54**: 9719.
24. Bennett AJ, Sinnott ML. *J. Am. Chem. Soc.* 1986; **108**: 7287.
25. Lee JK, Bain AD, Berti PJ. *J. Am. Chem. Soc.* 2004; **126**: 3769.
26. Amyes TL, Jencks WP. *J. Am. Chem. Soc.* 1989; **111**: 7888.
27. Zhu J, Bennett AJ. *J. Am. Chem. Soc.* 1998; **120**: 3887.
28. Gascon S, Neumann NP, Lampen JO. *J. Biol. Chem . 1968;* **243**: 1573.
29. Goldstein A, Lampen JO. *Methods Enzymol. C* 1975; **42**: 504.
30. O'Donnell AH, Yao X, Byers LD. *Biochim. Biophys. Acta* 2004; **1703**: 63.
31. Hamill WH, La Mer VK. *J. Chem. Phys.* 1936; **4**: 294.
32. Kosh K. *Curr. Opin. Plant Biol.* 2004; **7**: 235.
33. Badeck F, Tcherkez G, Nogués S, Piel C, Ghashghaie J. *Rapid Commun. Mass Spectrom.* 2005; **19**: 1381.
34. Cernusak L, Tcherkez G, Keitel C, Cornwell WK, Santiago LS, Knohl A, Barbour M, Williams DG, Reich PB, Ellsworth DS, Dawson TE, Griffiths HG, Farquhar GD, Wright IJ. *Funct. Plant Biol.* 2009; **36**: 199.
35. Brugnoli E, Farquhar GD. *Photosynthesis: Physiology and Metabolism*. Kluwer Academic Publishers: The Netherlands, 2000; 399–434.
36. Trouverie J, Thévenot C, Rocher JP, Sotta B, Prioul JP. *J. Exp. Bot.* 2003; **54**: 2177.
37. Tang X, Ruffner HP, Scholes JD, Rolfe SA. *Planta* 1996; **198**: 17.

DOI: 10.1002/rcm