Radio-Metabolite Analysis of Carbon-11 Biochemical Partitioning to Non-Structural Carbohydrates for Integrated Metabolism and Transport Studies

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Metabolism and phloem transport of carbohydrates are interactive processes, yet each is often studied in isolation from the other. Carbon-11 (11C) has been successfully used to study transport and allocation processes dynamically over time. There is a need for techniques to determine metabolic partitioning of newly fixed carbon that are compatible with existing non-invasive 11C-based methodologies for the study of phloem transport. In this report, we present methods using 11C-labeled CO2 to trace carbon partitioning to the major non-structural carbohydrates in leaves—sucrose, glucose, fructose and starch. High-performance thin-layer chromatography (HPTLC) was adapted to provide multisample throughput, raising the possibility of measuring different tissues of the same individual plant, or for screening multiple plants. An additional advantage of HPTLC was that phosphor plate imaging of radioactivity had a much higher sensitivity and broader range of sensitivity than radio-HPLC detection, allowing measurement of 11C partitioning to starch, which was previously not possible. Because of the high specific activity of 11C and high sensitivity of detection, our method may have additional applications in the study of rapid metabolic responses to environmental changes that occur on a time scale of minutes. The use of this method in tandem with other 11C assays for transport dynamics and whole-plant partitioning makes a powerful combination of tools to study carbohydrate metabolism and whole-plant transport as integrated processes.

Keywords: Carbohydrate metabolism • Carbon-11 • Leaf export • Phloem transport • Starch • Sugars.
Abbreviations: 11C, carbon-11; HPTLC, high-performance thin-layer chromatography; SNR, signal-to-noise ratio.

Introduction

Carbohydrates are critical as building blocks and signaling molecules, as well as for energy storage in plants. Much of the carbon assimilated by photosynthesis is converted to sucrose and hexoses, which may be processed directly for immediate energy needs, incorporated into the structural or non-structural glucose polymers cellulose or starch, respectively, or transported from ‘source’ tissues (i.e. leaves) to non-photosynthetic ‘sink’ tissues (e.g. roots, apical meristem and reproductive organs). Starch serves an important function as a storage molecule that protects against carbohydrate starvation, but also starch metabolism is increasingly viewed as important in the coordination of carbon supply with growth (Cross et al. 2006, Smith and Stitt 2007, Gibon et al. 2009, Graf et al. 2010).

Carbon-11-labeled carbon dioxide (11CO2) has been used as a tracer introduced into plants through photosynthesis to measure phloem transport dynamics and whole-plant carbon resource allocation. Recently non-invasive techniques using 11C have contributed to our understanding of the mechanics of phloem transport (Minchin and Thorpe 2003, Gould et al. 2012), whole-plant carbohydrate allocation (Babst et al. 2005, Schwachtje et al. 2006, Kawachi et al. 2011a, Kawachi et al. 2011b) and plant root physiology (Jahnke et al. 2009). Phloem transport is dependent on carbohydrate metabolism in both source and sink tissues. Sugar loading into the phloem in source tissues and sugar unloading in sink tissues generates a hydrostatic pressure gradient, which drives phloem sap flow (Jensen et al. 2012). Hence, there is a need to consider carbohydrate metabolism in studies of carbohydrate transport, particularly metabolic processes that would determine whether sugars are available in source tissues for loading into the phloem, as well as metabolic processes in sink tissues that would affect unloading of sugars. Recent metabolomics studies demonstrated a negative correlation between leaf starch concentrations and growth in Arabidopsis plants (e.g. Sulpice et al. 2009), but plant growth appears to be related more directly to metabolic fluxes, such as starch turnover rates, rather than starch concentrations per se (Cross et al. 2006, Gibon et al. 2009). Thus, there is also a need in studies of plant carbohydrate metabolism to measure...
phloem transport, and include both metabolism and transport in our conceptual models.

Carbon isotopes have been widely used to study carbohydrate partitioning in plants. However, carbon-14 (14C) methods to measure metabolic partitioning require relatively long administration periods (e.g. 30–60 min minimum) and then additional incubation time to attain the sensitivity required for accurate measurements. Recently, stable isotope, carbon-13 (13C) administration with mass spectrometry and nuclear magnetic resonance (NMR) analytical methodologies have been developed with the added benefit of flux modeling (Schwender 2008). However, 13C requires long labeling periods to achieve sufficient sensitivity, and most current metabolic flux models require steady-state conditions, which do not reflect the high variability in nature and are not compatible with 13C-labeled carbon dioxide as a tracer (Schwender 2008). Neither 14C nor 13C methods can be used like 11C to measure transport dynamics, and account for the substantial export or import of carbohydrates from or into the tissue, which is important not only for a full understanding of local tissue metabolism, but also for understanding the coordination of whole-plant growth processes (Ainsworth and Bush 2011).

Our goal was to develop a technique compatible with existing 11C methods to measure transport, utilizing a brief (i.e. 30 s) pulse of 11CO2 as a tracer, to a leaf to monitor partitioning of newly fixed carbon to the major non-structural carbohydrates in leaves. Because 11C has a 20.4 min half-life, rapid and careful analysis is required, and there is a limit to the number of samples that can be analyzed in series, for example by radio-HPLC. However, high-performance thin-layer chromatography (HPTLC) allows for rapid handling and analysis of multiple samples. Here we report facile methods to measure partitioning of newly fixed carbon to carbohydrates that can be done in tandem with transport measurements. After testing multiple species, including maize, sorghum, tobacco and Arabidopsis, we suggest that the methods have potential for high-throughput scaling for phenotype screening of mutant populations.

Results and Discussion

The size of a biochemical pool of a compound, such as a sugar, is dependent on both input fluxes (e.g. biosynthesis) and output fluxes, such as conversion to other compounds or transport out of a cell or tissue. Analysis of the partitioning of newly fixed carbon in plant tissues requires a simultaneous measurement of both [11C]sugar radioactivity, representing new carbon inputs into biochemical pools of sugars, and [13C]sugar concentrations, representing the total size of the biochemical pools of sugars accumulated over time (Fig. 1). Since the major abundant sugars in plants—sucrose, glucose and fructose—can be clearly separated on the NH2 HPTLC plates (Fig. 2), we tested the potential for simultaneous [13C]- and [12C]sugar analysis by HPTLC. First, we tested the capabilities and limitations of the HPTLC system for the quantification of 13C incorporation into sugar and starch. For sugar extraction, 7–10 mg of tissue were necessary, and 10–20 mg of tissue for starch analysis. Autoradiography provided more than sufficient sensitivity to quantify the radioactivity of 13C-labeled sugars in plant extracts. Although the signal-to-noise ratio (SNR) for [13C]sucrose was similar to that for radio-HPLC (>100), TLC was an improvement over radio-HPLC for [13C]hexoses (SNR >50 for TLC, but <40 for radio-HPLC). Further, [13C]glucose resulting from starch digestion was detectable using TLC (Fig. 3) with SNR >50, but was not detectable by radio-HPLC. The short half-life of 13C precludes buying or borrowing independently authenticated standards. To ensure accuracy, our procedure calls for calibration of the phosphor plate image of every TLC plate by measuring a 10 µl aliquot of each of the plant extracts in a calibrated well-type gamma radiation counter. The radioactivity of the entire sample lane on the TLC plate per microliter applied to the plate equals the radioactivity per microliter of the sample aliquot. Using image analysis of the autoradiogram, we determined the radioactivity of sucrose, glucose and fructose as the percentage of total lane radioactivity, and then multiplied the percentage radioactivity by the total sample radioactivity to determine the absolute radioactivity in megabecquerels (MBq) for each! For example, in 3-week-old wild-type maize plants (n = 8 plants) incubated for 1 h after the 11CO2 pulse, [11C]sucrose made up 70% of extract radioactivity for the soluble fraction, which was equivalent to 263 ± 111 MBq g FW−1 partitioning into sucrose, decay corrected to the time of 11CO2 administration (Fig. 4A). The precision of the measurements, based on technical replicates spotted on the same TLC plate, was 5.5 MBq g FW−1 (i.e. 2% technical error in MBq g FW−1 measurements). In the same experiment, glucose and fructose radioactivities were 5.2 MBq g FW−1 (1.5% of extract radioactivity) and 2.6 MBq g FW−1 (0.7% of extract radioactivity), with precisions of 0.74 and 0.37 MBq g FW−1, respectively, or about 14% error. As soluble biochemical components were first extracted prior to the starch digestion, the majority of the radioactivity of the starch digest was attributed to starch. Radioactivity of [11C]starch was 326 ± 144 MBq g FW−1 in the maize example, and was measured with a precision of 9.6 MBq g FW−1, or 3% error. Because the length of time of phosphor plate exposure to the radio-TLC can be adjusted, this radio-TLC method provides much higher potential sensitivity, as well as a much broader range of sensitivity than radio-HPLC, which has a short detection time, fixed by the HPLC flow rate. A rapid check of the TLC with a Geiger counter can be used empirically to estimate optimum plate exposure time.

Since measurements of [12C]sugars and [13C]starch were possible using our HPTLC system, we also tested the capabilities and limitations of HPTLC for quantification of [13C]sugar and [12C]starch concentrations. The sugar concentrations were quantifiable using standards of known concentration spotted...
Fig. 1 Rationale and protocol for [11C]- and [12C]carbohydrate measurement. Biochemical pool size is determined by input flux and output flux (A). Input flux and the size of the biochemical pool can be determined as 11C and 12C components of the biochemical pool, respectively, and the output flux can then be inferred. Protocol for [11C]- and [12C]carbohydrate measurement (B). Standard curves for TLC quantification of sucrose (C), glucose (D) and fructose (E). TLC of glucose resulting from digestion of starch in Nicotiana tabacum leaves (F), and glucose standards. Comparison of [12C]starch measurements with actual masses of corn starch standards (G). Each point on the standard curves (C, D and E) represents a single authentic standard of known concentration measured once. For (G), R^2 indicates fit of a regression line for all samples shown (0.5–5 mg), but the diagonal line represents 1:1 correspondence between the starch assay result and the actual starch mass, not a regression line. Each point in (G) represents the mass of a single corn starch standard as measured on an analytical balance and the amount of starch as measured by enzymatic assay and TLC quantification. Standard curves were determined from TLC using the naphthol sulfuric acid method.
onto each plate to make calibration curves ($R^2 > 0.99$; Fig. 1C–E). Sugar concentrations of maize and sorghum tissues were typically within the range of 2–30 mM (Fig. 4B), which would result in a tissue extract concentration of 1–10 mM. Occasionally, sample concentrations were outside of the range of our standards. For samples with concentrations too high for our standard curves, the samples were diluted and then re-analyzed by TLC. For samples with concentrations too low for our standard curve, we re-analyzed, spotting a higher volume (e.g. 2 or 4 ml) of sample onto the TLC plate. Any dilutions or changes in spotting volume were factored in when calculating sugar concentration on a tissue weight basis. The extraction volume for these experiments was selected based on preliminary analysis and was fairly robust for the species we tested, but could easily be modified for future experiments to accommodate different plant tissues with sugar concentrations outside of the range we tested. For example, 4 ml of extraction solvent per mg of leaf tissue was ideal for maize, but 2 ml of solvent per mg of leaf tissue may be better for Arabidopsis thaliana. We used repeated measures of 2.5 and 4 mM standards to determine the accuracy (0.15 mM) and precision (0.16 mM) of the TLC method, which indicated a technical error of 3–5%.

Starch must be digested to glucose before it can be analyzed by TLC. To accommodate the 20 min half-life of $^{11}$C, we streamlined the soluble sugar extraction and subsequent starch digestion as much as possible, in order to maximize the $^{11}$C radioactivity signal remaining in the resulting glucose at the time of measurement. Traditional methods of starch...
digestion required overnight incubations. Recent advances, particularly the isolation of heat-stable α-amylase from Bacillus licheniformis, now allow high reaction temperatures (i.e. 96–99°C) for the first enzymatic step in the starch digestion, which both helps to gelatinize the starch into solution and increases the rate of reaction without denaturing the α-amylase (Komolprasert and Ofili 1991). This high-speed starch digestion has been adopted previously for measurement of plant [12C]starch content (Fischer et al. 1997). After experiments testing the manufacturer’s protocol, sample mass and reagent volume were reduced by a factor of 5 to reduce the amount of tissue mass required and to accommodate a 2 ml microcentrifuge tube, and the α-amylase digestion time was increased from 5 min to 10 min, but otherwise the manufacturer’s recommended protocol was well optimized. Using our protocol, there was no evidence of any maltose or larger oligosaccharides remaining after digestion of corn starch standards or plant samples (e.g. Figs 1F, 3B). The starch assay had good accuracy (0.12 mg on average) from 0.04–4 mg (Fig. 1G), or a technical error of 7%. However, care must be taken not to overload the assay, because above 4–5 mg of starch the assay underestimated the amount of starch, presumably because the digestive enzymes became saturated. Most of the plant starch samples ranged from 30 to 300 μmol g FW⁻¹ (Fig. 4B), equivalent to 0.2–2 mg for 40 mg aliquots of sorghum, maize and A. thaliana fresh tissue, which was well within the range of good accuracy. The size of the plant tissue aliquot could easily be adjusted to accommodate starch contents outside of this range, if encountered in future experiments. For example, Nicotiana tabacum leaves, which were measured in high light (1,500 μmol m⁻² s⁻¹ photosynthetically active radiation), had starch contents up to 900 μmol g FW⁻¹, and required smaller tissue aliquots (e.g. 20 mg) to remain within the bounds of accuracy. These TLC measurements of sugar concentration and starch content are clearly adequate to test for the moderate or large differences that would be of biological relevance in most plant research.

The combination of [11C]- and [12C]carbohydrate assays will provide both a measure of the biochemical pool size (i.e. [12C]carbohydrate) and a measure of input flux to the biochemical pool (i.e. [11C]carbohydrate). As an example, [11C]- and [12C]starch exhibited different patterns in maize compared with N. tabacum (Fig. 4). The high [11C]starch and low [12C]starch in maize indicate a rapid incorporation of newly fixed carbon into starch, but a relatively rapid turnover. Conversely, in N. tabacum, [11C]starch was low, indicating
low starch biosynthesis, but [12C]starch was high. Taken together, a low rate of biosynthesis and a high biochemical pool size strongly suggest that starch turnover in the *N. tabacum* leaves was very low. This is not surprising, given that *N. tabacum* was measured under very high light conditions. Similarly, comparison among sugars can be made by comparing the specific activity, either as MBq mg^{-1} or as MBq μmol^{-1} sugar. In maize, the average specific activity of sucrose was 16 MBq μmol^{-1}, but for fructose it was only 1.5 MBq μmol^{-1}, indicating a much faster turnover rate for sucrose than for fructose. Given that sucrose is the primary transport sugar in maize (Slewinski et al. 2009), we would expect a much higher turnover rate for sucrose than for fructose, since export from the tissue contributes to ‘turnover’ in this case.

These [11C]carbohydrate methods may serve in studies of mutants and transgenics, as well as responses to environmental change to detect changes of in vivo activity of the carbohydrate biosynthetic machinery. For simple comparisons of different genotypes, pulse and chase times can be fixed for rapid comparison of multiple lines. However, photosynthetic processes and carbohydrate metabolism occur in rapidly and somewhat unpredictably changing environments. Studying the rapid plant metabolic responses to environmental changes will require the capability to determine metabolic partitioning of newly fixed carbon over short time scales. We have conducted measurements of [11C]sugars and starch with as little as a 5 min chase (Fig. 5) and up to a 2 h chase time (for 2 h, see sorghum in Fig. 4A) following the 30 s [11C]CO2 administration pulse. This time scale of minutes would capture rapid metabolic responses to environmental perturbations, and the time resolution of both pulse and chase could easily be further sharpened, if desired. For a time course, one approach is to hold constant the pulse and chase times, and perform the entire pulse, chase and measurement process at varying times of day or times after a treatment (e.g. Ferrieri et al. 2013), in order to have a time course of comparable instantaneous measurements of biochemical partitioning of [11C] at a given chase time. Alternatively, two or more chase times could be examined (Fig. 5), to follow the [11C] over time as it is transferred through different biochemical pools. In this experiment, [11C]sucrose decreased from the 5 min to 60 min chase time (Fig. 5), which is probably primarily due to phloem transport of [11C]sucrose out of the leaf. Although changes in metabolism may occur rapidly (e.g. nearly instantaneously in the case of allosteric regulation), changes in standing concentrations of soluble sugars and starch may require hours to become detectable (e.g. Lunn et al. 2006). Using [11C] for near instantaneous measurement of carbon partitioning may aid our ability to understand shorter term processes that may occur on the scale of minutes and the effects of common perturbations (e.g. light flecks, cloud cover) that may result during very large and rapid environmental changes throughout the day, potentially detecting even transient metabolic changes that

![Fig. 5](https://academic.oup.com/pcp/article-abstract/54/6/1016/1838202) Measurements of [11C]sugars and [11C]starch (A) and [12C]sugars and [12C]starch (B) conducted after 5 min and 1 h chase times. Administration of [11C]CO2 was done as a 30 s pulse to whole *Arabidopsis thaliana* shoots in a custom-made cuvette, and then leaf numbers 2 and 3 were harvested after 5 min and 1 h, respectively. Shown are means ± SE (n = 4 for sugars, n = 3 for starch).
might not manifest as detectable changes in biochemical concentrations (Hanik et al. 2010).

Transport measurements can complement these carbohydrate assays, providing other measures of whole-plant physiology, but also a more complete estimate of metabolism that includes metabolites exported from the leaf. For example, export of 11C-labeled biochemicals (‘photosynthate’) from a leaf by phloem transport can be measured in real time, and non-destructively, immediately up to the time when the leaf is harvested and flash-frozen in liquid nitrogen for carbohydrate analysis. By multiplying the total [11C]photosynthate export by the proportion of 11C that was transported as a particular biochemical, for example sucrose, we determine the amount of 11C that was both incorporated into that biochemical and then exported from the leaf. In the previous example of wild-type maize with a 1 h ‘chase’ period following the 11CO2 pulse administration, 684 MBq of 11C radioactivity had been exported from the region of leaf inside the cuvette where the 11CO2 was administered (load zone). So the amount of activity exported from the leaf was much higher than the amount of radioactivity as sucrose, glucose, fructose and starch combined, 104 MBq (or 57.5 MBq g FW\(^{-1}\)), that was remaining in the load zone at the end of the 1 h incubation period. We can also determine the biochemical form(s) of the 11C being transported in the phloem, which should be the same as what was loaded into the phloem, by analyzing leaf or petiole tissue basal to the load zone (Fig. 6A), because very little carbohydrate metabolic activity occurs inside the phloem sieve elements. Since basal leaf tissues were not exposed to 11CO2, the 11C present in the basal part of the leaf can only have originated from the load zone, and must be in transit within the phloem. For maize and sorghum, 11C in these basal tissues was primarily in the form of sucrose (about 85–98%); labeling of other metabolites was not detected (Fig 6B, D). This percentage composition within the phloem can be used to calculate the total 11C that was incorporated into sucrose within the load zone, including both the sucrose that was exported and the sucrose that remained in the load zone. In the case of wild-type maize, the amount of 11C:sucrose exported was 581 MBq, which was calculated by multiplying the total activity exported from the load zone (684 MBq) by the fraction of 11C exported as sucrose (85%). Together, the total retained [11C]:sucrose (47 MBq) plus exported [11C]:sucrose (581 MBq) that was biosynthesized in the load zone during the 1 h chase sums to 628 MBq. With an average load zone mass of 0.18 g, this would be the equivalent of 3,487 MBq g FW\(^{-1}\), which dwarfs the 57.5 MBq g FW\(^{-1}\) of the [11C]carbohydrates that were retained in the load zone after 1 h. The high ratio of exported to retained sucrose is not surprising since sucrose tends to be maintained at lower concentrations in leaves of species with apoplastic phloem loading, such as maize, sorghum, Arabidopsis and tobacco, which use sucrose/proton symporters to generate much higher sucrose concentrations inside the phloem than exist outside of the phloem (Turgeon 2010). Clearly, factoring in export can change the interpretation of this type of experiment. In the maize example, instead of concluding that partitioning to starch was slightly higher than to sucrose, we would now conclude that overall partitioning to sucrose was an order of magnitude higher than partitioning to starch, and that the majority of the labeled sucrose was exported from the leaf during the experiment.

In summary, the methodology demonstrated here provides a means to study very rapid metabolic responses, and is compatible with complementary techniques using 11CO2. For example, the high sensitivity of 11C affords the opportunity for real-time measurement of export from photosynthetic leaves, volatile organic emissions or import into sink tissues (Babst et al. 2005, Ferrieri et al. 2005, Gomez et al. 2010, Hanik et al. 2010, Kawachi et al. 2011b). Measuring 11C tracer partitioning to carbohydrates concurrently with previously developed 11C assays for transport dynamics and whole-plant partitioning makes a powerful combination of tools to address questions on phloem transport within the context of carbohydrate metabolism, and questions on carbohydrate metabolism in the context of whole-plant transport.

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**Fig. 6** Carbon-11 is transported in the phloem (A) primarily in the biochemical form of sucrose in maize (B, C) and sorghum (D and E). TLC chromatograms are shown for two different genotypes of maize, Y and Z, and two genotypes of sorghum, sweet (S) and grain (G), for [11C]sugars (B and D). Also shown are [12C]sugars (C and E), for comparison of R\(_f\) values. While [12C]sugars are a combination of vascular and non-vascular components, [11C]sugars are only present in the phloem, since these tissues basipetal to the load zone were not directly exposed to 11CO2. Plants were incubated for a 1 h (maize) or 2 h (sorghum) chase period after the 30 s 11CO2 pulse administration, and then basal leaf tissue, shown on an autoradiograph of a maize leaf (A), was harvested and analyzed. Sucrose (S), glucose (G) and fructose (F) were determined by TLC as described above, spotting 1 and 2 \(\mu\)l of each extract. For sorghum, there is one empty lane (e) between the 1 and 2 \(\mu\)l spots (D and E).

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Materials and Methods

Plant material

*Sorghum bicolor* and *Zea mays* plants were sown in potting mix (40% Promix BX™: 40% medium coarse sand: 20% medium perlite) in 14.9 liter pots. Maize plants were grown in a growth chamber with 800 μmol m⁻² s⁻¹ photosynthetically active radiation measured at the top of the plants, and a 15 h/9 h light/dark cycle, with temperatures maintained at 23°C during the daytime and 19°C during the night. Sorghum plants were grown in a greenhouse, and then moved into a growth chamber for approximately 1 week to acclimate to conditions of the isolate chamber prior to experiments. *Nicotiana tabacum* was sown in Promix in 0.75 liter square pots, and grown under 600 μmol m⁻² s⁻¹ for 5 weeks. *Arabidopsis thaliana* seeds were sown in Promix in 0.2 liter pots and grown under fluorescent lamps (250 μmol m⁻² s⁻¹) for 3 weeks.

¹¹CO₂ administration

An EBCO T19 cyclotron was used to produce C-11 as ¹¹CO₂ by the ¹⁴N(p,α)¹¹C nuclear transformation (Ferrieri and Wolf 1983). Briefly, a 1380 kPa N₂ gas target that includes trace O₂ (99.9995% purity N₂: MG Industries) was irradiated with a 17 MeV proton beam for 1–2 min, depending on the amount of radioactivity desired, and the resulting ¹¹CO₂ was captured on a molecular sieve. The ¹¹CO₂ was administered as a 30 s pulse in a continuous airflow, with a flow rate of 0.02–0.04 l min⁻¹, to a single leaf in a 0.06 liter leaf cuvette lit by red–blue LED arrays, with light intensity adjusted to suit the plant species and desired experimental conditions (e.g. as low as 100 μmol m⁻² s⁻¹ for *A. thaliana*, 800 μmol m⁻² s⁻¹ for maize or sorghum, 1,500 μmol m⁻² s⁻¹ for *N. tabacum*, and potentially up to 3,000 μmol m⁻² s⁻¹). After ¹¹CO₂ administration, plants were incubated with the leaf cuvette in continuous airflow harvested of tissues. The leaf cuvette was fitted with a PIN diode radiation detector for measuring the amount of radioactivity administered to the leaf, the amount fixed by photosynthesis and the rate of export of radioactivity away from the administration zone (Babst et al. 2005, Ferrieri et al. 2005, Gomez et al. 2010, Hanik et al. 2010).

Tissue extraction

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. Leaf tissues from the zone of ¹¹CO₂ administration were excised, and immediately frozen in liquid nitrogen. Tissues were ground to a fine powder under liquid nitrogen using a mortar and pestle, or using a ball mill grinder (Retsch), and aliquoted to frozen, pre-weighed plastic microcentrifuge tubes, which were weighed to obtain sample weights. Samples were extracted in cold 75%–25% methanol:water (200 μl per 100 mg of plant tissue powder) for 10 min in an ultrasonicator bath, kept at 4°C. Tissue residue was removed by centrifugation at 20,000 × g for 2 min, followed by filtration with a 20 μm syringe filter. For [¹³C]starch, soluble sugars were extracted from plant tissue and a STA20 starch assay kit (Sigma) was used to digest starch remaining in the residue, resulting in glucose, which was then measured using the same method as for soluble sugars. We followed the manufacturer’s suggested protocol, which we tested and found adequate (e.g. shorter and longer times), but with a smaller amount of plant tissue sample, and proportionally scaled down reagent volume, such that the procedure could be performed in 2 ml microcentrifuge tubes. Briefly, 30–40 mg of frozen plant tissue was extracted in 1 ml of 80% ethanol for 5 min at 85°C with constant shaking on a programmable thermal mixer (Fisher Scientific), and occasional vortexing. Any residue was rinsed down the walls of the centrifuge tube with 1 ml of additional 80% ethanol, centrifuged at high speed and the supernatant discarded. The residue was washed once more with 2 ml of 80% ethanol. Starch was sequentially digested, first using a heat-stable α-amylase at 98°C for 10 min to break large insoluble starch chains into soluble oligosaccharides, and then an aliquot was taken for amyloglucosidase digestion at 60°C for 15 min, which completed the digestion of oligosaccharides to glucose.

Measurement of [¹¹C]carbohydrates

Methanolic extracts were analyzed by HPTLC to determine sugar concentrations and metabolic partitioning of assimilated ¹¹C to soluble sugars. For initial testing, we used a calibrated micropipettor to apply either 1 or 2 µl of each sample. For later experiments, a semi-automatic Linomat 5 sample applicator (Camag Scientific Inc.) was used for high precision of spot size and sample volume applied. Authentic standards of glucose, fructose and sucrose (0.25–10 mM, depending on the experiment) were applied at 1 µl onto each TLC plate for quantification. Two TLC lanes were spotted for each plant sample, with 1 and 2 µl of methanolic extract, respectively, to ensure that sample concentrations and radioactivity were both within the range of quantification. For future experiments, it may be possible to spot only a single lane, depending on the plant species and environmental conditions. Sugars were separated on 5 × 10 cm NH₂-bonded silica 200 µm HPTLC plates (Sorbent Technologies), with 75% acetonitrile, 25% water (v/v) mobile phase for 15–20 min (based on Klaus et al. 1989). Immediately after developing, TLC plates were dried under a flow of room temperature air for several minutes prior to performing autoradiography by exposing the TLC plate to a phosphor plate for 2–10 min to quantify sugar radioactivity. Phosphor plates were scanned using either a BAS2500 (Fuji) or a Typhoon FLA 7000 scanner (GE Healthcare Life Sciences). The partitioning of ¹¹C radioactivity as a percentage of extract activity was determined using Fuji Image Gauge (ver 3.46) image analysis software, drawing regions of interest around the entire lane as well as the individual sugar spots (see Fig. 3A). The absolute radioactivity of an aliquot of each methanolic extract was also quantified on a calibrated well-type Picker gamma radiation counter, which was used as a calibration factor for the phosphor plate imaging of each TLC plate. All radioactivity values were decay corrected to the pulse time of ¹¹CO₂ administration.
Measurement of $^{12}$C carbohydrates

We tested two methods for determination of sugar concentrations, a naphthol sulfuric acid method and a thermal activation method. For the naphthol sulfuric acid method, each TLC plate was treated with a naphthol sulfuric acid reagent and heated to 100°C for about 5 min (Sherma and Zulick 1996, Payyavula et al. 2011). The naphthol sulfuric acid method results in a purple color for sucrose, pink for glucose and blue for fructose, which could be quantified by image analysis. The thermal activation method required no additional reagent, but only heat treatment of the dry TLC plate in a 200°C oven for 10 min, substantially reducing the hazardous waste output from the procedure (Klaus et al. 1989). The thermal activation method results in fluorescence of the sugar spots under a long-wave UV lamp (365 nm), which can be quantified (Klaus et al. 1990). Digital photographs of the TLC plates were analyzed using Fuji Image Gauge image analysis software to quantify soluble sugar concentrations, but could also be analyzed using other image analysis software (e.g. NIH Image). For starch, when converting the glucose-equivalent mass to mass of starch, we multiplied the glucose-equivalent mass by 0.9 (i.e. 162/180) to reflect the difference in molecular weight between free glucose monomers and polymerized glucose.

Statistics

Linear regression was used for standard curves. Where possible, accuracy was calculated as the average of the differences between the measured and actual values for known authentic standards that were not used to generate the standard curve. Precision is shown as the standard deviation, which was calculated using the pooled variances from repeated measurements of each individual of multiple samples. ‘Technical error’ was calculated as precision (standard deviation) as a percentage of the measured value. The SNR was calculated as the average signal [e.g. counts, mV, photostimulated luminescence (PSL)] of three data points at the chromatogram peak divided by the standard deviation of the same three data points.

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