

A New Substrate Cycle in Plants. Evidence for a High Glucose-Phosphate-to-Glucose Turnover from in Vivo Steady-State and Pulse-Labeling Experiments with [¹³C]Glucose and [¹⁴C]Glucose¹

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Substrate (futile) cycling involving carbohydrate turnover has been widely reported in plant tissues, although its extent, mechanisms, and functions are not well known. In this study, two complementary approaches, short and steady-state labeling experiments, were used to analyze glucose metabolism in maize (*Zea mays*) root tips. Unidirectional rates of synthesis for storage compounds (starch, Suc, and cell wall polysaccharides) were determined by short labeling experiments using [U-¹⁴C]glucose and compared with net synthesis fluxes to determine the rate of glucose production from these storage compounds. Steady-state labeling with [1-¹³C]glucose and [U-¹³C]glucose showed that the redistribution of label between carbon C-1 and C-6 in glucose is close to that in cytosolic hexose-P. These results indicate a high resynthesis flux of glucose from hexose-P that is not accounted for by glucose recycling from storage compounds, thus suggesting the occurrence of a direct glucose-P-to-glucose conversion. An enzyme assay confirmed the presence of substantial glucose-6-phosphatase activity in maize root tips. This new glucose-P-to-glucose cycle was shown to consume around 40% of the ATP generated in the cell, whereas Suc cycling consumes at most 3% to 6% of the ATP produced. The rate of glucose-P cycling differs by a factor of 3 between a maize W22 line and the hybrid maize cv Dea, and is significantly decreased by a carbohydrate starvation pretreatment.

The development of nonphotosynthetic tissues is closely related to Suc import. Suc is degraded by Suc synthase (SuSy) or invertase to provide UDP-Glc or hexoses for the biosynthesis of structural or storage compounds and for ATP production (Fig. 1). In 1988, Hargreaves and ap Rees used pulse-chase experiments to show the presence of a cycle of synthesis and degradation of Suc in pea roots. This cycle, consuming ATP without any apparent physiological function, was described as a futile cycle, according to Fell's definition (Fell, 1997), and, according to more recent terminology, which refers to a possible role for these processes, it can be described as a substrate cycle (the Suc cycle). Since then, the Suc cycle has been

found in many other tissues: chenopodium cells, potato (*Solanum tuberosum*) tubers, ripening banana (*Musa cavendishii*), maize (*Zea mays*) root tips, tomato (*Lycopersicon esculentum*) cells, and tomato fruit (Hatzfeld and Stitt, 1990; Hill and ap Rees, 1994; Dieuaide-Noubhani et al., 1995, 1997; N'tchobo et al., 1999; Rontein et al., 2002). One can estimate that, for the synthesis and degradation of one molecule of Suc, 1.5 to two molecules of ATP are consumed (Fig. 1). Depending on the tissue, this cycle could consume between 5% and 70% of the ATP produced by the cell (Hatzfeld and Stitt, 1990; Hill and ap Rees, 1994; Dieuaide-Noubhani et al., 1995; Fernie et al., 2002; Rontein et al., 2002).

Two other substrate cycles related to central carbohydrate metabolism have been described in plants, hexose-P ↔ triose-P cycling and the starch synthesis and degradation cycle. The occurrence of extensive cycling between starch or Suc and the triose-P has been confirmed by independent methods (Glawischnig et al., 2002), including the measurement of δ¹³C ratios in intact organs (Gleixner et al., 1998). However, the energy cost of these cycling phenomena has not been established. In a recent review, Portais and Delort (2002) underlined the need to distinguish between reversibility, metabolic cycles, and substrate cycles. Hexose-P ↔ triose-P cycling may be a substrate cycle or a reversibility flux, depending on the enzymes

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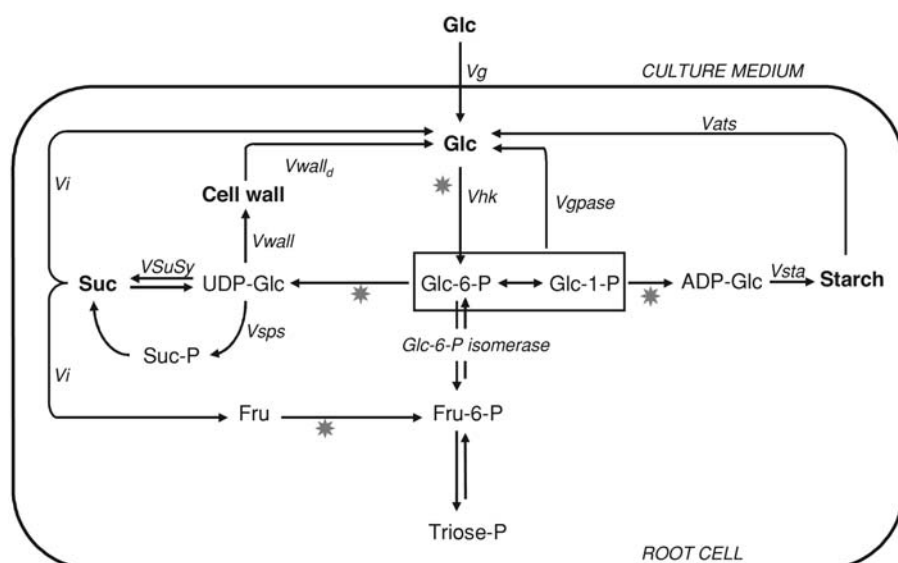


Figure 1. Metabolic scheme. The principal pathways considered in this study. The stars indicate the reactions consuming ATP or UTP and the flux names are indicated. *Vg*, Glc uptake; *Vhk*, hexokinase activity; *Vgpase*, Glc-6-phosphatase activity; *Vsta*, starch synthesis; *Vats*, starch degradation; *VSuSy*, Suc synthase activity; *Vsps*, Suc phosphate synthase activity; *Vi*, invertase activity; *Vwall*, cell wall polysaccharide synthesis; *Vwall_d*, cell wall polysaccharide degradation.

involved. It fits Fell's definition of a substrate cycle when phosphofruktokinase and Fru-bisphosphate are involved. The action of inorganic pyrophosphate (PPi):Fru-6-P phosphotransferase, which catalyzes a reversible reaction without consuming ATP, generates a reversibility cycle, not a substrate cycle. The operation of these alternative pathways has the same impact on label distribution patterns (Fernie et al., 2001). Since they are not distinguishable in labeling experiments, their relative importance and thus an evaluation of the energy cost remains difficult.

In fact, substrate cycles involving sugars also exist in microorganisms (for review, see Portais and Delort, 2002) and animal cells (Landau, 1999; Jones et al., 2002), and their roles are still a matter of debate. It has been suggested that they could serve to buffer metabolite concentrations, improve sensitivity in metabolic regulation, or rapidly change the direction of net flux (Newsholme et al., 1984; Fell, 1997). In the yeast *Pichia pastoris*, Teusink et al. (1998) and Blomberg (2000) have suggested that the cycling of trehalose could help to regenerate inorganic phosphate (Pi) sequestered in the hexose-P pool from the first part of glycolysis, thus avoiding phosphate depletion. In plants, a possible role for substrate cycles in allowing a rapid change in the direction of net flux has been suggested by the work of Geigenberger et al. (1997). These authors have shown that fluxes through the Suc and starch cycles favoring synthesis of starch from Suc in potato could be reversed in response to water deficit, thus allowing a net accumulation of Suc. Hill and ap Rees (1995) suggested that the modification of fluxes through different substrate cycles (an increase in hexose-P ↔ triose-P cycling and a decrease in the starch and Suc cycles) could participate in keeping the ATP-to-ADP ratio constant when oxygen pressure is lowered. In tomato fruit, Suc cycling may function to increase the efficiency of sugar storage in the cytosol and vacuole

(Nguyen-Quoc and Foyer, 2001). Interestingly, increasing Suc degradation activity in potato by transforming plants with a cytosolic invertase or a Suc phosphorylase leads to increases in Suc cycling and in the respiratory rate in the tuber (Trethewey et al., 1998, 1999, 2001; Fernie et al., 2002). The increase in glycolytic rate could result from an increase in the energy demand of Suc cycling, thus suggesting that glycolysis in plants is controlled by the rate of ATP utilization (Fernie et al., 2002).

A high degree of variation in flux around the Suc cycle has been reported. Two approaches for quantifying this flux have been described, both based on isotopic labeling and both having limitations that could lead to misrepresentation of the true flux. In one approach, the unidirectional flux of Suc synthesis was measured in a short time-labeling experiment from the rate of the incorporation of radioactivity into Suc (Hargeaves and ap Rees, 1988; Hatzfeld and Stitt, 1990; Hill and ap Rees, 1994, 1995). The unidirectional degradation flux was then calculated as the difference between the net flux of accumulation or degradation and the unidirectional flux of synthesis. The pitfall of this method, in the case of a substrate cycle, is that the unidirectional synthesis flux may be underestimated because of the loss of radioactivity in Suc due to degradation reactions. The second approach was based on steady-state labeling (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Schwender et al., 2003), where fluxes were calculated from equations describing the relation between input and output fluxes of label at the level of each atom (Katz and Grunnet, 1979). In contrast to the previous method, the sequence of reactions that link two metabolites cannot be determined from steady-state labeling; various sets of reaction may give the same distribution of label, and the reaction(s) actually involved must be deduced by other methods, such as enzyme identification and

compartmentation studies (Ferne et al., 2001). In maize root tips labeled with $[1-^{13}\text{C}]\text{Glc}$, the observation of label redistribution from carbon C-1 to C-6 of Glc indicated that the influx of Glc from the medium was not the only source of intracellular Glc, and that a flux from hexose-P to Glc should be considered (Dieuaide-Noubhani et al., 1995). Because the turnover of cell wall polysaccharides and starch was considered to be low in growing maize root tips, and in the absence of a clear indication of Glc-6-phosphatase activity in plant cells, the whole flux from hexose-P to Glc was attributed to the Suc cycle.

The high cost in ATP of hexose-P-to-Glc cycling (Fig. 1) could make it important for the regulation of plant cell metabolism. It is thus essential to identify and quantify the pathways leading to intracellular Glc accurately so as to understand both the role of substrate cycles in plant cells and their regulation. The goal of this work was to provide experimental data on the pathways potentially responsible for the hexose-P-to-Glc conversion. Since the established pathways were not sufficient to account for the redistribution of the labeling between carbon C-1 and C-6 observed in Glc, we conclude that this redistribution results mostly from Glc-phosphatase activity. These results are discussed in relation to the measurement of Glc-6-phosphatase activity in vitro and the potential localization of this activity in the cell.

RESULTS

Establishment of Isotopic and Metabolic Steady State upon Incubation with Glc

For steady-state labeling experiments, metabolic and isotopic steady state must be reached. Maize root tips were incubated with labeled substrates directly after excision. The time necessary to reach isotopic and metabolic steady state was determined by incubating maize roots with $[\text{U}-^{14}\text{C}]\text{Glc}$ and monitoring the evolution of the specific radioactivity (SR) in Glu and Asp in the last 3.5 mm of the roots. For these measurements, the roots were re-excised at 3.5 mm after incubation and labeling was monitored in these last 3.5 mm of the roots. The SR of Glu and Asp increased for 15 h and then reached a plateau, indicating that the intermediates of the glycolysis and those of the citric acid cycle had reached steady state (data not shown).

In order to further verify the time necessary to reach steady state, maize root tips were incubated with $[1-^{13}\text{C}]\text{Glc}$ for 25 and 30 h. The resonances assigned to Glc and Suc extracted from the last 3.5 mm of the roots showed the same enrichments after these two incubation times, thus confirming that sugars were at isotopic steady state after 25 h of incubation (data not shown). As a consequence, the time used for metabolic and isotopic steady-state labeling was set at 25 h.

Net Sugar Synthesis in Re-Excised Maize Root Tips Is Low

To determine the net fluxes of Suc, Glc, and starch synthesis, maize root tips were incubated 23 h in the nutritive solution containing Glc, re-excised to 3.5 mm, and further incubated for 2 or 4 h in the same medium. After 23-h incubation in the presence of Glc, re-excised maize root tips contained $72 \pm 14 \mu\text{mol}$ of Glc/g fresh weight (FW), $34 \pm 6 \mu\text{mol}$ of Suc/g FW, and $11.4 \pm 2.6 \mu\text{mol}$ of starch/g FW. No significant differences for Suc, Glc, and starch contents in the last 3.5 mm were observed over a period of 4 h. These results strongly support the existence of a metabolic steady state after 25 h of incubation and show that the rates of net synthesis of Suc, Glc, and starch are negligible.

Synthesis of Cell Wall Polysaccharides and Turnover of Suc and Starch

Short time-labeling experiments were conducted after an initial incubation period of 25 h with unlabeled Glc to allow the root tips to reach metabolic steady state. The flux values obtained are presented in Table I.

Glc Consumption Rate

Maize root tips were incubated with $[\text{U}-^{14}\text{C}]\text{Glc}$ for 5, 10, 15, and 20 min. The rate of Glc consumption (V_g) was determined by dividing the slope of the curve of radioactivity incorporation by the roots (Fig. 2) by the SR of extracellular Glc. The incubation times were selected to be as short as possible to lessen the underestimation of that flux due to the catabolism of Glc, leading to losses of labeling as CO_2 (by the pentose phosphate pathway, the UDP-glucuronate-decarboxylase reaction involved in synthesizing cell wall pentoses, and the citric acid cycle). V_g was found to be $394 \pm 73 \text{ nmol min}^{-1} \text{ g}^{-1} \text{ FW}$.

Rates of Starch and Suc Turnover

To quantify the unidirectional rates of Suc and starch synthesis, maize root tips were incubated for 15, 30, and 60 min with $[\text{U}-^{14}\text{C}]\text{Glc}$. The radioactivity incorporated into the glucosyl units of Suc and starch (data not shown), and the SR of their precursors (Fig. 3) were estimated. UDP-Glc and ADP-Glc are usually

Table I. Metabolic fluxes involved in Glc/hexose-P interconversion ($\text{nmol Glc min}^{-1} \text{ g}^{-1} \text{ FW}$) in the W22 maize line root tips

Intermediary Metabolite	Hexose-P Consumption	Toward Glc Pool
Glc influx		$V_g: 394 \pm 73$
Cell wall	$V_{\text{wall}}: 52 \pm 14$	$V_{\text{wall}_d}: \leq 52$
Starch	$V_{\text{sta}}: 30 \pm 11$	$V_{\text{ats}}: \leq 30$
Sucrose	$V_{\text{suc}}: 178 \pm 36$	$V_i: \leq 178$
Hexose-P	$V_{\text{res}}: 2,700 \pm 520$	$V_{\text{gpase}}: 2,500 \pm 520$

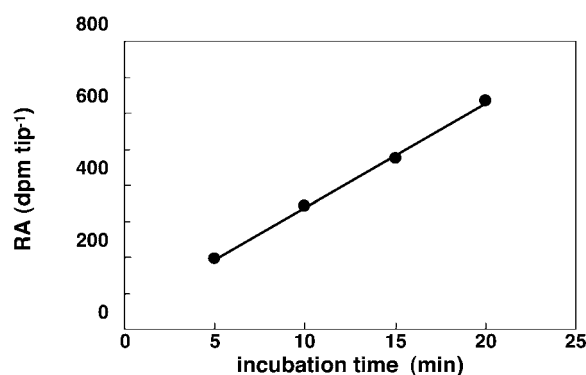


Figure 2. $[U-^{14}C]$ Glc uptake by maize root tips. Maize root tips were incubated with 200 mM Glc during 25 h and transferred into a medium containing 200 mM $[U-^{14}C]$ Glc (30 dpm nmol^{-1}) during 5, 10, 15, and 20 min. The maize root tips were then grounded and radioactivity was counted. These data are from one representative experiment, repeated four times.

rapidly exchanged with Glc-6-P through the UDP-Glc and ADP-Glc pyrophosphorylase reactions, so that Glc-6-P can be used as an indicator of the labeling of UDP-Glc and ADP-Glc. However, UDP-Glc is also produced by the SuSy reaction. Thus, in short time-labeling experiments, and if the SuSy reaction rate is high, UDP-Glc coming from Glc-6-P might be diluted by UDP-Glc coming from Suc. Using the SR of Glc-6-P instead of UDP-Glc could therefore lead to an underestimation of the rate of Suc synthesis. Figure 3 shows the increase of the SR of Glc-6-P and UDP-Glc. No significant differences were found between the SR of Glc-6-P and UDP-Glc. Therefore, Glc-6-P was considered a good indicator of the SR of UDP-Glc. This observation of labeling equilibrium agrees with previously published data (Geigenberger et al., 1997; Roscher et al. 1998) showing that many enzymes of sugar metabolism, including UDP-Glc pyrophosphorylase and hexose-P isomerase, catalyze near-equilibrium reactions.

The amounts of Suc and starch produced after 15, 30, and 60 min (Fig. 4, A and B) were calculated from the radioactivity incorporated into Suc and starch glucosyls, respectively, using the following equation:

$$N_{n+1} = N_n + (RA_{n+1} - RA_n) / G6P_n, \quad (1)$$

where N_n is the number of moles formed, RA_n is the amount of radioactivity incorporated into the compound studied (expressed in disintegrations per minute $[dpm] \text{g}^{-1} \text{FW}$), $G6P_n$ is the SR of Glc-6-P, and n indicates the n th time point of incubation.

We have emphasized in "Materials and Methods" that it is difficult to measure the flux of synthesis of a compound involved in a cycle of synthesis and degradation because of the loss of radioactivity by the degradation reaction. After 60 min, the SR of the glucosyl moiety of Suc reached $0.09 \text{ dpm nmol}^{-1}$, which is 21% of that of the precursor Glc-6-P ($0.43 \text{ dpm nmol}^{-1}$). This indicates that the pathway is still far

from isotopic steady state, which makes it reasonable to assume that losses of radioactivity are negligible. The same applies to starch, where the SR of glucosyl units was about $0.05 \text{ dpm nmol}^{-1}$. The linearity of the curves of Suc and starch synthesis (Fig. 4) confirms that losses of radioactivity due to turnover were weak enough to avoid underestimation of the rate of incorporation of the tracer into its product. Therefore, we can estimate the unidirectional rate of Suc synthesis, V_{suc} , which corresponds to the sum of the fluxes through the Suc-P synthase and the SuSy reactions, was estimated at $178 \pm 36 \text{ nmol min}^{-1} \text{g}^{-1} \text{FW}$. The unidirectional rate of starch synthesis, V_{sta} , was $30 \pm 11 \text{ nmol min}^{-1} \text{g}^{-1} \text{FW}$ (Table I).

These unidirectional rates of Suc and starch synthesis would, in the absence of degradation, lead to an increase over 4 h of approximately 43 and 7 μmol of Suc and starch, respectively, which are significant amounts compared to root tip contents of $34 \pm 6 \mu\text{mol}$ of Suc $\text{g}^{-1} \text{FW}$ and $11.4 \pm 2.6 \mu\text{mol}$ of starch $\text{g}^{-1} \text{FW}$. However, the content in Suc or starch did not change over 4 h (data not shown). These results indicate that the net flux to Suc was very low compared to the unidirectional flux of Suc synthesis, i.e. that the synthesis and degradation of Suc and starch occurred at the same rates.

Rate of Cell Wall Polysaccharide Synthesis

Maize root tips were incubated for 30, 60, and 120 min with $[U-^{14}C]$ Glc. Cell wall polysaccharides were separated from other compounds (see "Materials and Methods"). Using Equation 1, the unidirectional rate of synthesis of cell wall polysaccharides (Fig. 4C) was found to be $52 \pm 14 \text{ nmol min}^{-1} \text{g}^{-1} \text{FW}$.

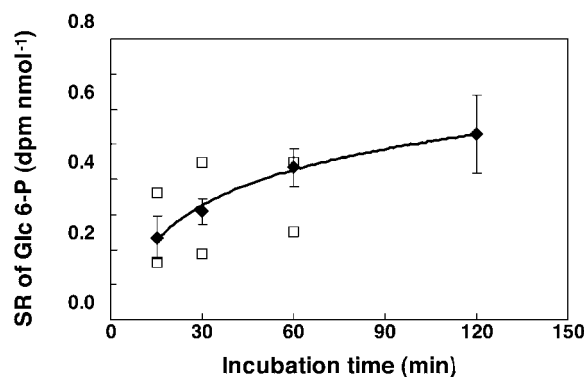


Figure 3. Evolution of the SR of Glc-6-P (\blacklozenge) and UDP-Glc (\square). Maize root tips were incubated with 200 mM Glc during 25 h and transferred into a medium containing 200 mM $[U-^{14}C]$ Glc ($100\text{--}200 \text{ dpm nmol}^{-1}$ according to the experiment) during 15, 30, 60, and 120 min. Following a perchloric extraction, the SR of Glc-6-P and UDP-Glc were obtained as described in "Materials and Methods." They were normalized by dividing the measured values by the SR of extracellular Glc. For Glc-6-P, the values indicated after 15, 30, 60, and 120 min are the mean of four or six values (\pm SD), obtained in three independent experiments. For UDP-Glc, the symbols correspond to SR values obtained in two independent experiments.

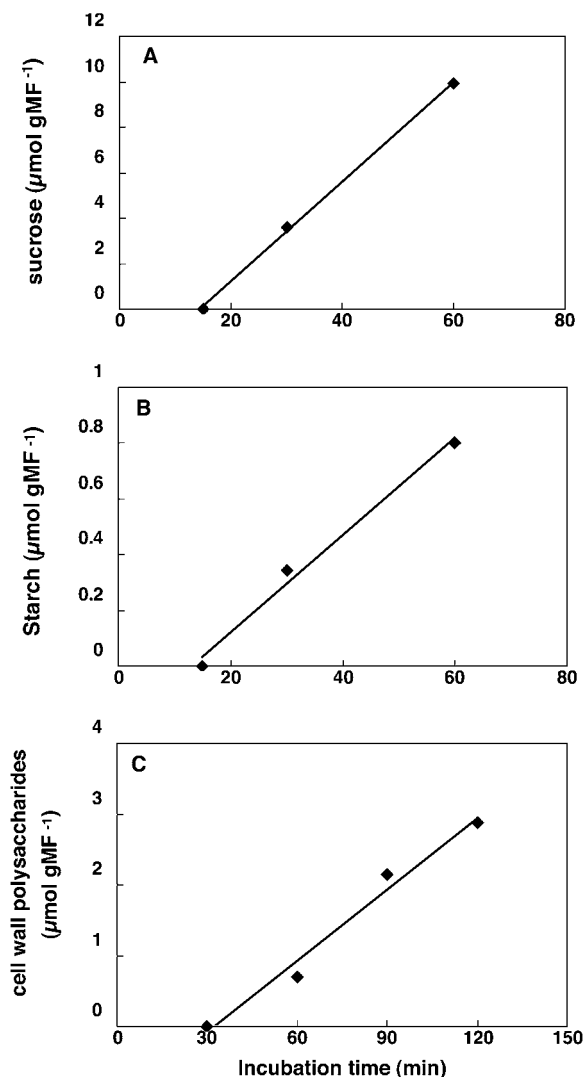


Figure 4. Determination of the unidirectional rates of Suc synthesis, starch, and cell wall polysaccharides. Maize root tips were incubated with 200 mM Glc during 25 h and transferred into a medium containing 200 mM [U-¹⁴C]Glc (30 to 380 dpm nmol⁻¹ according to the experiment) during 15, 30, and 60 min. A time of 90 min was added in experiments performed for cell wall polysaccharide studies. Because Suc was partially degraded by invertase activities in the grinding buffer used for the separation of cell wall polysaccharides, separate experiments were carried out for Suc and cell wall polysaccharide analysis (see “Materials and Methods”). Starch was purified as Glc, after ethanolic extraction as well as from samples allowing cell wall polysaccharide separation. Radioactivity incorporated into glucosyl moieties of Suc and starch and into polysaccharides was counted and normalized by dividing by the SR of external Glc. The quantities of radioactivity incorporated during a time interval were then converted to the amount of sugar produced, expressed in mol/g FW, using the equation $N_{n+1} = N_n + (RA_{n+1} - RA_n)/G6P_n$. N_n , RA_n , and $G6P_n$ correspond, respectively, to the number of moles, the radioactivity incorporated into the compound studied (expressed in dpm/g FW), and the SR of Glc-6-P; n indicates the n th time point of incubation. The results presented here correspond to one typical experiment, repeated five, five, and three times for Suc, starch, and cell wall polysaccharides, respectively. These data were used to quantify the unidirectional rate of Suc, starch, and cell wall polysaccharide syntheses.

Total Glc-P-to-Glc Recycling

Total Glc-P-to-Glc recycling was quantified after labeling to isotopic steady state and modeling, as described in “Materials and Methods.”

Analysis of Sugar Enrichments

Figure 5 shows typical NMR spectra of Glc and Suc. The ¹³C enrichments, measured and then corrected by the dilution factors (see “Materials and Methods”), are shown in Table II. At isotopic steady state, the labeling of the glucosyl and fructosyl moieties of Suc reflects the labeling of cytosolic Glc-6-P and Fru-6-P, respectively (Fig. 1). Compared to the carbon enrichment of external Glc (98% above natural abundance), we observed a dilution of the total enrichment of the hexose moieties of Suc ($Sg1 + Sg6 = 92.2\%$ above natural abundance), and a redistribution of label from the carbon C-1 position to C-6. Previous results in root tips of the maize cv Dea hybrid (Dieuaide-Noubhani et al., 1995) indicated that these effects result from a combination of the pentose phosphate pathway in the plastid, the reversibility of glycolysis between triose-P and hexose-P, and a cytosolic transaldolase activity. These data on the W22 line are consistent with the same metabolic scheme. However, it is notable that there was less redistribution from C-1 to C-6 in the glucosyl ($C-6/C-1 = 0.23 \pm 0.02$) than in the fructosyl moiety ($C-6/C-1 = 0.27 \pm 0.02$) of Suc ($P < 0.05$ in t test), suggesting that the hexose-P isomerase reaction is not at equilibrium. On the other hand, the C-1 to C-6 redistribution observed in Glc was close to that observed for the Suc glucosyl, which indicates that the Glc-P-to-Glc cycling is high compared to the influx of Glc.

The ratio of enrichment C-1 to C-6 of Fru was quantified in two independent experiments according to ¹³C-NMR spectra (data not shown). Its value, 3.9 ± 0.2 , was close to that measured for the fructosyl moiety of Suc (3.7 ± 0.3 ; see Table IV), thus indicating that Fru was produced mainly from the Suc turnover via invertase or through the reversibility of the SuSy reaction, or from Fru 6-P through a hexose-phosphatase reaction.

Calculation of the Glc-P-to-Glc Flux

Of the three pathways that may produce Glc from Glc-P, we considered here only the two routes involving invertase and Glc-phosphatase activities. This approximation could be made because the turnover of starch is low (see above) and because the turnover of cell wall polysaccharides is probably negligible in growing tissues (Haigler et al., 2001). Two equations can be written to describe the metabolic and isotopic steady-state labeling of the C-1 and C-6 carbons of intracellular Glc; these involve the fluxes V_g (rate of Glc uptake) and V_{res} (rate of Glc resynthesis) resulting from two enzymatic paths, invertase or Glc-phosphatase.

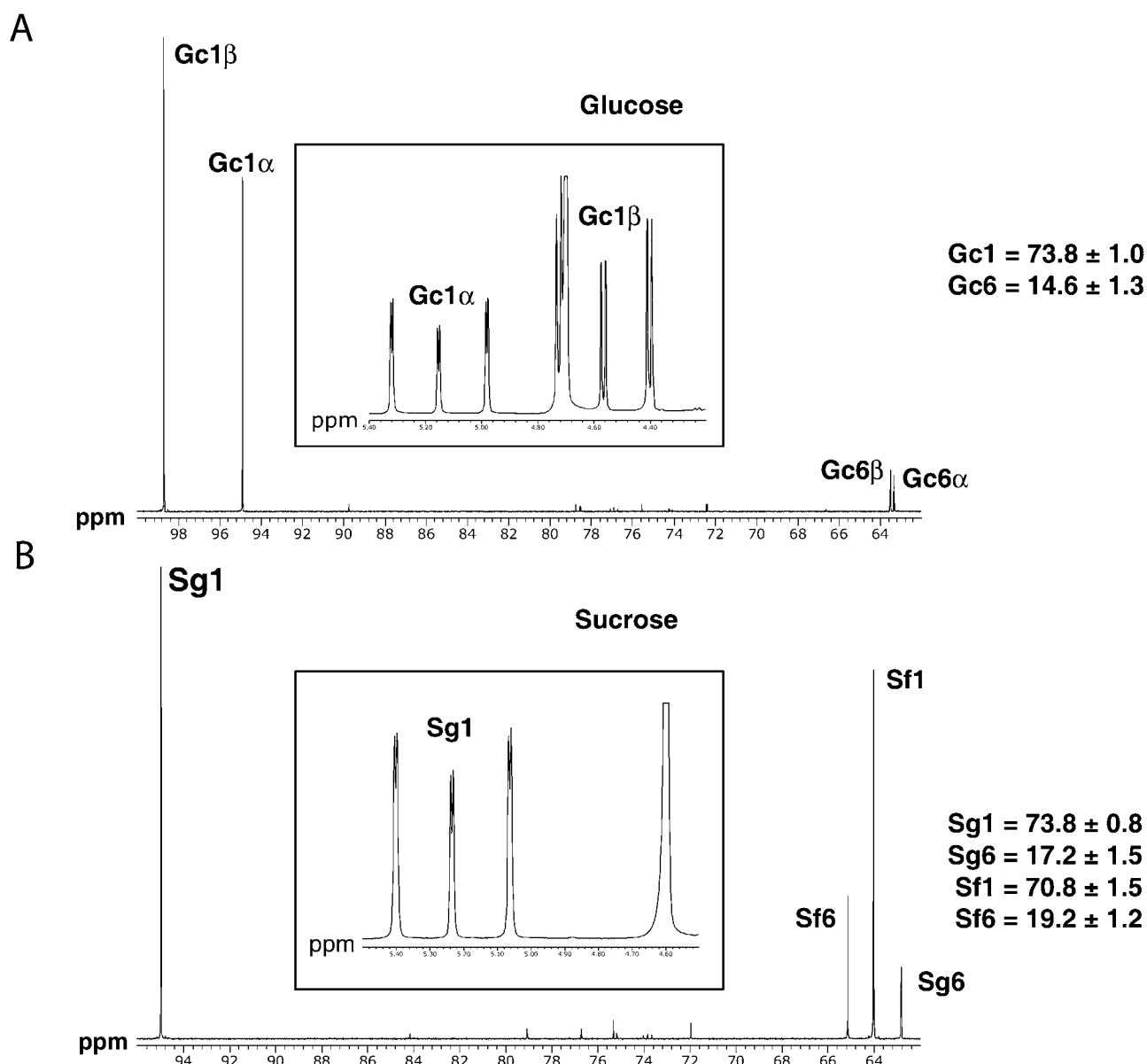


Figure 5. ^{13}C and ^1H (insert) spectra of cellular Glc (A) and Suc (B). Maize root tips were incubated for 25 h with $\text{D-}[1-^{13}\text{C}]\text{Glc}$ 200 mM. Glc and Suc were purified and analyzed by ^1H - and ^{13}C -NMR as described in "Materials and Methods." $\text{Gc}\alpha$, $\text{Gc}\beta$, Sg_i , and Sf_i correspond to the resonance of the carbon i of Glc- α and Glc- β , the glucosyl and fructosyl moieties of Suc, respectively. For the studied carbon atoms, the enrichment values were obtained as described in "Materials and Methods" and reported on the right of the spectra. The spectra are representative of one labeling experiment repeated four times.

$$V_g \times G_{\text{ex}1} + V_{\text{res}} \times \text{Sg}1 = (V_g + V_{\text{res}}) \times G_{\text{in}1} \quad (2)$$

$$V_g \times G_{\text{ex}6} + V_{\text{res}} \times \text{Sg}6 = (V_g + V_{\text{res}}) \times G_{\text{in}6} \quad (3)$$

$G_{\text{ex}1}$, $\text{Sg}1$, and $G_{\text{in}1}$ represent the enrichment of the C-1 of extracellular Glc, the glucosyl moiety of Suc, and intracellular Glc, respectively, and $G_{\text{ex}6}$, $\text{Sg}6$, and $G_{\text{in}6}$ represent the enrichments of C-6 in the same molecules. To solve these two equations, the enrichments of C-1 and C-6 of Suc and Glc must be determined. However, steady-state labeling of maize root tips with $[\text{U-}^{14}\text{C}]\text{Glc}$ reveals the presence of unlabeled

pools of sugars, probably located in the vacuole (see "Materials and Methods"). For each soluble sugar, a diluting factor was determined, allowing calculation of the enrichments of metabolically active pools (corrected enrichment; Table II) from the measured enrichments.

A new equation (Eq. 4) can be deduced from these two equations involving the enrichment ratios C-1 to C-6 for all sugars considered and the absolute enrichment of C-6 of Suc glucosyl. Using a ratio of enrichments in flux calculation eliminates the error introduced by the Glc diluting factors.

Table II. Specific ^{13}C enrichments of the carbon atoms of soluble sugars

Maize root tips were incubated for 25 h with 200 mM D-[1- ^{13}C]Glc. The Glc and Suc were purified by HPLC and analyzed by ^1H - and ^{13}C -NMR. The enrichment values of the C-1 carbons were calculated from ^1H -NMR spectra, those of the C-2 to C-6 carbons, from ^{13}C -NMR spectra. The results reported as measured enrichments are the average \pm SD of five independent experiments. For Glc and Suc, as the enrichments of carbons C-2 to C-5 are close to natural abundance, they are not reported in the table. The measured enrichments obtained after a steady-state labeling with [1- ^{13}C]Glc were corrected by the diluting factor according to the following formula: $E_{\text{corrected}} = (E_{\text{measured}} - 1.1) \cdot \text{DF} + 1.1$, where DF is the diluting factor (see "Materials and Methods"), 1.066 and 1.038 for Glc and Suc, respectively. These values are reported as corrected enrichments.

	Glucose	Sucrose	
		Glucosyl	Fructosyl
Measured enrichments (%)			
C-1	73.8 \pm 1.0	73.8 \pm 0.8	70.8 \pm 1.5
C-6	14.6 \pm 1.3	17.2 \pm 1.5	19.2 \pm 1.2
Corrected enrichments (%)			
C-1	78.6 \pm 2.7	76.6 \pm 1.1	73.4 \pm 1.8
C-6	15.5 \pm 1.7	17.8 \pm 1.6	19.9 \pm 1.3

$$V_{\text{res}} = V_{\text{g}} \times (G_{\text{ex}1}/S_{\text{g}6} - G_{\text{in}1}/G_{\text{in}6} \times G_{\text{ex}6}/S_{\text{g}6}) / (G_{\text{in}1}/G_{\text{in}6} - S_{\text{g}1}/S_{\text{g}6}) \quad (4)$$

Using the enrichments presented in Table II to solve Equation 4, V_{res} was found to be $2.7 \pm 0.5 \mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$. This flux must then be compared to the flux through Suc cycling, estimated at $178 \text{ nmol min}^{-1} \text{g}^{-1} \text{FW}$ from ^{14}C pulse labeling (Table I). The large difference between these two values implies that the Suc cycle cannot account for the observed labeling of Glc. Therefore, a flux through a Glc-phosphatase must be considered. By subtraction of the flux through the Suc cycle, the flux catalyzed by the Glc-phosphatase was estimated at $2.5 \pm 0.5 \mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$.

Phosphatase Activities in Maize Root Tips

The existence of this new cycle requires substantial phosphatase activity. Phosphatase activity was therefore measured in extracts obtained from maize Dea and W22 root tips, using Glc-6-P as substrate (Table III). Because of the uncertainty concerning the sub-cellular localization of Glc-6-phosphatase in plant cells, we measured the activity at pH 5.5 and pH 7.5, corresponding to the vacuolar and cytosolic pH in maize root tips (R.M. Brouquisse, D. Rolin, and C. Roby, unpublished data). At both pHs, the activity measured in W22 root tips was higher than in Dea root tips. It is interesting to note that the activities measured at pH 7.5 were low compared to the flux measured in vivo. On the other hand, the activities measured at the vacuolar pH were of the same order as the fluxes measured in vivo.

Fluxes through the Substrate Cycle Depend on the Maize Variety and Are Sensitive to Starvation Pretreatment

In order to compare these data on W22 with those obtained on the Dea hybrid (Dieuaide-Noubhani et al., 1995), steady-state labeling with [1- ^{13}C]Glc was performed (1) with W22 according to the protocol previously used for Dea, i.e. after starvation pretreatment (4 h without Glc in medium before feeding with [1- ^{13}C]Glc) and (2) with Dea according to this protocol (see "Materials and Methods"), i.e. without starvation pretreatment. The ratios of enrichments C-1 to C-6 were determined for the intracellular Glc and the glucosyl and fructosyl moieties of Suc, and the relative Glc-P-to-Glc conversion fluxes (V_{res}) calculated from these data and Eq. (4). Results are shown in Table IV.

Effects of Starvation Pretreatment

For both lines, starvation pretreatment induced a substantial decrease of the randomization of labeling from C-1 to C-6 in Glc and in Suc. Moreover, it appears that starvation led to a significant reduction of the recycling of Glc-P to Glc in the W22 variety, but had little or no effect in Dea.

Comparison of W22 and Dea Metabolism

The flux of Glc-P-to-Glc conversion was higher in W22 than in Dea, regardless of Glc starvation pretreatment. In order to estimate the Glc-phosphatase flux (V_{gpase}) in Dea maize, we determined the flux of Suc synthesis (V_{suc}) and the Glc influx (V_{g}) after 25 h of incubation with Glc (Table IV). In Dea, V_{g} ($350 \text{ nmol min}^{-1} \text{g}^{-1} \text{FW}$) was similar to that observed in W22 (Table I). In the absence of Suc, accumulation in Dea (Dieuaide-Noubhani et al., 1995), V_i , the unidirectional flux of Suc degradation, is equal to V_{suc} , thus indicating that the maximal flux through invertase was $V_i = 0.2 \mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$, whereas V_{gpase} was around $0.8 \mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$. These data clearly show that the difference measured in V_{res} was a consequence of the increase of Glc-phosphatase activity in W22 compared to Dea, the Suc hydrolysis rate being comparatively low in both genotypes.

Table III. Glc-6-phosphatase activities

Glc-6-phosphatase activities were measured in maize Dea and W22 root tips at pH 5.5 (vacuolar pH) and pH 7.5 (cytosolic pH) as described in "Materials and Methods." The values represent the mean (\pm SD) of two (activity measured at pH 5.5) and four (activity measured at pH 7.5) separate experiments.

	pH 5.5	pH 7.5
	Glc-6-phosphatase activity ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$)	
Dea	1.5 \pm 0.3	0.55 \pm 0.1
W22	1.5 \pm 0.2	0.84 \pm 0.1

Table IV. Comparison between maize Dea and W22 incubated with Glc with or without starvation pretreatment

Maize root tips were incubated with [U-¹⁴C]Glc and [1-¹³C]Glc (see "Materials and Methods") after starvation pretreatment (maize root tips were incubated 4 h without Glc and then 25 h plus labeled Glc) or not. For Dea, with starvation pretreatment, the ratio C-1 to C-6 and the fluxes were calculated from data published by Dieuaide-Noubhani et al. (1995). All fluxes, V_{res} , V_{gpase} , and V_i were determined as for W22 (see "Results"). The values of relative fluxes were calculated for a rate of Glc consumption (V_g) equal to 1. The values of absolute fluxes were then calculated for V_g equal to 350 and 394 nmol min⁻¹ g⁻¹ FW for Dea and W22, respectively. Results are given as mean \pm SD ($n = 5$ for W22 without starvation pretreatment, and $n = 2$ for W22 with starvation pretreatment). For Dea without starvation pretreatment, results are from one experiment.

	Without Starvation Pretreatment		With Starvation Pretreatment	
	Dea	W22	Dea	W22
Enrichment ratio C-1 to C-6				
Sucrose glucosyl	4.6	4.3 \pm 0.4	5.7	5.9 \pm 0.4
Sucrose fructosyl	3.8	3.7 \pm 0.3	5.4	5.0 \pm 0.2
Glucose	6.5	5.1 \pm 0.5	8.6	8.1 \pm 0.4
Relative fluxes				
V_{res}	2.9	6.86 \pm 1.33	2.1	3.0 \pm 0.2
Absolute fluxes (μ mol min ⁻¹ g ⁻¹ FW)				
V_{res}	1.0	2.70 \pm 0.52		
V_i	0.2	0.15 \pm 0.03		
V_{gpase}	0.8	2.55 \pm 0.52		

DISCUSSION

Carbohydrate Starvation Reduces Substrate Cycling and Perturbs Metabolism

Internal pools of sugars can limit metabolic studies in plants because they delay the time necessary for reaching steady-state labeling. In a previous study, Dieuaide-Noubhani et al. (1995) starved maize root tips for 4 h before labeling. This treatment reduced the intracellular pools of sugars and the time required to reach isotopic steady state at 12 h, but it could potentially alter metabolic rates.

The prestarvation treatment significantly affects carbon enrichments (data not shown) and increases the C-1 to C-6 ratios (Table IV) in the hexose units of Suc and starch and in free Glc. The increase of the C-1 to C-6 ratio in free Glc indicates that the prestarvation treatment lowers hexose-P cycling by a factor of 2 in the W22 line (see Table IV). The C1-C6 randomization in Suc and starch, i.e. in hexose-P, has been observed in most plant materials (Hatzfeld and Stitt, 1990, Viola et al., 1991, Dieuaide-Noubhani et al., 1995, Rontein et al., 2002). It has been attributed to triose-P recycling through the PPI-dependent Fru-6-P phosphotransferase reaction (Hatzfeld and Stitt, 1990, Krook et al. 1998). In addition, it is established that the enrichment of C-1 is decreased by flux of hexose-P through the oxidative pentose phosphate pathway, and a careful examination of the enrichments of the C-1 and C-6 Glc carbons showed that part of this randomization was due to transaldolase activity (Dieuaide-Noubhani et al., 1995). The lower C-1 to C-6 ratio observed in control, compared to starvation pretreated root tips (Table IV), indicates that one or more of these fluxes of

central metabolism are decreased by the starvation pretreatment relative to Glc uptake rate. In the absence of a complete analysis of fluxes, it is difficult to determine whether the variation of the C-1 to C-6 ratio is due to an increase in V_g , a decrease in the other fluxes, or both. However, it has been established that a number of treatments leading to an increased demand for carbohydrates induce the expression of Glc transporters at the plasma membrane (Sakr et al., 1997; Truernit et al., 1996). According to our data, Dea maize roots incubated immediately after excision absorbed Glc at a rate estimated at 1.1 nmol min⁻¹ per root, which is 3 times lower than the value measured by Dieuaide-Noubhani et al. (1995). Thus, the higher C-1 to C-6 ratio observed after prestarvation treatment is likely to result, at least in part, from an increased Glc uptake.

Glc-P-to-Glc Conversion Cannot Be Explained by the Suc Cycle Alone and Suggests Glc-Phosphatase Activity

In steady-state labeling experiments, C-1 to C-6 scrambling of the intracellular Glc pool indicates that it is formed not only from exogenous Glc, but also from molecules derived from hexose-P. Using steady-state modeling, the total flux from hexose-P to Glc (V_{res}) was found to be 3 and 6 times the Glc influx in the Dea hybrid and the W22 line, respectively. This confirms the importance of hexose-P recycling in maize root tips (Dieuaide-Noubhani et al., 1995).

The hexose-P could have been directly degraded to Glc by a Glc-phosphatase or incorporated into Suc, starch, and cell wall polysaccharides before being returned to the Glc pool (see Fig. 1). In previous

studies (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002), the simplifying hypothesis was that the Glc-P-to-Glc conversion occurred only via Suc cycling. In this work, the possible contribution of turnover of different cellular sugar fractions to Glc resynthesis was evaluated by measuring their rate of synthesis using pulse-labeling experiments and calculating the reverse flux (Glc production) from the net accumulation. According to our data, Suc turnover accounts for only 7% of the total flux of Glc-P-to-Glc conversion. This low value is probably an overestimate because Suc is not degraded by invertase only, but also by SuSy, whose activity is included in the total Suc turnover measurement but does not contribute to *V_{res}*.

Starch and cell wall polysaccharide synthesis and turnover rates are low, and their minor contribution to Glc resynthesis was neglected. To confirm whether these turnovers contribute significantly to *V_{res}*, and to evaluate the possible error in *V_{gppase}* flux caused by ignoring them, we considered the unidirectional rates of starch and cell wall polysaccharides syntheses as the maximum rate of their Glc-P-to-Glc conversion. From this, it may be seen that starch and cell wall polysaccharide turnover contribute little to *V_{res}*, reducing the flux *V_{gppase}* by at most 6% and 2%, respectively. Furthermore, the amylolytic pathway leading to Glc may contribute to *V_{res}*, but the phosphorylytic pathway leading to Glc-1-P cannot; the relative contribution of these two pathways to starch degradation in maize root tips is not known.

Another possible source of Glc with C-1 to C-6 scrambling might be degradation of Suc to UDP-Glc and Fru by SuSy, followed by the direct conversion of Fru to Glc by a Glc-Fru isomerase. In microorganisms, a Xyl isomerase able to catalyze the isomerization reaction between Glc and Fru has been described (Bhosale et al., 1996). In Dea maize, a consensus sequence (TC 260043; www.tigr.org) showing 89% identity with a cDNA (EMBL accession no. X95257) coding for a Xyl isomerase of barley was described. However, according to Kristo et al. (1996), the barley enzyme does not catalyze Glc isomerization. The absence of a Glc-Fru isomerase activity in maize root tips is supported by our data. (1) In an attempt to test whether Glc and Fru might be directly interconverted, desalted protein extracts from maize root tips were incubated with 10 mM [U-¹⁴C]Fru or 10 mM [U-¹⁴C]Glc as substrates. No radioactivity transfer between Glc and Fru pools was detected (data not shown). The sensitivity of this assay was sufficient to detect a Glc/Fru isomerase activity of less than 1% of the calculated *V_{res}* flux. (2) In the hypothesis of rapid exchange between Glc and Fru through an isomerase reaction, the labeling of Fru would be intermediate between those of Glc and Suc fructosyl, or even identical to that of Glc. The similarity of the C-1 to C-6 ratios in Fru and Suc indicates that Fru is mainly formed from Suc, thus excluding the role of a Glc-Fru isomerase in Glc labeling in maize root tips.

Is There Molecular Evidence for Glc-Phosphatase in Plant Cells?

No Glc-phosphatase has yet been characterized in plants. However, several reports described phosphatase activity with Glc-6-P or Glc-1-P as substrates. In a recent study of maize root tips, Cortès et al. (2003) established the operation of such a phosphatase by measuring the dephosphorylation of the Glc-6-P analog, 3-O-methylglucose-6-P, using in vivo ³¹P-NMR spectroscopy. A Suc-6(F)-P phosphohydrolase (EC 3.1.3.24) was purified from rice leaves. The activity is dependent on Mg²⁺ and is highly specific for Suc 6(F)-P (Lunn et al., 2000). Of particular interest is the acidic phosphoenolpyruvate (PEP) phosphatase purified by Duff et al. (1989). It was designated as a PEP phosphatase owing to the high *V_{max}* to *K_m* ratio for this substrate, compared to 14 other natural substrates. However, its activity and affinity toward Glc-1-P and Glc-6-P are significant when the concentration of these metabolites in plant tissues is taken into account. This enzyme was found to be localized in the vacuole (Duff et al., 1991), and was associated with the bypass of the pyruvate kinase step of glycolysis that may be useful during phosphate starvation. Using a nonaqueous extraction method in cells of potato tubers, Farré et al. (2001) have shown that most PEP and Glc-6-P are cytosolic, but a small proportion of PEP, and as much as 34% of Glc-1-P, is located in the vacuole of potato tubers, making it plausible that the PEP phosphatase could hydrolyze Glc-1-P under physiological conditions.

Energy Cost and Possible Role of the Glc-P Cycle in Plant Cells

The cycling of each Glc via Glc-6-phosphatase costs one ATP, meaning that $2.5 \pm 0.5 \mu\text{mol}$ of ATP are consumed/ min g^{-1} FW. In W22, the respiratory rate was $1.2 \pm 0.2 \mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$ FW. Assuming that 30 molecules of ATP are produced from the degradation of each Glc molecule (Geigenberger and Stitt, 1991), the rate of ATP synthesis can be estimated as $6 \pm 1 \mu\text{mol min}^{-1} \text{ g}^{-1}$ FW. Thus, 41% of the ATP generated in the cell seems to be wasted by the Glc-P cycle. As a comparison, the Suc cycle requires one to three molecules of ATP per molecule of Suc, depending on the enzymes involved in Suc synthesis, and the equivalence of PPi with ATP as phosphate donor (Dieuaide-Noubhani et al., 1995; Trethewey et al., 1999). The attribution of Glc-P-to-Glc recycling to a phosphatase reaction rather than to the Suc cycle decreases the ATP cost about 2-fold. According to these data, the Suc cycle only consumes 3% to 6% of the ATP produced by respiration, which is in agreement with previously measured values (Trethewey et al., 1999).

In animal cells, Glc-6-phosphatases are involved in extracellular transport. By analogy, Moore and McClellan (1985) have suggested that Glc-6-phosphatase could be involved in mucilage secretion by root cap

cells. However, this function is probably limited to a specific root domain and is probably achieved by an extracellular phosphatase. The turnover of Glc-P described in this work is certainly not associated with this process. Indeed, after correction by the diluting factors, Glc and Suc were found to be identically labeled in the mature domain and in the root tip (data not shown), thus indicating that the Glc-P turnover occurs in all root tissues, not only in the cap cells. It has been shown that phosphatase activities increase in response to Pi starvation and are thus implied in the remobilization of Pi (Plaxton, 1998; Raghotama, 1999). However, because Pi starvation inhibits growth (Plaxton 1998), it is unlikely to occur in maize root tips that are continuously growing during the 25 h of incubation. On the other hand, we cannot exclude the possibility that synthesis and degradation of hexose-P occur successively and not simultaneously: Active synthesis could decrease the Pi content in cytosol and would be followed by degradation of hexose-P in order to regenerate Pi. Recently, Urbanczyk-Wochniak et al. (2003) have suggested that glycolytic flux in plant cells is controlled by the rate of ATP utilization and that Suc turnover could increase the glycolytic rate by increasing the energy demand. A similar role could be ascribed to the hexose-P cycle.

CONCLUSION

Of the three established pathways that may introduce glucosyl units from Glc-P into the intracellular Glc pool, namely, turnover of cell wall, starch, or Suc, the major one appears to be Suc hydrolysis by invertase. However, the quantitative evaluation of these fluxes shows that they only account for a small fraction of total Glc-P turnover. This leads us to hypothesize the occurrence of a new substrate cycle between hexose-P and Glc in maize root tips, the simplest hypothesis being a Glc-phosphatase catalyzed reaction. The high activity measured *in vivo* (Table I) suggests an important role for this pathway of carbohydrate metabolism both in terms of carbon flux and energy cost.

MATERIALS AND METHODS

Materials and Incubations

Maize seeds (*Zea mays* L. cv Dea, Pioneer France Mais, or W22) were germinated for 3 d in darkness at 25°C as described by Brouquisse et al. (1991). The 3.5-mm tips of primary roots were excised.

Short-Term Labeling Experiments

Maize root tips (10 roots/mL) were incubated for 25 h in medium A, as described by Brouquisse et al. (1991), supplemented with 200 mM Glc, and bubbled with a N₂/O₂ mixture (50:50, v/v). After incubation, the root tips were washed with abundant water to eliminate exogenous Glc, and then further incubated with 200 mM D-[U-¹⁴C]Glc for 5 to 60 min. Roots were harvested by filtration, washed with ice-cold water, and re-excised at 3.5 mm before being frozen in liquid N₂.

Steady-State Labeling Experiments

In ¹³C-labeling experiments, 150 to 200 excised root tips were incubated for 25 h with 200 mM D-[U-¹³C]Glc (99% enrichment) or D-[1-¹³C]Glc (99% enrichment) in medium A, washed with abundant ice-cold water, re-excised at 3.5 mm, and frozen in liquid N₂. When indicated, a starvation pretreatment (4 h in medium A) was performed before incubating maize roots with labeled Glc.

Preparation of Extracts

Acid Extracts of Glc-6-P and UDP-Glc

Water-soluble metabolites, including Glc-6-P and UDP-Glc, were extracted using perchloric acid as described by Brouquisse et al. (2001). After extraction, the extracts were dried.

Ethanolic Extraction and Fractionation of Ethanol-Soluble Compounds

The extraction of soluble components was performed using boiling aqueous solutions of ethanol (successively 80% and 50%), as described by Salon et al. (1988). The extract was resuspended in 1 mL water after evaporation. A neutral fraction containing soluble sugars was then prepared by passing the sample through cationic (AG 50W-X8 Superfine, H⁺ form; Bio-Rad Laboratories, Hercules, CA) and anionic (AG 1-X8 Superfine, formate form; Bio-Rad) columns, according to the manufacturer's instructions. Amino acids were eluted from the cationic column with 1.4 M NH₄OH.

Starch Extraction

The residue remaining after ethanolic extraction was used for the analysis of starch. It was washed with 20% ethanol (v/v) and with water (1 mL/10 roots), and starch was converted to Glc enzymatically as described by Moing et al. (1994).

Cell Wall Polysaccharide Extraction

We used a protocol described by Gibeaut and Carpita (1991), with some modifications. Maize root tips were homogenized in 50 mM TES buffer, pH 5.5, containing 50 mM NaCl and 30 mM ascorbate. The extract was centrifuged for 10 min at 4,000 rpm. The pellet was washed successively with 0.5 M NaCl (3×), water (2×), methanol:chloroform (1:1, v/v; 2× for 30 min at 45°C), methanol (1×), and water (1×). Proteins were degraded by incubating the pellet with pronase, as described by Dieuaide-Noubhani et al. (1997). Starch was removed as described previously. The remaining pellet contained the cell wall polysaccharides.

Analysis of Metabolites

Normalization of the Results

According to the [1-¹⁴C]Glc labeling experiment, the SR of the external Glc varied between 20 and 150 dpm/nmol. To allow comparison of data from different experiments, all values are given after normalization of the SR of external Glc to 1, i.e. after dividing the radioactivity measured by the SR of the external Glc.

Purification of Sugar by HPLC

The neutral fraction was separated using a PL Hi-Plex calcium column (Polymer Laboratories, Amherst, MA), as described by Moing et al. (1994). Sugar detection was performed with an ERMA ERC-7515 refractometer. Sugar quantification was achieved with Millenium software (Waters, Milford, MA), after external calibration. Each peak was collected. In the case of ¹⁴C-labeling experiments, radioactivity was counted to determine the SR of each sugar. For the quantification of radioactivity incorporated in Suc glucosyl, the repartition of the labeling between the glucosyl and fructosyl moieties was determined in the first experiments. The purified Suc was hydrolyzed by yeast invertase

in 0.2 M acetate buffer, pH 4.8. The sample was passed through anionic and cationic columns as described previously and the SR of the glucosyl and fructosyl moieties of Suc were determined after HPLC separation. The results showed that the glucosyl moiety accounted for 53% of the radioactivity measured in Suc independently of the incubation time (data not shown). The radioactivity incorporated into glucosyl was then estimated from the radioactivity incorporated into Suc, multiplied by 0.53. In the case of ^{13}C -labeling experiments, fractions were dried before NMR analysis.

Purification of Amino Acids by HPLC

Amino acids from ethanolic extract were analyzed by reverse-phase HPLC (Thermo Separation Products, Riviera Beach, FL) on a Nova-Pack C18 column after *o*-phthalaldehyde derivatization (Salon et al., 1988). Detection was performed with a Waters 486 spectrophotometer by following the absorption at 350 nm. Only Asp and Glu peaks were collected and counted. SR of Asp and Glu was determined as in a previous study (Salon et al., 1988).

Quantification and Determination of the SR of Glc-6-P

The SR of Glc-6-P was determined from the perchloric extracts, resuspended in water. First, the quantity of Glc-6-P present in the perchloric extract was determined by using an enzymatic assay described by Michal (1984), after some modifications. The sample was incubated in 100 mM KH_2PO_4 buffer at pH 7.5, containing 3 mM NADP. The reaction, carried out at 25°C, was started by adding 3 units of Glc-6-P dehydrogenase and the production of NADH was monitored at 340 nm. In a second step, the radioactivity incorporated into Glc-6-P was determined by following a procedure similar to that described by Dieuaide-Noubhani et al. (1995) for Glc analysis. The reaction was performed in a Warburg flask containing 100 mM KH_2PO_4 buffer at pH 7.5, containing 3 mM NADP, 3 units of Glc-6-P dehydrogenase, and 2 units of 6-phosphoglucuronate dehydrogenase. Whatman paper soaked with KOH 2% (w/v) was placed in the central well and 0.1 M HCl in the sidearm of the flask. The reaction was started by adding the extract and the flask was hermetically closed for 100 min. At the end of the incubation time, HCl was mixed with the reaction medium in order to stop the reaction and to liberate CO_2 . After 30 min, the paper filter was recovered, the central well was rinsed twice, and the radioactivity contained in CO_2 (corresponding to carbon C-1 of Glc-6-P) was counted. Glc-6-P radioactivity was calculated by multiplying CO_2 radioactivity by 6. Quantitative degradation and recovery of CO_2 were confirmed with defined solutions of $[\text{U-}^{14}\text{C}]\text{Glc-6-P}$.

Quantification and Determination of the SR of UDP-Glc

The analysis of the UDP-Glc was performed from perchloric extracts, according to the protocol of Răbina et al. (2001). After analysis of Glc-6-P, the extracts were dried and resuspended in 1 mL of 10 mM NH_4HCO_3 . The sugar nucleotides were partially purified on Supelclean Envi-Carb SPE columns (Supelco, Bellefonte, PA), previously conditioned with 3 mL of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid, and washed with 2 mL water. After loading the sample, the column was successively washed with 2 mL each of water, 25% (v/v) acetonitrile-water, and 50 mM aqueous triethylammonium acetate (TEAA) buffer at pH 7.0. The sugar nucleotides were then eluted with 3 mL of 25% (v/v) acetonitrile-water containing 50 mM TEAA at pH 7.0. The sugar nucleotides were then separated by HPLC on a Satisfaction RP18-AB 5- μm column, 250 \times 4.6 mm (CIL, Cluzeau, France). The mobile phase was a gradient system with acetonitrile (A) and 20 mM TEAA buffer, pH 6.0 (B). The flow rate was 1 mL min^{-1} . The separation was performed for 51 min, with 100% solvent B for 15 min, from 100% to 98% solvent B in 20 min, 98% to 96% solvent B in 5 min, 96% solvent B for 10 min, 96% to 100% solvent B in 1 min. A re-equilibration period of 9 min was used between individual runs. The detection wavelength was set at 254 nm. Identification and quantification of UDP-Glc were performed after external calibration. UDP-Glc was collected and the radioactivity was counted.

Determination of Glc and Suc Enrichments

NMR analyses were performed at 24°C with a Bruker Avance 500 spectrometer equipped with a 5-mm cryoprobe optimized for detecting ^{13}C . ^1H -NMR spectra were obtained at 500.16 MHz with a pulse of 10 μs

(corresponding to an angle of 90°) using a recycling time greater than $6T_1$. ^{13}C -NMR spectra were obtained at 125.77 MHz with a pulse of 6.7 μs (corresponding to an angle of 90°) using a recycling time greater than $6T_1$. For Suc and Glc, T_1 was measured with an inversion-recovery sequence and found to be, respectively, 1.7 and 2.7 s for ^1H carried by the C-1, 0.6 and 1.3 s for ^{13}C -1, and 0.3 and 0.63 s for ^{13}C -6. Peak assignment was performed according to previous studies (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002) and from spectra of pure compounds. The scan number was 128 and 512 for ^1H and ^{13}C spectra, respectively.

The absolute ^{13}C enrichments of Glc- α and Glc- β C-1, Suc glucosyl C-1, and starch glucosyl C-1 were determined from ^1H -NMR spectra, as the ratio of the area of the satellites (^1H - ^{13}C J-coupling) area to the total area of the multiplet. The relative enrichments of Glc, Suc, and starch glucosyl carbons C-2 to C-6 were determined by ^{13}C -NMR in comparison with the absolute enrichment of Glc, Suc glucosyl, and starch glucosyl C-1 measured by ^1H -NMR.

Preliminary experiments of labeling with $[\text{1-}^{13}\text{C}]\text{Glc}$ showed that the enrichment of the Glc C-1 carbon was lower than that of the Suc glucosyl C-1 carbon. However, Suc glucosyl C-1 comes from internal Glc C-1, so it should be labeled as more than or the same as internal Glc. This discrepancy between the theoretical and the experimental values is a good argument in favor of the existence of an unlabeled pool of Glc. In the same way, unlabeled pools of Suc and starch could also exist. To estimate the quantitative importance of these unlabeled pools of sugars, maize root tips were incubated with $[\text{U-}^{13}\text{C}]\text{Glc}$. The enrichments of the C-1 of purified Glc, Suc, and starch glucosyl units were determined by ^1H -NMR. A diluting factor was determined (see Table I) and applied to the enrichment determined in $[\text{1-}^{13}\text{C}]\text{Glc}$ labeling experiments to obtain the corrected enrichments.

Quantification of Glc-6-Phosphatase Activity

The enzymatic activity was measured in extracts obtained from 30 to 50 maize root tips re-excised to 3.5 mm after 25-h incubation. Thus, the roots were ground at 4°C in 50 mM Tris buffer (pH 7.5) containing 2.6 mM dithiothreitol, 10 mM EDTA, 10 μM chymostatine, 1% (w/v) Triton X-100, and 1% (w/v) bovine serum albumin. The homogenate was centrifuged for 5 min at 15,000 rpm. The supernatant was then desalted through a G25 column equilibrated with 50 mM Tris buffer, pH 7.5, containing 2.6 mM dithiothreitol and 10 mM EDTA. After desalting, chymostatine (10 μM final concentration) was added to the extract.

Neutral and acidic activities were measured by incubating the extract in a 50 mM acetate, 50 mM MES, 50 mM Tris buffer, pH 7.5 or pH 5.5. The reaction, measured at 25°C, was initiated with 9 mM $[\text{U-}^{14}\text{C}]\text{Glc-6-P}$ (100 dpm/nmol). Aliquots of 200 μL were sampled after 20 and 40 min, and the reaction was stopped by adding 500 μL of boiling water and incubating 5 min at 95°C. The samples were then applied to an anionic column (AG 1-X8 Superfine, formate form; Bio-Rad), and the resin was washed with 5 volumes of water. The effluent, containing the $[\text{U-}^{14}\text{C}]\text{Glc}$ produced by Glc-6-phosphatase activity, was counted. The phosphatase activity was deduced from the production of labeled Glc between 20 and 40 min. In these experimental conditions, the activity was linear over a time period of 60 min.

Flux Measurements

Short Time-Labeling Method

The unidirectional rate of synthesis of a cellular compound can be measured after short time labeling by dividing the rate of radioactivity incorporated by the SR of the precursor. Several precautions have to be taken to measure accurate fluxes with this approach: (1) The labeled substrate must enter the tissue rapidly and uniformly; (2) the SR of the precursor, UDP-Glc, and the ADP-Glc for Suc and starch, respectively, have to be determined; and (3) the incubation time has to be as short as possible to ensure that the SR of the product is low, compared to that of its precursor, in order to avoid underestimating the flux. This last point is crucial in the case of starch and Suc, both involved in substrate cycles, because radioactivity could be lost by the degradation reaction (invertase and SuSy activities).

Steady-State Labeling Method

When isotopic labeling is long enough to reach steady state, both the concentration and labeling of metabolic intermediates are constant. For each compound X located at a metabolic crossroads, equations can be written

describing metabolic steady state ($\Sigma V_s = \Sigma V_d$, where V_s and V_d represent rates of synthesis and degradation, respectively, expressed per mass of tissue) and isotopic steady state ($\Sigma(V_{s_i} * SL_i) = SL_X * \Sigma V_d$, where V_{s_i} corresponds to the rate of synthesis of the compound X from a precursor i , SL_i and SL_X are the specific labeling of compounds i and X , respectively). Such equations can be written for each carbon of a metabolite. SL corresponds to SR or to ^{13}C enrichment in the case of radioactive or ^{13}C -enriched compounds, respectively. Determining the specific labeling of the compound involved allows quantification of relative fluxes. Absolute fluxes can be deduced after the quantification of one flux rate using an independent method. In this work, fluxes were determined relative to the rate of Glc uptake (V_g), which was quantified by ^{14}C -labeling experiments. All fluxes were expressed as hexose $\text{nmol min}^{-1} \text{g}^{-1} \text{FW}$, except for Suc synthesis and degradation expressed as Suc $\text{nmol min}^{-1} \text{g}^{-1} \text{FW}$.

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