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Substrate cycles in the central metabolism of maize root tips under hypoxia

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Abstract

Substrate cycles, also called "futile" cycles, are ubiquitous and lead to a net consumption of ATP which, in the normoxic maize root, have been estimated at about 50% of the total ATP produced [Alonso, A.P., Vigeolas, H., Raymond, P., Rolin, D., Dieuaide-Noubhani, M., 2005. 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 [^{13}C] glucose and [^{14}C] glucose. Plant Physiol. 138, 2220–2232]. To evaluate their role we studied the substrate cycles of maize root tips under an oxygen limitation of respiration (3% O_2). Short-time labeling experiments with [U-^{14}C]-Glc were performed to quantify the fluxes through sucrose and starch cycles of synthesis and degradation. Steady-state labeling with [1-^{13}C]-Glc followed by ^{1}H NMR and ^{13}C NMR analysis of sugars and free alanine was used to quantify fluxes in the central metabolic pathways, including the Glc-P/Glc cycle and the fructose-P/triose-P cycle of glycolysis. Comparison with results previously obtained in normoxia [Alonso et al., as mentioned above] showed that 3% O_2 induced fermentation and reduced respiration, which led to a lesser amount of ATP produced. The rates of Glc consumption, glycolytic flux and all substrate cycles were lower, but the proportion of ATP consumed in the substrate cycles remained unchanged. These findings suggest that substrate cycles are not a luxury but an integral part of the organization of the plant central metabolism.

Keywords: Metabolic flux analysis; Maize root; Isotopic labeling; NMR; Hypoxia; Substrate cycle

1. Introduction

A substrate cycle, also called a futile cycle, is a cycle of synthesis and degradation of a compound resulting in ATP consumption (Portais and Delort, 2002). These cycles appear to waste energy without any apparent physiological reason. In plants, several substrate cycles have been characterized (Geigenberger and Stitt, 1991; Hill and ap Rees, 1994; Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Alonso et al., 2005) such as (i) cycling between hexose-P and hexoses, both via sucrose cycling through the invertase and sucrose phosphate synthase reactions, and Glc-P (Glc-1-P or Glc-6-P)/Glc cycling via the Glc phosphatase and hexokinase reactions; (ii) synthesis and degradation of starch in the plastid; and (iii) cycling between triose-P and hexose-P. Sucrose cycling has been found to be present in all tissues studied although at different rates. Its energy cost as a percentage of the total ATP produced by respiration varies from 5% in the cotyledons of *Ricinus* (Geigenberger and Stitt, 1991) to 62% in banana (Hill and ap Rees, 1994). In maize root tips, cycling between Glc-P and free Glc was first attributed to sucrose cycling with a calculated energy cost of around 70% of the ATP produced by respiration. More recently, a direct estimation of the rate of sucrose turnover by short-time labeling (Alonso et al., 2005) showed that sucrose cycling was a minor contributor to this cycle, most of which was tentatively attributed to a direct hydrolysis of Glc-P by a Glc phosphatase with an energy cost of 40% of ATP produced by respiration.

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Substrate cycles are not specific to plants since they have also been reported in mammalian cells (Jones et al., 2002), yeasts (Blomberg, 2000; Teusink et al., 2000) and bacteria (Sauer et al., 1999; Christiansen et al., 2002). Many studies have proposed various physiological roles for this dissipation of energy, as reviewed in Portais and Delort (2002). For instance, in animals they are useful for generating heat. They allow relatively low net fluxes of substrates to be maintained compared with the high enzymatic activities which are required for quickly increasing the flux in a pathway in response to stress. For example, sucrose and starch cycling in potato tubers allows rapid adjustments of the fluxes towards sucrose or starch accumulation in the short-term response to water deficit (Geigenberger et al., 1997). The hexose-P and sucrose cycles may act as "safety valves" that avoid excessive accumulation of hexose-P and ATP and phosphate depletion as a result of imbalanced functioning of glycolysis, which might lead to cell death (Blomberg, 2000; Teusink et al., 2000). Together with near equilibrium reactions, substrate cycles may be useful in balancing metabolite concentrations, thus contributing to the flexibility of plant metabolism (Rontein et al., 2002).

Higher plants are strict aerobic organisms that may often be exposed to oxygen deficit when the diffusion of oxygen is restricted, as in seeds and roots in flooded soil, and in the core of compact organs like tubers or fruits. Decreasing the oxygen concentration below a limit called the critical oxygen pressure (COP) results in a decrease in the rate of respiration. Fermentation starts under oxygen levels well below the COP. In banana, a reduction in the O_2 concentration from 21% oxygen to 15 or 10% results in a reduction of the respiration rate but in no induction of fermentation (Hill and ap Rees, 1995). In potato tubers, fermentation, i.e. lactate production, occurs only below 1% oxygen (Geigenberger et al., 2000; Geigenberger, 2003). Hypoxia is more common in nature than true anoxia. When the oxygen partial pressure decreases around maize roots, the cells of the core become hypoxic, whereas the cells of the cortex are still in normoxia (Thomson and Greenway, 1991). Similarly, the center of potato tuber is less oxygenated and has a lower energy charge than the periphery (Geigenberger et al., 2000).

Careful control of oxygen concentrations in experimental media demonstrates strikingly different responses under hypoxia and anoxia (Drew, 1997). A pretreatment under hypoxia increases the resistance to anoxia in various cereals (Saglio et al., 1988; Waters et al., 1991) and in Arabidopsis seedlings (Ellis and Setter, 1999). In maize roots, hypoxic acclimation extends survival under anoxia from 7 h to about 3 d (Saglio et al., 1988) and is obtained after only 2 h at an oxygen partial pressure of 3% (Chang et al., 2000). Protein synthesis is severely reduced under anoxia: only 20 "anaerobic proteins" are synthesized, most of which are enzymes of the fermentative pathway. In contrast, the pattern of protein synthesis in the first hours of acclimation was found to present more similarities than differences with the aerobic pattern (Chang et al., 2000), with only few proteins accumulating faster than in normoxia. A detailed study of sucrose synthase and invertase expression and activities in maize root tips showed that hypoxia leads to rapid repression of invertases with a global decrease in the sucrolytic capacity and a change in the invertase/sucrose synthase activity ratio (Zeng et al., 1999), which was suggested to affect profoundly the metabolic pathways in the central metabolism.

The aim of the present work was to gain insight into changes in pathways of the central metabolism that are induced by hypoxia, with a particular focus on the effect of decreased ATP production and modified sucrolytic activities on the fluxes through the substrate cycles.

2. Results

2.1. Isotopic steady state

To determine the time necessary to reach steady state in hypoxia, maize root tips were incubated with [1-^{14}C]-Glc for 17–26 h. The specific radioactivity of free sugars was stable during this time period (Fig. 1) whereas that of glutamate was still increasing at 26 h. This indicates that free sugars were already close to isotopic steady state but not the tricarboxylic acid cycle intermediates. To establish when glycolytic intermediates reached isotopic steady state, maize root tips were incubated with [1-^{13}C]-Glc for up to 33 h and carbon enrichments of Glc, sucrose, starch glucosyl and alanine were determined. Between 24 and 28 h of incubation, the total labeling of sucrose, Glc and starch did not vary whereas an increasing transfer of label between the carbons C-1 and C-6 of sucrose, Glc and starch was observed (Table 1). No difference was found between 28 h and 33 h of incubation, indicating that free sugars and hexose-P or sugar nucleotides like ADP-Glc and UDP-Glc were steady state-labeled after 28 h of incubation. Alanine C-3 enrichment was also stable after 28 h of incubation,

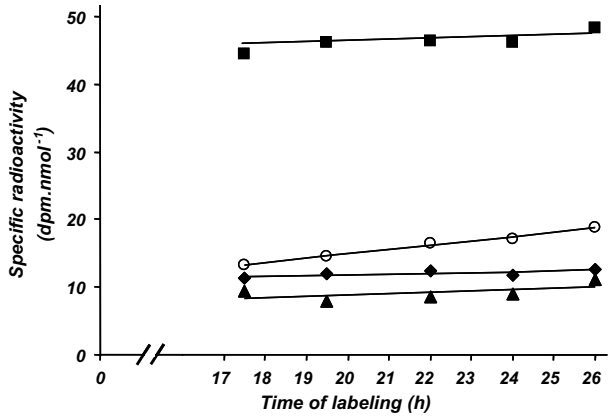

Fig. 1. Specific radioactivity of sucrose (■), Glc (◆), fructose (▲) and glutamate (○) during incubation of maize root tips with [U-^{13}C]-Glc, under 3% O_2.

Table 1
Enrichments of Glc, sucrose, starch and alanine during hypoxia

Carbon atoms	Measured enrichments[a]			Corrected enrichments[b]	Calculated enrichments[e]
	24 h	28 h[c]	33 h	28 h	28 h
Glucose					
C1	41.6	39.6–44.7	41.2	97.4–110	37.3–36.2
C6	2.6	3.3–4.8	4.2	6.6–10.4	3.3–4.0
C6/C1	0.06	0.08–0.1	0.08		
Sucrose					
Glucosyl					
C1	83.4	83.7–82.1	83.0	89.1–87.4	
C6	10.3	11.7–11.9	12.5	12.4–12.6	9.8–10.4
Fructosyl					
C1	85.2	81.3–79.4	81.1	86.5–84.5	
C6	11.3	12.0–12.5	12.8	12.7–13.2	
C6/C1	0.13	0.15–0.16	0.16		
Starch					
C1	9.6	6.7–9.1	7.7	40.4–57.2	
C6	ND	1.3–ND[d]	1.9	2.4–ND[d]	3–4
Alanine					
C2	1.4	ND[d] – 1.2	ND[d]		
C3	41.6	42.4–43[f]	43.4		

[a] Enrichments were determined from ^{1}H and ^{13}C NMR spectra as defined in Section 4. For carbons C-2 to C-5 of sugars, the enrichments were close to natural abundance and are not reported.
[b] Corrected enrichments were calculated from measured enrichment using the formula: $E_{corrected} = (E_{measured} - 1.1)DF + 1.1$. DF is a diluting factor specific for each sugar and determined from [U-13C]-Glc labeling experiments (Alonso et al., 2005).
[c] Values from two independent experiments.
[d] Not detected.
[e] Carbon enrichments set as unknowns in the model and re-calculated from the determined fluxes. The values corrected from the diluted factor are to be compared to the measured enrichments after 28 h of incubation.
[f] Enrichments used to calculate the fluxes without correction.

which indicates that pyruvate and all glycolytic intermediates were steady state-labeled after 28 h of incubation under hypoxia.

2.2. Physiological and biochemical characterization of W22 in hypoxia

Maize root tips incubated in normoxic conditions grew from 3.5 mm to 8.6 mm. After 28 h incubation with 3% O_2, the length of maize root tips only increased from 3.5 mm to 4.3 mm, which represents 15% of the growth in normoxia. The respiration rate was 85% lower in hypoxia than in normoxia and the energy charge was significantly decreased (Table 2). Free sugar levels were not significantly affected while starch was reduced by 69% in maize root tips incubated in hypoxia. Hypoxia led to 2.5- and 5.5-fold increases in the levels of Glc-6-P and UDP-Glc, respectively. Enzyme activities involved in sucrose metabolism and Glc-P/Glc cycling were also measured (Table 2). Sucrose synthase and acid invertase activities remained unchanged and decreased 4-fold, respectively. The decreases in the invertase/sucrose synthase ratio and in the total sucrolytic activity are consistent with previous findings (Zeng et al., 1999). Glucokinase activity was remained unchanged whereas Glc-6-phosphatase activity was significantly lower in hypoxia.

2.3. The metabolic network

The scheme of the maize root central metabolism network used in the present work (Fig. 2) is essentially similar to that described previously (Dieuaide-Noubhani et al., 1995). Briefly, the metabolic network includes cytosolic and plastidial glycolysis, the plastidial pentose phosphate pathway (PPP), and the sucrose and starch cycles. The starch precursors are shown to have a plastidial origin, based on both their lower total ^{13}C enrichment and the higher transfer of label from C-1 to C-6 in starch glucosyl compared to sucrose glucosyl (which reflects cytosolic Glc-6-P). These differences between starch and sucrose labeling indicate the following: (i) starch is not formed from cytosolic hexose-P but essentially from ADP-Glc produced from plastidial Glc-P imported into the plastid and further metabolized in the plastidial PPP; (ii) cytosolic and plastidial hexose-P are not rapidly exchanged (Dieuaide-Noubhani et al., 1995). These results indicate that the hypothesis of starch formation from ADP-Glc synthesized in the cytosol proposed by Baroja-Fernandez et al. (2003) does not apply to maize root tips. In the present work, the lower enrichment of starch glucosyl C-1 compared to sucrose glucosyl C-1 also indicates that most hexose-P molecules imported into the plastid go through the plastidial PPP before being incorporated into starch. No cytosolic PPP

Table 2
Physiological and biochemical characterization of W22 in hypoxia

	Normoxia (25 h, 50% O_2)	Hypoxia (28 h, 3% O_2)
Length (mm tip^{-1})	8.6 ± 1.1^{6b}	4.3 ± 0.3
	Re-excised tip (3.5 mm) [a]	Not re-excised tips
Fresh weight (mg tip^{-1})	2.8 ± 0.4^{9b}	3.8 ± 0.3^{5}
Respiratory rate (μmol O_2 min^{-1} gFW^{-1})	1.20 ± 0.18^{5b}	0.18 ± 0.02^{3}
Ethanol production rate (μmol min^{-1} gFW^{-1})[c]		0.460 ± 0.040^{2}
Lactate production rate (μmol min^{-1} gFW^{-1})[c]		0.007
Intracellular metabolites (μmol gFW^{-1})		
Glucose	72 ± 14^{16b}	52 ± 7^{6}
Fructose	30 ± 9^{16}	17 ± 7^{6}
Sucrose	34 ± 6^{16b}	46 ± 11^{6}
Starch[d]	11.4 ± 2.6^{7b}	3.5 ± 1.2^{5}
Glc-6-P	0.59 ± 0.04^{8}	1.52 ± 0.22^{6}
UDP-Glc	0.17 ± 0.01^{4}	0.94 ± 0.10^{3}
Lactate		5.3 ± 1.6^{3}
Ethanol		10.6 ± 1.6^{3}
Energy charge	0.88 ± 0.01^{2}	0.76 ± 0.04^{3}
ATP/ADP ratio	6.9–7.1	2.2 ± 0.01^{3}
Enzymatic activities (μmol hexose min^{-1} gFW^{-1})		
Neutral invertase	0.34 ± 0.07^{4}	0.19 ± 0.05^{4}
Acidic invertase	4.39 ± 0.37^{3}	0.92 ± 0.56^{4}
Sucrose synthase	0.38 ± 0.09^{3}	0.36 ± 0.09^{4}
Glucokinase	0.81 ± 0.23^{3}	0.80 ± 0.18^{4}
Glucose 6-phosphatase	0.84 ± 0.14^{3b}	0.28 ± 0.04^{4}

Data represent means ± SD, the superscript number specifies the number of experiments.

[a] Maize root tips incubated with 50% O_2 grew and were re-excised to 3.5 mm prior to further analyses.

[b] Data from Alonso et al. (2005).

[c] Corresponding to ethanol and lactate secreted in the incubating medium and measured as described in Section 4.

[d] Starch was expressed as μmol of hexose unit per gFW.

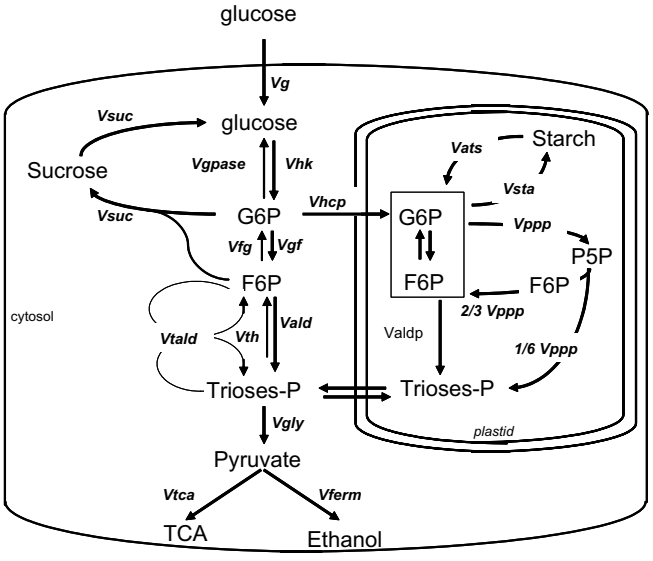

Fig. 2. Metabolic scheme in maize root tips.

was included because the low dilution of the hexosyl moieties of sucrose (sum of the enrichments of carbons C-1 + C-6) compared to the [1-^{13}C]-Glc substrate indicates that cytosolic PPP, if present, is almost inactive. The label randomization between carbons C-1 and C-6 observed in all sugars indicates that the reversibility of glycolysis between triose-P and hexose-P is still active in hypoxia. Major differences with the previous network are the following: first, the inclusion of the Glc-P/Glc cycle recently described by Alonso et al. (2005); second, the absence of cell wall polysaccharide synthesis, which was considered negligible due to the very slow growth rate; third, the PEP carboxylase (PEPC) flux was also assumed to be negligible owing to the reduction in growth. In addition, given that the TCA cycle intermediates did not reach steady state, it was not possible to calculate the partition of glycolysis between anaplerotic (PEPC) and oxidative (pyruvate dehydrogenase) fluxes. This does not contradict the evidence for active protein synthesis described in hypoxic root tips (Chang et al., 2000) since protein turnover may provide the amino acids incorporated into the new proteins. Fourth, the fermentation flux was included to account for the measurements of lactate and ethanol. The glycolytic flux was then divided between oxidative and fermentative fluxes.

2.4. Flux quantification

Short-time labeling experiments allowed the Glc uptake (V_g) and unidirectional fluxes into carbohydrate pools, sucrose (V_{suc}) and starch (V_{sta}), to be measured (Table 3). In hypoxia, the rate of Glc consumption was determined after incubation of the root tips with [U-^{14}C]-Glc for up

Table 3
Metabolic fluxes in maize root tips after 28 h of hypoxia

	Flux (nmol hexose min^{-1} gFW^{-1})
V_g	298 ± 6 [3a]
V_{suc}	57 ± 8 [5a]
V_{sta}	0.75 ± 0.25^{b}
V_{ats}	5[c]
V_{ferm}	230 ± 20^{2b}
V_{hk}	724–1168[d]
V_{gpase}	369–813[d]
V_{hcp}	94–124[d]
V_{gf}	1360–1379[d]
V_{fg}	1157–1206[d]
V_{ald}	281–269[d]
V_{th}	79–95[d]
V_{tald}	30–16[d]
V_{gly}	273–284[d]
V_{ppp}	173–104[d]
V_{aldp}	41–84[d]
V_{tca}	43–34[d]

[a] Means ± SD, the superscript number indicating the number of experiments.

[b] Mean of two experimental determinations ± the range.

[c] Estimated from starch content measurement.

[d] Calculated from ^{13}C labeling data. Two values are given, calculated from each of the two datasets given in Table 1.

to 20 min (Fig. 3a). The rate of Glc consumption, 298 ± 6 nmol hexose min^{-1} gFW^{-1}, was significantly lower than that measured in normoxia (394 ± 73 nmol hexose min^{-1} gFW^{-1}; Alonso et al., 2005). Longer incubation times of up to 60 min had to be used to quantify the unidirectional rates of starch and sucrose synthesis. The amount of starch and sucrose produced after 15, 30 and 60 min (Fig. 3c and d) was calculated as described in Section 4 from the amount of radioactivity incorporated into starch and sucrose glucosyl units respectively and from the specific radioactivity of their precursor, Glc-6-P (Fig. 3b). In hypoxia, the unidirectional rate of sucrose synthesis was estimated at 57 ± 8 nmol min^{-1} gFW^{-1}. The unidirectional rate of starch synthesis was between 0.5 and 1 nmol min^{-1} gFW^{-1}. These data are significantly lower than those obtained in normoxia (178 and 30 nmol min^{-1} gFW^{-1} for the sucrose and starch synthesis rates, respectively; Alonso et al., 2005).

The unidirectional rates of sucrose and starch degradation can be determined as the difference between the net flux measured biochemically and the unidirectional flux of synthesis measured by short-time labeling (net flux = unidirectional rate of synthesis − unidirectional rate of degradation). By comparing the starch content at the beginning of incubation (10.4 ± 2 μmol gFW^{-1}; data not shown) and after 28 h in hypoxia (3.5 ± 1.2 μmol gFW^{-1}; Table 2), starch was found to be degraded at a net rate of 4.1 nmol hexose min^{-1} gFW^{-1} (net flux = −4.1 μmol gFW^{-1}), which is relatively high in comparison with the unidirectional rate of synthesis of 0.7 nmol hexose min^{-1} gFW^{-1} (see above). The unidirectional rate of starch degradation was therefore 4.8 nmol hexose min^{-1} gFW^{-1}. This represents a mean rate over the 28 h incubation under hypoxia. We believe this rate may be lower at 28 h because the amount of starch remaining in the root tips was still significant after 33 h (not shown). The present data suggest that the metabolism of starch is a relatively minor pathway in terms of energy metabolism, at least quantitatively. Concerning sucrose, no significant variation was noted between the beginning and the end of incubation with 45 ± 12 (data not shown) and 46 ± 11 μmol gFW^{-1} (Table 2), respectively. According to the rate of sucrose synthesis (Fig. 3d), a significant increase would have been measured in the absence of degradation. The stability of the level of sucrose therefore indicates that its rates of synthesis and degradation were equal.

The fermentation products alanine and lactate rapidly increase and then level off in the first minutes of anoxic

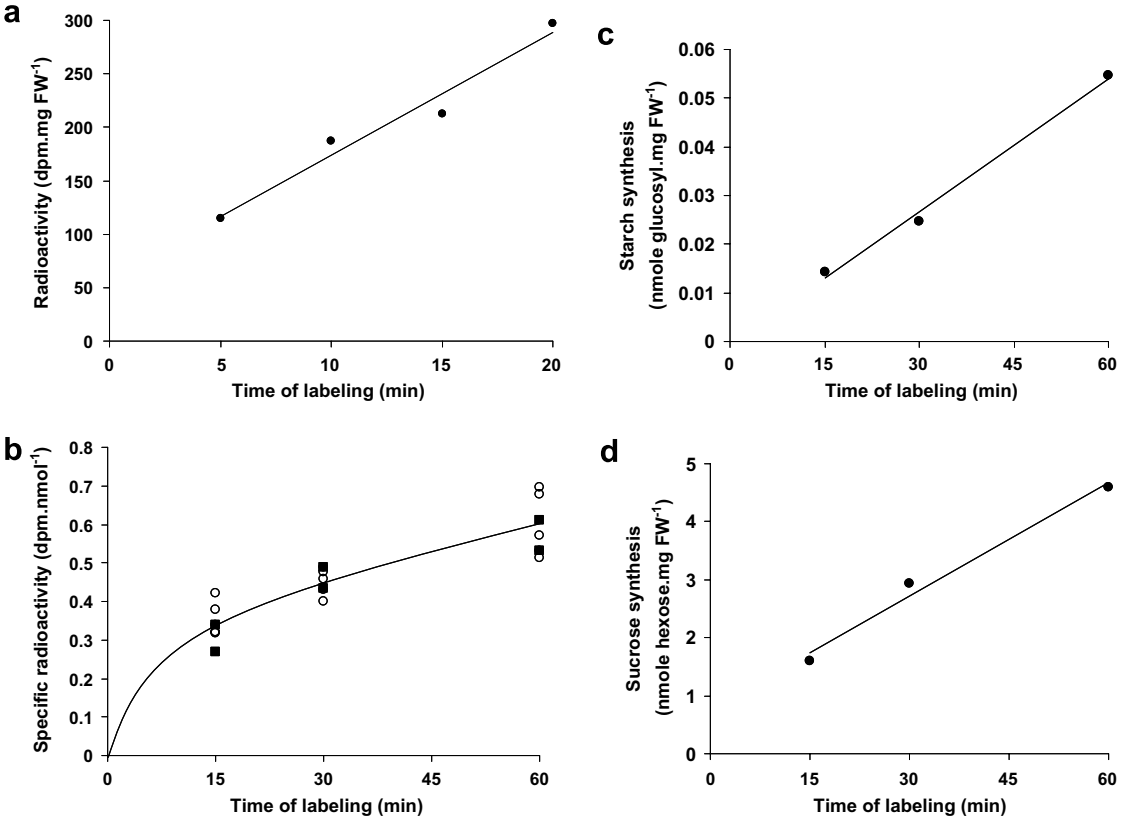

Fig. 3. Data for unidirectional flux measurements by short-time labeling with [U-14C]-Glc. Incorporation of [U-^{14}C]-Glc for calculation of Glc uptake rate (a); specific radioactivities of Glc-6-P (○) and UDP-Glc (■) (b) for calculating the unidirectional rates of synthesis of starch (c) and sucrose (d). The amount of sucrose and starch produced during a time interval was determined from the quantities of radioactivity incorporated during the interval using the equation: $N_{n+1} = N_n + (RA_{n+1} - RA_n)/(SR_{Glc\text{-}6\text{-}P})_n$. N_n and N_{n+1} are the number of moles of labeled product, nth and $(n+1)$th the time points of incubation, RA_n is the amount of radioactivity incorporated into the compound studied (expressed in dpm gFW^{-1}), and $(SR_{Glc\text{-}6\text{-}P})_n$ is the SR of Glc-6-P (b). The results presented here correspond to one typical experiment, repeated 2, 4 and 1 times for Glc uptake and for sucrose and starch synthesis, respectively. These data were used to quantify Glc uptake and the unidirectional rates of sucrose and starch.

treatment, whereas ethanol is produced over a longer period, particularly in acclimated roots (Saglio et al., 1980; Xia and Saglio, 1992). Ethanol is known to diffuse freely to the medium and lactic acid is also secreted into the medium in acclimated root tips (Xia and Saglio, 1992). The fermentation products were therefore measured in both the root tips and the medium during incubation under hypoxia as described in Section 4 (see values after 28 h of hypoxia, Table 2). Alanine and lactate levels remained constant but ethanol increased steadily particularly in the incubation medium (data not shown). The fermentation rate under hypoxia was therefore 230–250 μmol hexose min^{-1} gFW^{-1}, which is in the range of values reported for maize root tips under anoxia in Glc-supplemented media (Xia and Saglio, 1992).

The intermediary fluxes of carbon metabolism were calculated (Table 3) from each of the two data sets obtained after steady state labeling (28h of incubation, Table 1). First, Eq. (4) from Alonso et al. (2005) was used to quantify the flux V_{res}, corresponding to the sum of V_{gpase}, the direct conversion of Glc-P to Glc and Vsuc, the hydrolysis of sucrose by invertase. The system of 14 equations of the steady state model (see Section 4) was then solved using the Mathematica software: the previously determined fluxes V_g, V_{suc}, V_{sta}, V_{ats}, V_{ferm} and V_{gpase} were entered as parameters together with the experimental enrichments (sucrose glucosyl C-1, sucrose fructosyl C-1 and C-6, starch glucosyl C-1 and alanine C-3) and fluxes in the central metabolism were calculated (Fig. 3). As a control, the enrichments of the Glc C-1 and C-6, sucrose glucosyl C-6 and starch glucosyl C-6 were then re-calculated from the determined fluxes (Table 1). Their reasonable proximity with the experimental enrichments was used as an initial validation of the calculated flux values. The discrepancy between experimental and re-calculated enrichments of intracellular Glc probably results from an underestimation of the diluting pool of Glc. Considering starch, the relatively large error on the C-6 enrichment is more likely due to the error made in its experimental determination: the peak area corresponding to starch C-6 was close to background due to the low amount of starch in hypoxia and the low enrichment of this carbon.

2.5. Validation of the model

The model was validated first by comparing the respiratory rate from the calculated fluxes with the direct measurements of oxygen uptake, and second by verifying that the balance of ATP production and its consumption by the substrate cycles was positive.

The model calculates that between 34 and 43 nmol hexose min^{-1} gFW^{-1} enter the tricarboxylic acid cycle. Assuming that PEPC flux is negligible and that all glycolytic products enter the tricarboxylic acid cycle through pyruvate dehydrogenase, oxygen consumption varying between 204 and 258 nmol O_2 min^{-1} gFW^{-1} can be deduced. These values are close to the measured respiratory rate (Table 2).

Since respiration and fermentation were both active at 3% oxygen, both sources of ATP were considered. ATP production could be estimated from the respiration and fermentation rates assuming that the ATP yield of respiration is 30 ATP per Glc (Geigenberger and Stitt, 1991) and that of glycolysis + fermentation is 2 ATP per Glc. The value thus obtained was 1380 nmol ATP min^{-1} gFW^{-1}. ATP consumption could be estimated for each substrate cycle: (i) the Glc-P/Glc cycle involving Glc-phosphatase activity (Alonso et al., 2005) costs 1 ATP per hexose; (ii) the sucrose cycle (V_{suc}) requires 1–3 molecules of ATP per molecule of sucrose, depending on the enzymes involved. Considering sucrose degradation by invertase and sucrose synthesis by sucrose phosphate synthase, with the PPi produced considered as an ATP equivalent, sucrose cycling costs 2 ATP per sucrose molecule (Hill and ap Rees, 1994; Dieuaide-Noubhani et al., 1995; Trethewey et al., 1999); (iii) a cycle of starch synthesis (V_{sta}) and degradation (V_{ats}) requires 1 ATP for each transformation of Glc-1-P into ADP-Glc via ADP-Glc pyrophosphorylase; (iv) for the triose-P/hexose-P cycle (V_{ald}), a maximum consumption of ATP was determined by considering that ATP-dependent phosphofructokinase and fructose 1,6-bisphosphatase were involved. According to the flux values in Fig. 3, the Glc-P/Glc, sucrose, starch and triose-P/hexose-P cycles consumed approximately 600, 114, 1 and 87 nmol of ATP, respectively. Altogether, these substrate cycles were found to degrade 58% of the ATP produced by the hypoxic root tips. Although considerably reduced by hypoxia, the ATP production rate was high enough to sustain the substrate cycles.

3. Discussion

3.1. Hypoxia at 3% O_2 leads to reduced respiration and high fermentation with a total glycolytic flux similar to that under normoxia

An oxygen concentration of 3% was found to induce acclimation to anoxia in maize root tips (Saglio et al., 1988) and has since been commonly used in studies of hypoxia. The respiration of maize root tips in a liquid medium decreases at an oxygen partial pressure lower than 30%, i.e. the critical oxygen pressure (Saglio et al., 1884). In agreement with previous studies (Pradet and Raymond, 1983; Geigenberger, 2003), the decrease in respiration is accompanied by a reduction in the energy charge and ATP/ADP ratio, indicating lower ATP supply by mitochondria. Fermentation was already activated at a rate close to that measured under anoxia in similar root tips (Xia and Saglio, 1992). As a result of the contrasting effects of the decreased respiration rate and the induction of fermentation, the rate of Glc uptake and the glycolytic fluxes at 3% oxygen were found to be very similar to those under normoxia, with nearly 90% of the glycolytic flux going to fermentation. This differs from the induction of fermentation in potato tubers where glycolysis decreases under

hypoxia to a minimum rate when oxygen falls near 1% O_2 and then returns to its aerobic rate under anoxia (Geigenberger et al., 2000; Geigenberger, 2003).

Unlike in previous studies of acclimation to anoxia where the whole seedling was exposed to hypoxia (Saglio et al., 1988; Chang et al., 2000; Zeng et al., 1999), we incubated only the excised maize root tips in hypoxic conditions. Since the production of ethanol under anoxia is severely decreased after about 2 h in non-acclimated root tips while it continues at a more constant rate for hours in acclimated roots, the high rate of ethanol production after 28 h under hypoxia (Table 2) suggests that the roots had become acclimated. However, we have no further evidence for acclimation.

3.2. The oxidative pentose phosphate pathway remains highly active in hypoxia

The flux of hexose units entering the OPPP in the hypoxic W22 root tips was 40–60% of the Glc influx to the roots. This OPPP flux value is about twice that reported in aerobic root tips of the DEA variety (27% of the Glc influx, Dieuaide-Noubhani et al., 1995) but similar to that found in aerobic W22 root tips (data not shown). Similarly, OPPP was not modified in ripening banana placed in 10% O_2 (Hill and ap Rees, 1995). The major role of the OPPP in non-green cells is the production of NADPH for biosyntheses and defense against reactive oxygen species (ROS). Since biosyntheses are strongly reduced in hypoxia, the proportion of NADPH available against oxidative stress under hypoxia is increased compared to normoxia. ROS are known to be produced in response to many stresses, including hypoxia. Damage to plants by ROS as a consequence of anoxia was first attributed to ''post-anoxic injury'', which results from re-exposure of the tissues to oxygen (Drew, 1997 and refs therein). More recently, increased hydrogen peroxide production was detected in tissues exposed to O_2 concentrations lower than 12 μM, i.e., lower than 5% oxygen (Blokhina et al., 2001). Hydrogen peroxide production by a NADPH oxidase was involved in the hypoxic induction of ADH mRNA in Arabidopsis (Baxter-Burrell et al., 2002), and the early expression of the NADPH oxidase RbohD mRNA in Arabidopsis was interpreted as supporting the role of ROS in mediating anaerobic responses (Liu et al., 2005). Our labeling data indicate that maize roots are able to produce the NADPH required for protection against an excess of ROS produced under hypoxia and in the production of H_2O_2 as a signaling molecule.

3.3. The rate of substrate cycles decreases proportionally to the ATP production rate

The apparent waste of ATP in substrate cycles, first called futile cycles, raises the question of their physiological function. Under limiting capacities of ATP production, metabolic regulations are expected to inhibit selectively different pathways in relation to their usefulness for the cell or organism. It has been suggested that substrate cycles may be useful to increase the flexibility of plant metabolism (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002). The regulation of substrate cycles might depend on their importance for the plant cell: if substrate cycles are useful but inessential, they would be drastically restricted with decreasing ATP availability; conversely if they are essential, they would be maintained while other ATP consuming processes, like growth or storage, would be restricted. Comparing the effect of hypoxia on the rate of these pathways is a means to evaluate the importance of substrate cycles in the central metabolism.

Three substrate cycles led to a significant waste of ATP: the Glc-P/Glc cycle through the Glc 6-phosphatase and glucokinase reactions; the sucrose cycle through the invertase and sucrose-phosphate synthase reactions; and the triose-P/hexose-P cycle of glycolysis. The loss of ATP through starch turnover was comparatively very small. The fluxes through these three cycles decreased to 23%, 32% and 35%, respectively, of their values in normoxia. The reduced rates of the Glc-P/Glc and the sucrose cycles are associated with decreases in the Glc-6-phosphatase and invertase activities, respectively (Table 2) and may explain the 2.6-fold increase in the Glc-6-P level in hypoxia compared to normoxia. The energy cost of the substrate cycles is reduced by the hypoxic treatment, but since the rate of ATP production under hypoxia is also reduced to 23% of the normoxic rate, a similar percentage of the ATP is consumed for the operation of the substrate cycles in both conditions.

On the other hand, the increase in length under hypoxia and the rate of starch synthesis were decreased to 14% and 3%, respectively, of their rates in normoxia (see Sections 2.2 and 2.4). The comparatively low decrease in substrate cycle activity compared to growth or starch synthesis suggests that substrate cycles have priority over the biosynthetic processes. Therefore, they appear to be an integral feature of the normal functioning of the central metabolism, at least in maize root tips.

4. Materials and methods

4.1. Materials and incubation conditions

Maize seeds were germinated for 3 days in darkness at 25 °C as described by Brouquisse et al. (1991). The 3.5 mm tips of primary roots were excised and incubated with 200 mM Glc in medium A described by Brouquisse et al. (1991). The incubation medium was bubbled with a N_2/O_2 mixture, 50/50, (v/v) for normoxia (50% O_2) or 97/3 (v/v) for hypoxia (3% O_2).

For steady state labeling experiments, 150 to 200 excised root tips were incubated for 28 h with 200 mM D-[U-^{13}C]-Glc (99% enrichment) or D-[1-^{13}C]-Glc (99% enrichment), washed with abundant ice-cold water and frozen in liquid N_2. While apparently high, the Glc concentration used in

this work has been shown to be necessary to sustain the normal respiration of root tips (Saglio et al., 1980) and to avoid the induction of proteolytic activities due to sugar starvation (James et al., 1993). Moreover, it is intermediate between the sugar concentration in the phloem (0.5 M; Bret-Harte and Silk, 1994) and that in the root tip cell (about 100 mM) and is probably close to that around the root cells.

For short-time labeling experiments, maize root tips (10 roots/ml) were incubated for 28 h in medium A supplemented with 200 mM Glc. After incubation, the root tips were washed with abundant water to eliminate exogenous Glc, and then further incubated with 200 mM D-[U-^{14}C]-Glc (Specific radioactivity (SR) varying between 34 and 380 dpm nmol^{-1}) for 5–60 min. Roots were harvested by filtration, then washed with ice-cold water before being frozen in liquid N_2.

4.2. Preparation of extracts

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

Soluble components were extracted according to the procedure of Salon et al. (1988). The extract was evaporated and resuspended in 1 ml water. The extracted metabolites were separated as neutral, acidic and cationic fractions and free sugars (Glc, fructose and sucrose) were purified by HPLC from the neutral fraction (Moing et al., 1994).

Starch was extracted from the residue of ethanolic extraction and further purified as Glc by HPLC, as described by Moing et al. (1994).

4.3. Analysis of metabolites

To compare data from experiments with different SR of supplied Glc, all SR values were normalized to that of external Glc i.e. the specific radioactivities measured are divided by the SR of external Glc in the corresponding experiment (see Fig. 2: SR of Glc-6-P).

The SR of soluble sugars, amino acids and UDP-Glc were determined after HPLC purification as described by Alonso et al. (2005). The SR of Glc-6-P was determined from the perchloric extracts as described by Alonso et al. (2005).

The carbon enrichments of Glc, sucrose, starch and alanine were determined from NMR analysis. Dried samples were dissolved in D_2O. NMR analyses were performed at 24 °C with a Bruker Avance 500 spectrometer equipped with a 5-mm cryoprobe optimized for detecting ^{13}C. ^{1}H 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 sucrose and Glc, T_1 was measured with an inversion-recovery sequence and found to be, respectively, 1.7 and 2.7 s for ^{1}H carried by the C1, 0.6 and 1.3 s for ^{13}C1, 10.6 and 1.3 for ^{13}C2, 0.6 and 1.4 for ^{13}C3, 0.6 and 1.2 for ^{13}C4, 0.5 and 1.3 for ^{13}C5 and 0.3 and 0.6 for ^{13}C6. For alanine T_1 was 6.6 for ^{1}H carried by the C3, 3.8 for ^{13}C2 and 1.6 for ^{13}C3. Peak assignment was performed according to previous studies (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002) and from spectra of pure compounds.

The absolute ^{13}C enrichments of Glc α and β C1, sucrose glucosyl C1, starch glucosyl C1 and alanine C3 were determined from ^{1}H NMR, the other enrichment being determined from ^{13}C NMR spectra as described by Dieuaide-Noubhani et al. (1995). Alonso et al. (2005) determined diluting factors for sugars from [U-^{13}C]-Glc labeling experiments. Similarly, for hypoxic maize root tips, the diluting factors of Glc, sucrose and starch were determined to be 2.52, 1.07 and 6.7 respectively. These diluting factors were applied to the enrichments determined in [1-^{13}C]-Glc labeling experiments in order to correct the measurements (Table 1) with the formula: $E_{corrected} = (E_{measured} - 1.1) \cdot DF + 1.1$ where DF is the diluting factor.

4.4. Enzyme assays

Enzymatic activities were measured in extracts obtained from 30 to 50 maize root tips after 28 h incubation (Alonso et al., 2005). Glc-6-phosphatase (*EC 3.1.3.9*) activity was measured on a crude extract as described in Alonso et al. (2005). The activities of neutral invertase (*EC 3.2.1.26*) and total sucrose synthase (*EC 2.4.1.13*) were successively measured essentially according to Pelleschi et al. (1997) at 30 °C by monitoring NAD reduction at 340 nm. The assay mix contained 0.3 M TEA buffer (pH 7.5), 30 mM $MgSO_4$, 110 mM sucrose, 2.5 mM DTT, 1 mM ATP, 0.4 mM NAD, 2 U/ml hexokinase, 2 U/ml Glc-6-P isomerase and 1 U/ml Glc-6-P dehydrogenase. Neutral invertase activity was measured first. Sucrose synthase activity was measured after the addition of 0.2 mM UDP from the increase in the rate of NAD reduction. Acid invertase assays were adapted from Lowell et al. (1989). Extract (25 μl) was added to 35 μl of 0.2 M sodium acetate buffer (pH 4.8) containing 110 mM sucrose and incubated for 15 min at 30 °C, The reaction was stopped by adding 50 μl of 0.6 M sodium phosphate buffer (pH 7.0) and boiling the extract for 5 min. After cooling to room temperature, Glc produced by sucrose hydrolysis was measured by adding 743 μl 0.3 M TEA buffer (pH 7.5) containing 30 mM $MgSO_4$, 1.3 mM ATP, 0.5 mM NAD, 2.7 U/ml hexokinase, 2.7 U/ml Glc-6-P isomerase and 1.3 U/ml Glc-6-P dehydrogenase was added. After 30 min incubation at 30 °C, the supernatant was clarified by centrifugation (14,000 rpm, 3 min) and its OD was measured at 340 nm. Glucokinase activity was assayed as described by Bouny and Saglio (1996).

4.5. Respiration rate

Ten maize root tips were transferred to 1.5 ml incubation medium previously bubbled with 50% O_2 as control or with 3% O_2 for hypoxia. O_2 consumption was measured

at 25 °C using an O_2 Clark electrode (Model 1302; Strathkelvin Instruments, Glasgow, Scotland) connected to an O_2 analyzer (Chemical microsensor 1201; Diamond General Corp., Ann Arbor, MI, USA).

4.6. Fermentation products

Lactate and ethanol were extracted from maize root tips incubated for 22–28 h in hypoxia as described in Xia and Saglio (1992). In the same experiment, lactate accumulated outside the roots was also quantified.

To avoid losses of ethanol, ethanol secretion was measured on maize root tips pre-incubated for 28 h in Glc-supplemented medium A (see above) bubbled with 3% O_2. Roots were then transferred to 50 ml penicillin vials containing 2 ml of the incubation medium bubbled with 3% O_2 (10 roots per flask). The vials were sealed and equilibrated for 1 min with 3% O_2 using two needles. Needle removal was time zero of incubation. Agitation was maintained to ensure a homogeneous concentration of O_2 in the incubation medium. Incubation was stopped after 20, 45 and 70 min. Ethanol and lactate extracted from the roots or dissolved in the incubation medium were immediately measured by an enzymatic method using commercial kits (Boehringer Mannheim).

4.7. 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 incorporation by the SR of the precursor. 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 quantities of radioactivity incorporated into glucosyl moieties of sucrose and starch during a time interval were converted to the amount of sugar produced and expressed in mol gFW^{-1} by using the equation:

$$N_{n+1} = N_n + (RA_{n+1} - RA_n)/(SR_{Glc\text{-}6\text{-}P})_n,$$

where N_n, RA_n and $(SR_{Glc\text{-}6\text{-}P})_n$ correspond, respectively, to the number of moles, the radioactivity incorporated into the compound studied (expressed in dpm gFW^{-1}), and the SR of Glc-6-P; n indicates the nth time point (see Alonso et al., 2005). The slopes of the curves thus obtained were used to calculate the rate of sucrose (Vsuc) and starch (Vsta) biosynthesis.

Steady state model – A mathematical model of the metabolic network presented in Fig. 3 was generated through equations describing the labeling of free Glc, cytosolic and plastidial hexose-P, and triose-P.

Isotopic steady-state equations:

Glucose:

$$G1\ (V_g + V_{suc} + V_{gpase}) = Gl1\ V_g + H1\ V_{suc} + H1\ V_{gpase} \quad (1)$$

$$G6\ (V_g + V_{suc} + V_{gpase}) = Gl6\ V_g + H6\ V_{suc} + H6\ V_{gpase} \quad (2)$$

Cytosolic Glc-6-P:

$$H1\ (V_{hk} + V_{fg}) = G1\ V_{hk} + F1\ V_{fg} \quad (3)$$

$$H6\ (V_{hk} + V_{fg}) = G6\ V_{hk} + F6\ V_{fg} \quad (4)$$

Cytosolic Fru-6-P:

$$F1\ (V_{gf} + V_{th}) = H1\ V_{gf} + T3\ V_{th} \quad (5)$$

$$F6\ (V_{gf} + V_{th} + V_{tald}) = H6\ V_{gf} + T3\ V_{th} + T3\ V_{tald} \quad (6)$$

Plastidial Glc-6-P:

$$HP1\ (V_{hcp} + (2/3)V_{ppp}) = H1\ V_{hcp} + (2/3)\ V_{ppp}\ HP2 \quad (7)$$

$$HP6\ (V_{hcp} + (2/3)V_{ppp}) = H6\ V_{hcp} + (2/3)V_{ppp}\ (T3 + HP6)/2 \quad (8)$$

Trioses-P:

$$T3\ (2V_{ald} + (2/3)V_{ppp} + V_{tald} + 2V_{aldp}) = (F1 + F6)\ V_{ald} + (2/3)\ HP6\ V_{ppp} + F6\ V_{tald} + (HP1 + HP6)\ V_{aldp} \quad (9)$$

Metabolic steady-state equations:

$$V_g + V_{suc} + V_{gpase} = V_{hk} \quad (10)$$

$$V_{hk} + V_{fg} = V_{gf} + V_{hcp} + V_{suc} + V_{gpase} \quad (11)$$

$$V_{gf} + V_{th} = V_{fg} + V_{ald} \quad (12)$$

$$V_{hcp} + V_{ats} = (1/3)V_{ppp} + V_{sta} + V_{aldp} \quad (13)$$

$$V_{ald} + (1/6)\ (V_{ppp}) + V_{aldp} = V_{gly} + V_{th}. \quad (14)$$

With V_g, the rate of Glc uptake; V_{hk}, the flux through hexokinase; V_{gpase}, the rate of Glc-P/Glc cycling through Glc phosphatase activity; V_{suc}, the flux through sucrose cycling; V_{hcp}, the import of hexose-P into the plastid; V_{gf} and V_{fg}, the fluxes catalyzed by Glc-6-P isomerase; V_{ald}, the flux catalyzed by cytosolic aldolase; V_{tald}, the flux catalyzed by the cytosolic transaldolase; V_{th}, the reversibility of glycolysis between triose-P and Fru-6-P; V_{aldp}, the flux catalyzed by plastidic aldolase; V_{ppp}, the flux through the pentose phosphate pathway; V_{sta}, the rate of starch synthesis; V_{ats}, the rate of starch degradation; V_{gly}, the glycolytic flux. Gl designates external Glc; G, internal Glc; H, cytosolic Glc-6-P (identically labeled to sucrose glucosyl); F, cytosolic Fru-6-P (identically labeled to sucrose fructosyl); HP, plastidial hexose-P (identically labeled to starch); and T, triose-P. Numbers indicate the carbon positions in the molecule. The resolution of simultaneous algebraic equations was performed using the Mathematica software (Wolfram Research, Champaign, IL).

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