Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 2189–2196
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Vacuolar compartmentation complicates the steady-state analysis of glucose metabolism and forces reappraisal of sucrose cycling in plants

Nicholas J. Kruger *, Pascaline Le Lay, R. George Ratcliffe *

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

Received 21 February 2007; received in revised form 30 March 2007
Available online 23 May 2007

Abstract

Steady-state stable isotope labelling provides a method for generating flux maps of the compartmented network of central metabolism in heterotrophic plant tissues. Theoretical analysis of the contribution of the vacuole to the regeneration of glucose by endogenous processes shows that numerical fitting of isotopomeric data will only generate an accurate map of the fluxes involving intracellular glucose if information is available on the labelling of both the cytosolic and vacuolar glucose pools. In the absence of this information many of the calculated fluxes are at best unreliable or at worst indeterminate. This result suggests that the anomalously high rates of sucrose cycling and glucose resynthesis that have been reported in earlier steady-state analyses of tissues labelled with ^{13}C-glucose precursors may be an artefact of assuming that the labelling pattern of extracted glucose reflected the labelling of the cytosolic pool. The analysis emphasises that although subcellular information can sometimes be deduced from a steady-state analysis without recourse to subcellular fractionation, the success of this procedure depends critically on the structure of the metabolic network. It is concluded that methods need to be implemented that will allow measurement of the subcellular labelling pattern of glucose and other metabolites, as part of the routine analysis of the redistribution of label in steady-state stable isotope labelling experiments, if the true potential of network flux analysis for generating metabolic phenotypes is to be realized.

Keywords: Metabolic modelling; Network flux analysis; Steady-state stable isotope labelling; Subcellular compartmentation; Sucrose cycling; Vacuole

1. Introduction

The metabolic network in eukaryotes spans several subcellular compartments and arguably the resulting complexity is at its greatest in plant cells (Kruger and Ratcliffe, in press). Here the existence of several metabolically important compartments, including the cytosol, mitochondria, plastids and the vacuole, together with the existence of equivalent steps and pathways in two or more compartments, creates a severe challenge for network flux analysis. In the absence of subcellular fractionation, compartmented fluxes can be deduced in favourable cases from the labelling patterns of metabolites that are synthesised in only one compartment (Ratcliffe and Shachar-Hill, 2005, 2006), and typically flux maps based on steady-state analysis include mitochondrial and/or plastidic fluxes as well as those in the cytosol (Dieuaide-Noubhani et al., 1995; Schwender et al., 2006; Sriram et al., 2004). The objective of using these flux maps to provide functional metabolic phenotypes for the network encourages the development of more complex models that might capture the operation of the network more effectively, but at the same time model building needs to be conservative since the quality of the data may not support a more elaborate model (Ratcliffe and Shachar-Hill, 2006). Exchange processes across internal membranes also work against the physical isolation

* Corresponding authors. Tel.: +44 1865 275000; fax: +44 1865 275074.
E-mail addresses: nick.kruger@plants.ox.ac.uk (N.J. Kruger), george.ratcliffe@plants.ox.ac.uk (R.G. Ratcliffe).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.04.004

of pools and enzymes with the result that there may be no justification for modelling equivalent processes in separate compartments independently (Schwender et al., 2003).

One compartment that has generally been ignored in the steady-state network flux analysis of plant metabolism is the vacuole. At first sight this might seem justifiable, since the participation of the vacuole in metabolism is largely based on the accumulation and storage of products derived from cytosolic metabolism. If significant accumulation occurs during a steady-state labelling experiment, then this can be accounted for as one of the output fluxes. Moreover, if the uptake of the vacuolar metabolite from the cytosol is reversible, then this process will have no effect on the labelling of the cytosolic pool since this is the pool from which the vacuolar metabolite originated. However, the situation becomes more complicated if the vacuolar metabolite is generated in such a way that the cytosolic and vacuolar pools of the metabolite have different labelling patterns in the steady-state. The analytical approaches commonly used in network flux analysis make little or no attempt to distinguish the labelling patterns of identical metabolites from separate compartments by direct measurement. Instead, this information is either inferred from the labelling pattern of downstream metabolites, or an assumption is made that the metabolite is effectively uncompartmented as a result of fast exchange processes, or that the analysed pool will be predominantly from a single compartment (Ratcliffe and Shachar-Hill, 2006).

This paper presents a theoretical analysis, and associated simulations, of the impact of glucose storage in the vacuole on the steady-state flux analysis of carbohydrate metabolism in plant cells. The analysis provides an alternative explanation for the surprisingly large rates of sucrose cycling that have been reported in some ^{13}C-labelling experiments, and it suggests that an accurate measure of this process can only be obtained from a steady-state analysis if the labelling pattern is known for both the cytosolic and vacuolar glucose pools.

2. Results

The models of carbohydrate metabolism that are commonly used for steady-state flux analysis in plants generally ignore the impact of the vacuolar compartmentation of metabolites, and metabolite interconversions, on the redistribution of label in the system. For a typical steady-state labelling experiment with [1-^{13}C]glucose (Alonso et al., 2005; Dieuaide-Noubhani et al., 1995; Rontein et al., 2002), the redistribution of label from C1 to C6 in the endogenous glucose pool might be analysed using the scheme shown in Fig. 1. Here the resynthesis of glucose from the hexose phosphate pool leads to the redistribution of label in the glucose pool because the labelling of the hexose phosphate pool is itself scrambled as a result of hexose phosphate/triose phosphate recycling and multiple fluxes through the pentose phosphate pathway (Fernie et al., 2001; Roscher et al., 2000). Assuming an isotopic and metabolic steady-state for cytosolic glucose (Glc_{cyt}), and equating Glc_{cyt} in Fig. 1 with an endogenous glucose pool (G_{int}) gives two equations:

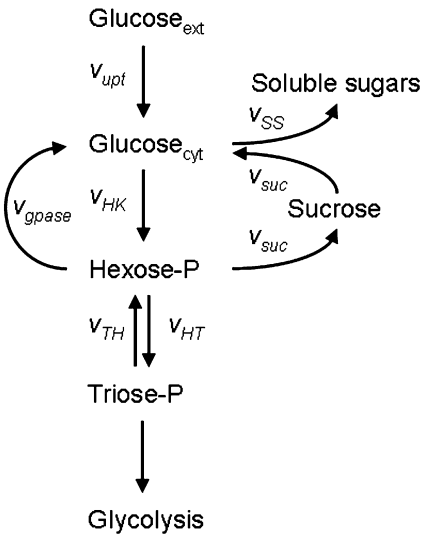

Fig. 1. Metabolic network commonly used to analyse the labelling of the cytosolic glucose pool in steady-state labelling experiments with an external supply of ^{13}C-labelled glucose. The diagram only shows processes directly relevant to the labelling of cytosolic glucose and it is assumed that resynthesis of glucose occurs in the cytosol. Recycling between hexose phosphates and triose phosphates is also included to allow for redistribution of label between C1 and C6 of the hexose phosphate pool. The defined fluxes (v) involve the following enzymes/transport processes: v_{upt}, glucose uptake across the plasma membrane; v_{HK}, hexokinase; v_{HT}, phosphofructokinase/PPi-fructose 6-phosphate phosphotransferase/aldolase/triose phosphate isomerase; v_{TH}, triose phosphate isomerise/aldolase/PPi-fructose 6-phosphate phosphotransferase/fructose-1,6-bisphosphatase; v_{gpase}, glucose-6-phosphatase; v_{suc}, sucrose cycling through sucrose phosphate synthase/sucrose phosphatase and invertase; v_{SS}, accumulation of intracellular glucose.

$$v_{upt} f^{C1}_{Glc\text{-}ext} + (v_{gpase} + v_{suc}) f^{C1}_{HexP} = (v_{HK} + v_{SS}) f^{C1}_{Glc\text{-}int} \quad (1)$$

$$v_{upt} + (v_{gpase} + v_{suc}) = v_{HK} + v_{SS} \quad (2)$$

where $f^{position}_{molecule}$ is the fractional abundance of ^{13}C at a specified atom in the specified molecule and v_x is a flux between specified points in the network (Fig. 1). Eqs. (1) and (2) can be combined to provide a relation between the fluxes into the Glc_{int} pool:

$$\frac{v_{gpase} + v_{suc}}{v_{upt}} = \frac{f^{C1}_{Glc\text{-}int} - f^{C1}_{Glc\text{-}ext}}{f^{C1}_{HexP} - f^{C1}_{Glc\text{-}int}} \quad (3)$$

The flux v_{gpase} has only recently been proposed (Alonso et al., 2005) and Eq. (3) is more commonly written (Alonso et al., 2005; Dieuaide-Noubhani et al., 1995; Rontein et al., 2002):

$$\frac{v_{suc}}{v_{upt}} = \frac{f^{C1}_{Glc\text{-}int} - f^{C1}_{Glc\text{-}ext}}{f^{C1}_{HexP} - f^{C1}_{Glc\text{-}int}} \quad (4)$$

Note that Eqs. (1)–(4) are written in terms of Glc_{int}, rather than the Glc_{cyt}, because this is the form in which they have been used, i.e. information on the label distribution in the

cytosolic glucose pool is inferred from information on the labelling of the glucose in an unfractionated extract. This approximation is only valid if the other pools of glucose are derived directly from the cytosolic glucose pool, and if these other pools are empty prior to the start of the labelling experiment. In principle the second condition can be relaxed by measuring an appropriate dilution factor (Alonso et al., 2005), but the first condition is much more problematic because the vacuole is not just a compartment for receiving the products of cytosolic metabolism.

The simplified network in Fig. 1 ignores the fact that glucose regeneration is likely to be a vacuolar process whether promoted by invertase activity (Leigh et al., 1979; Richardson et al., 1990; Sergeeva et al., 2006) or by the putative glucose phosphatase activity (Alonso et al., 2005). The network in Fig. 2, which is related to one discussed in detail elsewhere (Malone et al., 2006) allows these processes to occur in the vacuole, and it can be analysed in exactly the same way as before. Thus assuming an isotopic and metabolic steady-state for Glc_{cyt} leads to:

$$v_{upt} f^{C1}_{Glc\text{-}ext} + (v_{release}) f^{C1}_{Glc\text{-}vac} = (v_{HK} + v_{store}) f^{C1}_{Glc\text{-}cyt} \quad (5)$$

$$v_{upt} + v_{release} = v_{HK} + v_{store} \quad (6)$$

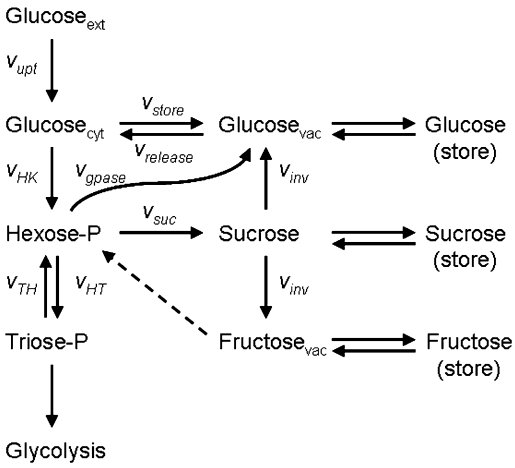

Fig. 2. Metabolic network for the analysis of the steady-state labelling of cytosolic glucose assuming that regeneration of glucose occurs in the vacuole. Processes directly relevant to the labelling of cytosolic glucose are shown with solid arrows; the return of vacuolar fructose to the cytosolic hexose phosphate pool, which has no impact on the redistribution of label, is shown with a dotted arrow. Recycling between hexose phosphates and triose phosphates is included to allow for redistribution of label between C1 and C6 of the hexose phosphate pool. Output fluxes to sucrose(store), glucose(store) and fructose(store) are included to allow for accumulation of soluble sugars in the vacuole. The defined fluxes (v) involve the following enzymes/transport processes: v_{upt}, glucose uptake across the plasma membrane; v_{HK}, hexokinase; v_{HT}, phosphofructokinase/PPi-fructose 6-phosphate phosphotransferase/aldolase/triose phosphate isomerase; v_{TH}, triose phosphate isomerise/aldolase/PPi-fructose 6-phosphate phosphotransferase/fructose-1,6-bisphosphatase; v_{suc}, sucrose phosphate synthase/sucrose phosphatase/sucrose transport across the tonoplast; v_{inv}, vacuolar invertase; $v_{release}$, glucose transport from vacuole to cytosol across the tonoplast; v_{store}, glucose transport from cytosol to vacuole across the tonoplast. Details of carbon atom rearrangements occurring in these steps are presented in Supplementary Information.

which can be combined to give a new equation for the relative contributions to the labelling of the Glc_{cyt} pool:

$$\frac{v_{release}}{v_{upt}} = \frac{f^{C1}_{Glc\text{-}cyt} - f^{C1}_{Glc\text{-}ext}}{f^{C1}_{Glc\text{-}vac} - f^{C1}_{Glc\text{-}cyt}} \quad (7)$$

This equation has two implications for the analysis of the resynthesis of glucose in steady-state labelling experiments. First, in a system in which glucose can accumulate in the vacuole by direct transfer from the cytosol ($v_{store} > 0$), as would occur for example with a non-zero v_{SS} in the scheme shown in Fig. 1, the return of label to the cytosolic pool via the vacuolar degradation of sucrose or glucose 6-phosphate depends on $v_{release}$ rather than simply on the metabolic processes that lead to the formation of glucose in the vacuole. Thus even if one of these processes is thought to dominate the resynthesis of glucose, the rate of that process cannot be determined from Eq. (7) without also knowing both v_{store} and the net rate of glucose accumulation in the vacuole. Secondly, only in a situation where glucose is released irreversibly from the vacuole ($v_{store} = 0$), and where there is also no net accumulation of vacuolar glucose or sucrose ($v_{suc} = v_{inv}$), does Eq. (7) reduce to the simple form:

$$\frac{v_{gpase} + v_{suc}}{v_{upt}} = \frac{f^{C1}_{Glc\text{-}cyt} - f^{C1}_{Glc\text{-}ext}}{f^{C1}_{Glc\text{-}vac} - f^{C1}_{Glc\text{-}cyt}} \quad (8)$$

Since $f^{C1}_{Glc\text{-}vac} = f^{C1}_{HexP}$ when $v_{store} = 0$, Eq. (8) is identical with Eq. (3) except for the explicit inclusion of $f^{C1}_{Glc\text{-}cyt}$ in place of $f^{C1}_{Glc\text{-}int}$. However this emphasises the importance of distinguishing between $f^{C1}_{Glc\text{-}cyt}$ and $f^{C1}_{Glc\text{-}int}$. While $f^{C1}_{Glc\text{-}cyt} = f^{C1}_{Glc\text{-}int}$ under the conditions required to derive (8), these two quantities will not usually be the same, and approximating $f^{C1}_{Glc\text{-}cyt}$ to $f^{C1}_{Glc\text{-}int}$ because the latter is easily measurable will lead to significant errors in the estimation of ($v_{gpase} + v_{suc}$).

The impact of neglecting vacuolar compartmentation in the analysis of the redistribution of label from a glucose precursor can be modelled using ^{13}C-Flux (Wiechert et al., 2001). Fig. 3a shows that the apparent rate of recycling of hexose phosphate to cytosolic glucose ($v_{inv} + v_{gpase}$) becomes much greater as exchange between the cytosolic and vacuolar glucose pools is restricted. The ratio $v_{store}/v_{release}$ is identical to the disequilibrium ratio (Fell, 1997) for the efflux of glucose from the vacuole, and the modelling exercise shows that the error in the rate of recycling increases as the extent of equilibration of the newly synthesised vacuolar glucose with the cytosolic pool decreases. The magnitude of this error also depends on the distribution of glucose between the two compartments, and the analysis shows that even relatively small amounts of vacuolar glucose may have an appreciable effect on the apparent flux. In fact, current estimates suggest that the vacuolar volume is typically 5–10 times that of the cytosol (Farré et al., 2001; Leidreiter et al., 1995; Winter et al., 1993, 1994) and, assuming that the concentration of glucose is the same in these two compartments (Farré et al., 2001), the extent of conversion of hexose phosphates to

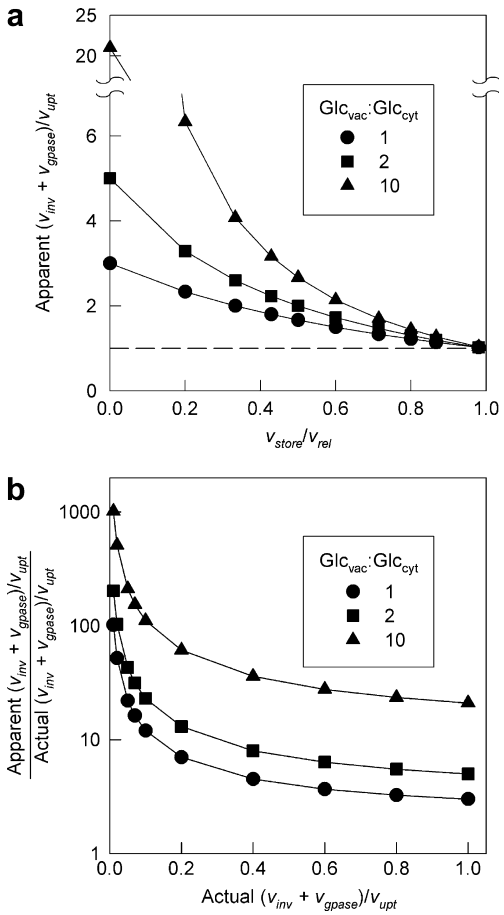

Fig. 3. Influence of the vacuolar compartmentation of glucose on estimates of the recycling of hexose phosphates to cytosolic glucose for the network defined in Fig. 2. (a) The dependence of the apparent rate of hexose phosphate recycling ($v_{inv} + v_{gpase}$) on the flux ratio $v_{store}/v_{release}$, assuming a ratio of vacuolar to cytosolic glucose content of 1 (●), 2 (■) or 10 (▲). The recycling rate was calculated from Eq. (3) using values for $f^{C1}_{Glc\text{-}int}$ calculated from the steady-state values of $f^{C1}_{Glc\text{-}cyt}$ and $f^{C1}_{Glc\text{-}vac}$, and it is expressed relative to the uptake of external glucose (v_{upt}). The actual ratio $(v_{inv} + v_{gpase})/v_{upt}$ was held constant at 1.0 and is shown by the dashed line. (b) The deviation of the apparent recycling rate from the true recycling rate as a function of the true recycling rate. The calculations were performed for $v_{store}/v_{release} = 0$ and the results are plotted on a logarithmic scale.

glucose could be over-estimated by up to 20-fold if the effect of vacuolar compartmentation is ignored. While the simulations in Fig. 3a correspond to a situation in which the true rate of recycling and v_{upt} are identical, the same effect is observed at other ratios of these two fluxes (Fig. 3b). Here the ratio of the apparent recycling rate to the true recycling rate increases to very high values as the recycling rate becomes slow relative to the uptake rate. In both cases the ultimate origin of the effect is the lack of equilibration of the vacuolar pool with the cytosolic pool.

The significance of the cytosolic and vacuolar glucose pools can also be demonstrated by determining the reliability of flux estimates based on measurements of the fractional abundance of ^{13}C in positions C1 and C6 of selected metabolites. Distribution of label within the external, cytosolic and vacuolar glucose pools and the hexose phosphate pool is sufficient to obtain reliable estimates of the fluxes involving glucose in the network defined in Fig. 2. However, the precision of the estimated fluxes varies between reactions, and it depends on the labelling data used and the errors associated with them (Table 1). Thus flux estimates based on f^{C1} and f^{C6} are better than those based on f^{C1} alone, or those based solely on f^{C6} (data not shown). The impact of measurement error varies between reactions and is dependent on the isotopomer set used to define the network, but restricting the input of labelling information can decrease the reliability of flux estimates profoundly, even when the isotopomer abundances are determined with high precision. Analysis of the network at an experimentally exacting degree of measurement error (1%) indicates that information on labelling is required for both the cytosolic and vacuolar glucose pools to obtain informative estimates of flux through the network (Table 2). In the absence of labelling information for the vacuolar glucose pool, the fluxes between the cytosolic and vacuolar pools of glucose (determined by v_{rel} and v_{store}) are essentially indeterminate. Similarly, under most conditions, when the labelling of the cytosolic pool is unknown, the extent of sucrose recycling ($v_{inv} + v_{gpase}$) as well as v_{rel} and v_{HK} are poorly defined. Moreover, if no information is available from either of the internal glucose pools, none of the fluxes are determinable (data not shown). Hence, knowledge of labelling in both the cytosolic and vacuolar glucose pools is required to define fluxes in the compartmented model adequately.

3. Discussion

The continuous synthesis and degradation of sucrose that is frequently observed in heterotrophic plant tissues gives rise to an energy consuming substrate cycle (Geigenberger and Stitt, 1991b). This process was first quantified by comparing the rate of sucrose synthesis, measured in a short-term ^{14}C-labelling experiment, with the rate of sucrose accumulation and results have been obtained for many tissues, including roots (Hargreaves and ap Rees, 1988), heterotrophic cell suspension cultures (Dancer et al., 1990; Wendler et al., 1991), cotyledons (Geigenberger and Stitt, 1991a), tubers (Geigenberger and Stitt, 1993; Trethewey et al., 1999), and ripening fruits (Hill and ap Rees, 1994; MacRae et al., 1992). The occurrence of sucrose cycling in sugar cane is also well documented (Whittaker and Botha, 1997), and the factors determining its extent have been modelled *in silico* on the basis of *in vitro* kinetic data (Rohwer and Botha, 2001; Schafer et al., 2004). Sucrose cycling has an associated energy cost and this can be calculated on the basis of experimentally justifiable assumptions about the relative contributions of sucrose phosphate synthase and sucrose synthase to synthesis, and sucrose synthase and invertase to degradation. The result is usually expressed as a % of ATP turnover,

Table 1
Influence of measurement error on estimates of glucose recycling

Reaction	Flux	Standard deviation of flux estimate calculated from defined fractional enrichments with specified measurement errors							
		f^{C1}				f^{C1} and f^{C6}			
		1%	2%	5%	10%	1%	2%	5%	10%
v_{upt}	100	0.77	0.92	0.99	1.00	0.39	0.65	0.91	0.97
v_{HK}	110	5.53	10.13	24.19	47.99	0.53	0.90	1.43	2.07
$v_{inv} + v_{gpase}$	10	5.95	10.47	24.36	48.08	0.20	0.38	0.86	1.67
v_{rel}	10	5.75	10.36	24.49	48.49	0.27	0.55	1.37	2.73
v_{store}	0	0.79	1.43	3.40	6.72	0.24	0.46	1.11	2.19

The dependence of the standard deviation of the flux estimates on the precision of the isotopomer measurements was determined for the metabolic network shown in Fig. 2. The analysis was based on the fractional enrichments in positions C1 (f^{C1}) and C6 (f^{C6}) of Glc_{ext}, Glc_{cyt}, Glc_{vac} and Hexose-P for a network in which $(v_{inv} + v_{gpase}) = 0.1(v_{upt})$ and $v_{store} = 0$.

Table 2
Influence of glucose labelling information on flux estimates

$\frac{(v_{inv} + v_{gpase})}{v_{upt}}$	Reaction	Flux	Standard deviation of flux estimate determined from:					
			f^{C1}			f^{C1} and f^{C6}		
			All metabolites	Without Glc_{vac}	Without Glc_{cyt}	All metabolites	Without Glc_{vac}	Without Glc_{cyt}
0.01	v_{upt}	100	**0.77**	**1.00**	*	**0.39**	**0.53**	**0.51**
	v_{HK}	101	**4.68**	**14.8**	*	**0.43**	**4.39**	468
	$v_{inv} + v_{gpase}$	1	5.11	14.4	*	**0.14**	4.63	468
	v_{rel}	1	5.09	1.60×10^6	*	**0.14**	4.62×10^5	468
	v_{store}	0	0.07	1.60×10^6	*	0.02	4.62×10^5	0.03
0.1	v_{upt}	100	**0.77**	**1.00**	*	**0.39**	**0.53**	**0.51**
	v_{HK}	110	**5.53**	**16.5**	*	**0.53**	**4.86**	4.68×10^4
	$v_{inv} + v_{gpase}$	10	5.95	16.0	*	**0.20**	5.09	4.68×10^4
	v_{rel}	10	5.75	1.74×10^7	*	**0.27**	5.04×10^6	4.68×10^4
	v_{store}	0	0.79	1.74×10^7	*	0.24	5.04×10^6	0.29
0.2	v_{upt}	100	**0.77**	*	*	**0.39**	*	**0.51**
	v_{HK}	120	**6.46**	*	*	**0.66**	*	1.87×10^5
	$v_{inv} + v_{gpase}$	20	6.89	*	*	**0.30**	*	1.87×10^5
	v_{rel}	20	6.55	*	*	**0.51**	*	1.87×10^5
	v_{store}	0	1.73	*	*	0.52	*	0.63
0.5	v_{upt}	100	**0.77**	*	*	**0.39**	*	**0.51**
	v_{HK}	150	**10.18**	*	*	**1.09**	*	1.17×10^6
	$v_{inv} + v_{gpase}$	50	10.58	*	*	**0.72**	*	1.17×10^6
	v_{rel}	50	10.39	*	*	**1.52**	*	1.17×10^6
	v_{store}	0	5.42	*	*	1.62	*	1.94
1	v_{upt}	100	**0.95**	*	**1.00**	**0.59**	**0.81**	**0.94**
	v_{HK}	200	**2.68**	*	**3.01**	**0.57**	**1.05**	**0.87**
	$v_{inv} + v_{gpase}$	100	**3.14**	*	**3.32**	**1.04**	**1.15**	**1.05**
	v_{rel}	100	**18.08**	*	**19.49**	**4.32**	1.29×10^7	**4.38**
	v_{store}	0	16.06	*	17.22	4.82	1.29×10^7	4.88

The impact of omitting the labelling information for the intracellular pools of glucose was determined for the metabolic network shown in Fig. 2. The analysis was based on the fractional abundance in positions C1 (f^{C1}) and C6 (f^{C6}) of Glc_{ext}, Glc_{cyt}, Glc_{vac} and hexose-P with measurement errors of 1%. Values in bold indicate standard deviation <0.2(flux) for non-zero fluxes. * Indicates standard deviations of flux estimates that are indeterminate without further constraints on the network.

and typically the result is of the order of 5% (Bindon and Botha, 2002; Dancer et al., 1990; Geigenberger and Stitt, 1991a; Trethewey et al., 1999). However there are instances where sucrose cycling consumes a much greater proportion of the available ATP, for example more than 60% in ripening banana fruit (Hill and ap Rees, 1994).

Steady-state ^{13}C-labelling provides an alternative method for measuring sucrose cycling, and applications have been reported for maize root tips (Alonso et al., 2005; Dieuaide-Noubhani et al., 1995) and tomato cell suspension cultures (Rontein et al., 2002). It appeared from this work that the energy consumption for sucrose cycling was much higher than most of the early measurements, with values of 69% of ATP turnover in maize root tips (Dieuaide-Noubhani et al., 1995), and values of 62%, 82% and 43% in cultured tomato cells at days 5, 6 and

7.5, respectively (Rontein et al., 2002). This discrepancy has since been addressed by performing both short term ^{14}C-labelling experiments and a steady-state ^{13}C analysis on the same tissue (Alonso et al., 2005). These experiments showed that sucrose cycling was not the primary mechanism for the apparent resynthesis of glucose in maize root tips, that sucrose cycling in this system only consumed 3–6% of the available ATP, and that therefore some other mechanism, thought to be the action of a glucose phosphatase, must be responsible for the resynthesis of glucose (Alonso et al., 2005). As well as providing an alternative explanation for the redistribution of label in the internal glucose pool, this proposal also had the advantage of reducing the ATP cost of glucose resynthesis to approximately 40% of ATP turnover (Alonso et al., 2005).

The analysis presented here suggests that the rate of glucose recycling has been overestimated in these steady-state labelling experiments. Theory and modelling show that apparently high rates of glucose resynthesis can be obtained by ignoring the likely vacuolar location of the process, or by assuming that the labelling of the cytosolic and vacuolar glucose pools is equivalent (Figs. 2 and 3). In contrast if the role of the vacuole in the network is taken into account, then it is no longer necessary to invoke high rates for the degradation of sucrose or glucose 6-phosphate. Several lines of evidence suggest that the conclusions of this analysis are relevant to the steady-state analysis of the pathways of carbohydrate metabolism. First, invertase contributes to sucrose turnover in many heterotrophic tissues, and much of this turnover is considered to be vacuolar (Leigh et al., 1979; Richardson et al., 1990; Whittaker and Botha, 1997). Secondly, soluble sugars certainly accumulate in tissues during steady-state labelling experiments with glucose (18% of glucose uptake in maize root tips, (Dieuaide-Noubhani et al., 1995); 4–18% of glucose uptake in tomato cells, (Rontein et al., 2002)) and this will be partitioned between cytoplasm and vacuole. Thirdly, starvation decreased the redistribution of label from C1 to C6 of endogenous glucose in maize root tips (Alonso et al., 2005), and while there is insufficient data in the paper to provide a definitive explanation, this observation is consistent with a reduced contribution from vacuolar glucose to the fractional enrichment of the extracted glucose.

It can be concluded that there is no need to invoke a glucose phosphatase activity to explain the labelling data for the endogenous glucose pool in maize root tips (Alonso et al., 2005), and this in turn further reduces the apparent energy cost attributed to glucose resynthesis. It would certainly be reassuring if expenditure on the recycling of carbohydrate turned out to be of the order of only a few % of ATP turnover in all but the most exceptional cases (Hill and ap Rees, 1994). In passing it may be noted that whatever the origin of the redistribution of label in the cytosolic glucose pool, it seems unlikely that it could be attributed to the reversibility of hexokinase. This energy-neutral option does not appear to have been considered explicitly in earlier analyses, but it seems likely that the step catalysed by hexokinase is sufficiently far from equilibrium (Cornish-Bowden and Cardenas, 2001) for the reverse step to provide only a very limited input into the glucose pool.

The theoretical analysis presented here also has wider implications for the scope and practice of network flux analysis in compartmented systems. First, the network analysed in Fig. 2 provides an example of a system in which knowledge of subcellular labelling patterns is mandatory if the flux map is to be deduced correctly. One of the advantages of the steady-state labelling approach is that it allows subcellular information to be obtained without subcellular fractionation (Ratcliffe and Shachar-Hill, 2005, 2006), but the success of this strategy clearly depends on the structure of the network. Thus, as suggested elsewhere (Fernie et al., 2005), it would be useful to investigate the practicality of using subcellular fractionation, or potentially other direct measurement methods, to generate labelling information for specific pools of compartmented metabolites. Secondly, although this paper focuses on a problem that arises through the vacuolar compartmentation of glucose, similar problems may be encountered in other sections of the metabolic network where interconversions occur between compartmented pools of metabolites that are generated through different routes and which may therefore differ in their isotopomer composition. We emphasise that this problem is not confined to highly abundant metabolites, and that it is the potential lack of isotopic equilibration between compartmented pools, not the relative size of the pools, that is the cause of the problem. Assuming fast exchange between such pools, or assuming that the measured labelling pattern reflects a specific compartment for convenience, may lead to an erroneous flux map and a misleading metabolic phenotype. At the very least the absence of specific subcellular information where it is required can be expected to lead to poorer definition of the true fluxes as demonstrated here for glucose cycling. Finally, we note that the analysis presented here could be further complicated by the increasing evidence for two parallel pathways of glucose uptake into heterotrophic plant cells, the conventional pathway through a plasma membrane transporter into the cytosol and a vesicular pathway linking the external medium and the vacuole (Baroja-Fernandez et al., 2006; Etxeberria et al., 2005; Junker et al., 2006). It would be a straightforward task to modify the network shown in Fig. 2 to allow for this process, by introducing another unknown flux (two if the process is considered to be reversible), but the potential existence of this pathway further emphasises the need to obtain direct experimental evidence for the labelling of the cytosolic and vacuolar glucose pools.

4. Experimental

The impact of the vacuolar compartmentation of glucose on estimates of the rate of recycling of hexose phosphates was analysed in the network defined in Fig. 2 using ^{13}C-Flux (Wiechert et al., 2001). The details of the model are presented as Supplementary Information.

Sucrose synthase is specifically excluded from this scheme since UDPglucose and fructose generated from sucrose will retain the isotopomer composition of the hexose phosphates from which they were synthesised and thus recycling of these metabolites will not influence the labelling pattern of the hexose phosphate pool. In addition, other major outputs from the hexose phosphate pool (such as cell wall components) are excluded since their synthesis does not affect the isotopomer composition of the intermediates within the defined network. The CumoNet routine in ^{13}C-Flux was used to simulate the steady-state redistribution of label from an exogenous supply of [1-^{13}C]glucose for defined fluxes through the network, and the predicted abundance of the isotopomers was then used to calculate the fractional enrichment of particular carbon atoms in particular metabolites. In these simulations the net accumulation of vacuolar glucose and sucrose were constrained to zero, the extent of recycling of hexose phosphates was adjusted by systematically varying v_{HK}, and the degree of equilibration between the cytosolic and vacuolar glucose pools was varied by adjusting v_{store}. Redistribution of label from C1 to C6 in the hexose phosphate pool was simulated by setting $v_{TH} = 100$. $f^{C1}_{Glc\text{-}int}$ was obtained from the values of $f^{C1}_{Glc\text{-}cyt}$ and $f^{C1}_{Glc\text{-}vac}$ for specified ratios of the glucose content in the two compartments, and the apparent rate of glucose resynthesis was calculated from Eq. (3).

The reliability of the flux estimates through the network defined in Fig. 2 was determined from a sensitivity analysis of isotopomer abundance using ^{13}C-Flux (Wiechert et al., 2001). The analysis was based on the fractional abundance of ^{13}C in positions C1 and C6 of the external, cytosolic and vacuolar pools of glucose, and the hexose phosphate pool. The standard deviations of the estimated fluxes were calculated using the EstimateStat routine in ^{13}C-Flux assuming constant measurement errors of 1% unless noted otherwise. In these simulations, fluxes were set to the following values: v_{upt}, 100; v_{TH}, 100; v_{store}, 0. The net accumulation of vacuolar glucose and sucrose were also constrained to 0, and the extent of glucose recycling was adjusted by systematically varying v_{HK}. In some simulations involving reduced sets of isotopomer measurements, standard deviations of the estimated fluxes could not be calculated by EstimateStat without further constraints in the network, and these fluxes were considered to be indeterminate.

Acknowledgement

This research was supported by the Biotechnology and Biological Sciences Research Council of the United Kingdom (Grant # B17210).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.04.004.

References

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.

Baroja-Fernandez, E., Etxeberria, E., Munoz, F.J., Moran-Zorzano, M.T., Alonso-Casajus, N., Gonzalez, P., Pozueta-Romero, J., 2006. An important pool of sucrose linked to starch biosynthesis is taken up by endocytosis in heterotrophic cells. Plant Cell Physiol. 47, 447–456.

Bindon, K.A., Botha, F.C., 2002. Carbon allocation to the insoluble fraction, respiration and triose-phosphate cycling in the sugarcane culm. Physiol. Plant 116, 12–19.

Cornish-Bowden, A., Cardenas, M.L., 2001. Information transfer in metabolic pathways. Effects of irreversible steps in computer models. Eur. J. Biochem. 268, 6616–6624.

Dancer, J., Hatzfeld, W.D., Stitt, M., 1990. Cytosolic cycles regulate the turnover of sucrose in heterotrophic cell suspension cultures of *Chenopodium rubrum* L. Planta 182, 223–231.

Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A., Raymond, P., 1995. Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from ^{13}C-labeled or ^{14}C-labeled glucose. J. Biol. Chem. 270, 13147–13159.

Etxeberria, E., Gonzalez, P., Tomlinson, P., Pozueta-Romero, J., 2005. Existence of two parallel mechanisms for glucose uptake in heterotrophic plant cells. J. Exp. Bot. 56, 1905–1912.

Farré, E.M., Tiessen, A., Roessner, U., Geigenberger, P., Trethewey, R.N., Willmitzer, L., 2001. Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol. 127, 685–700.

Fell, D., 1997. Understanding the Control of Metabolism. Portland Press, London, pp. 15–16.

Fernie, A.R., Geigenberger, P., Stitt, M., 2005. Flux an important, but neglected, component of functional genomics. Curr. Opin. Plant Biol. 8, 174–182.

Fernie, A.R., Roscher, A., Ratcliffe, R.G., Kruger, N.J., 2001. Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. Planta 212, 250–263.

Geigenberger, P., Stitt, M., 1991a. A futile cycle of sucrose synthesis and degradation is involved in regulating partitioning between sucrose, starch and respiration in cotyledons of germinating *Ricinus communis* L seedlings when phloem transport is inhibited. Planta 185, 81–90.

Geigenberger, P., Stitt, M., 1991b. The role of futile cycles in sucrose metabolism. Bio. Chem. Hoppe-Seyler 372, 660.

Geigenberger, P., Stitt, M., 1993. Sucrose synthase catalyzes a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. Planta 189, 329–339.

Hargreaves, J.A., ap Rees, T., 1988. Turnover of starch and sucrose in roots of *Pisum sativum*. Phytochemistry 27, 1627–1629.

Hill, S.A., ap Rees, T., 1994. Fluxes of carbohydrate metabolism in ripening bananas. Planta 192, 52–60.

Junker, B.H., Wuttke, R., Nunes-Nesi, A., Steinhauser, D., Schauer, N., Bussis, D., Willmitzer, L., Fernie, A.R., 2006. Enhancing vacuolar sucrose cleavage within the developing potato tuber has only minor effects on metabolism. Plant Cell Physiol. 47, 277–289.

Kruger, N.J., Ratcliffe, R.G., (in press) Metabolic organization in plants: a challenge for the metabolic engineer. In: Bohnert, H.J. Nguyen, H.T. (Eds.), Advances in Plant Biochemistry and Molecular Biology, vol. 1. Bioengineering and Molecular Biology of Plant Pathways. Elsevier, Amsterdam.

Leidreiter, K., Kruse, A., Heineke, D., Robinson, D.G., Heldt, H.W., 1995. Subcellular volumes and metabolite concentrations in potato (*Solanum tuberosum* cv Desirée) leaves. Bot. Acta 108, 439–444.

Leigh, R.A., ap Rees, T., Fuller, W.A., Banfield, J., 1979. Location of acid invertase activity and sucrose in the vacuoles of storage roots of beetroot (*Beta vulgaris*). Biochem. J. 178, 539–547.

MacRae, E., Quick, W.P., Benker, C., Stitt, M., 1992. Carbohydrate metabolism during postharvest ripening in kiwifruit. Planta 188, 314–323.

Malone, J.G., Mittova, V., Ratcliffe, R.G., Kruger, N.J., 2006. The response of carbohydrate metabolism in potato tubers to low temperature. Plant Cell Physiol. 47, 1309–1322.

Ratcliffe, R.G., Shachar-Hill, Y., 2005. Revealing metabolic phenotypes in plants: inputs from NMR analysis. Biol. Rev. 80, 27–43.

Ratcliffe, R.G., Shachar-Hill, Y., 2006. Measuring multiple fluxes through plant metabolic networks. Plant J. 45, 490–511.

Richardson, D.L., Davies, H.V., Ross, H.A., Mackay, G.R., 1990. Invertase activity and its relation to hexose accumulation in potato tubers. J. Exp. Bot. 41, 95–99.

Rohwer, J.M., Botha, F.C., 2001. Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. Biochem. J. 358, 437–445.

Rontein, D., Dieuaide-Noubhani, M., Dufourc, E.J., Raymond, P., Rolin, D., 2002. The metabolic architecture of plant cells – stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J. Biol. Chem. 277, 43948–43960.

Roscher, A., Kruger, N.J., Ratcliffe, R.G., 2000. Strategies for metabolic flux analysis in plants using isotope labelling. J. Biotechnol. 77, 81–102.

Schafer, W.E., Rohwer, J.M., Botha, F.C., 2004. A kinetic study of sugarcane sucrose synthase. Eur. J. Biochem. 271, 3971–3977.

Schwender, J., Ohlrogge, J.B., Shachar-Hill, Y., 2003. A flux model of glycolysis and the oxidative pentose phosphate pathway in developing *Brassica napus* embryos. J. Biol. Chem. 278, 29442–29453.

Schwender, J., Shachar-Hill, Y., Ohlrogge, J.B., 2006. Mitochondrial metabolism in developing embryos of *Brassica napus*. J. Biol. Chem. 281, 34040–34047.

Sergeeva, L.I., Keurentjes, J.J.B., Bentsink, L., Vonk, J., van der Plas, L.H.W., Koornneef, M., Vreugdenhil, D., 2006. Vacuolar invertase regulates elongation of *Arabidopsis thaliana* roots as revealed by QTL and mutant analysis. Proc. Natl. Acad. Sci. USA 103, 2994–2999.

Sriram, G., Fulton, D.B., Iyer, V.V., Peterson, J.M., Zhou, R.L., Westgate, M.E., Spalding, M.H., Shanks, J.V., 2004. Quantification of compartmented metabolic fluxes in developing soybean embryos by employing biosynthetically directed fractional ^{13}C labeling, [^{13}C, ^{1}H] two-dimensional nuclear magnetic resonance, and comprehensive isotopomer balancing. Plant Physiol. 136, 3043–3057.

Trethewey, R.N., Riesmeier, J.W., Willmitzer, L., Stitt, M., Geigenberger, P., 1999. Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. Planta 208, 227–238.

Wendler, R., Veith, R., Dancer, J., Stitt, M., Komor, E., 1991. Sucrose storage in cell-suspension cultures of *Saccharum* sp (sugarcane) is regulated by a cycle of synthesis and degradation. Planta 183, 31–39.

Whittaker, A., Botha, F.C., 1997. Carbon partitioning during sucrose accumulation in sugarcane internodal tissue. Plant Physiol. 115, 1651–1659.

Wiechert, W., Mollney, M., Petersen, S., de Graaf, A.A., 2001. A universal framework for ^{13}C metabolic flux analysis. Metabol. Eng. 3, 265–283.

Winter, H., Robinson, D.G., Heldt, H.W., 1993. Subcellular volumes and metabolite concentrations in barley leaves. Planta 191, 180–190.

Winter, H., Robinson, D.G., Heldt, H.W., 1994. Subcellular volumes and metabolite concentrations in spinach leaves. Planta 193, 530–535.