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Determination of metabolic fluxes in a non-steady-state system

C.J. Baxter [a,1], J.L. Liu [b,1], A.R. Fernie [c], L.J. Sweetlove [a,*]

[a] Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
[b] School of Biological and Biomedical Sciences, Durham University, Durham DH1 3LE, UK
[c] Max-Planck Institute for Molecular Plant Physiology, Am Mühlenberg 14476, Potsdam-Golm, Germany

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Abstract

Estimation of fluxes through metabolic networks from redistribution patterns of ^{13}C has become a well developed technique in recent years. However, the approach is currently limited to systems at metabolic steady-state; dynamic changes in metabolic fluxes cannot be assessed. This is a major impediment to understanding the behaviour of metabolic networks, because steady-state is not always experimentally achievable and a great deal of information about the control hierarchy of the network can be derived from the analysis of flux dynamics. To address this issue, we have developed a method for estimating non-steady-state fluxes based on the mass-balance of mass isotopomers. This approach allows multiple mass-balance equations to be written for the change in labelling of a given metabolite pool and thereby permits over-determination of fluxes. We demonstrate how linear regression methods can be used to estimate non-steady-state fluxes from these mass balance equations. The approach can be used to calculate fluxes from both mass isotopomer and positional isotopomer labelling information and thus has general applicability to data generated from common spectrometry- or NMR-based analytical platforms. The approach is applied to a GC–MS time-series dataset of ^{13}C-labelling of metabolites in a heterotrophic Arabidopsis cell suspension culture. Threonine biosynthesis is used to demonstrate that non-steady-state fluxes can be successfully estimated from such data while organic acid metabolism is used to highlight some common issues that can complicate flux estimation. These include multiple pools of the same metabolite that label at different rates and carbon skeleton rearrangements.

Keywords: Non-steady-state; Flux; Arabidopsis

1. Introduction

It is well established that metabolic networks operate at a quasi steady state and a complex array of regulatory systems are present that maintain the network at that steady state. Nevertheless, in order to adapt to changes in the external environment or to respond to different demands throughout development of the organism, the metabolic network must shift from one steady state to another (Bohnert and Sheveleva, 1998). In order to understand the underlying control structure of the metabolic network, it is necessary to quantify the behaviour of the network during this dynamic phase (Ratcliffe and Shachar-Hill, 2005). The development of metabolite profiling technology platforms means that it is now possible to analyse metabolic change at an unprecedented level of detail (Fernie et al., 2004). By analysing metabolite abundances in a time-series of samples, it is possible to gain detailed information on the nature and extent of metabolic perturbation following a change in conditions (Baxter et al., 2007). However, the interpretation of metabolite abundance changes is not simple (Harada et al., 2006). For example, an increase in a metabolite pool may straightforwardly be taken as an indication of an increase in flux through the pathway to which that metabolite belongs. However, it is equally possible that flux through the pathway is decreased, but the metabolite still accumulates, because the efflux from that metabolite pool has decreased more than the influx into it.

* Corresponding author. Tel.: +44 0 1865 275000.
E-mail address: lee.sweetlove@plants.ox.ac.uk (L.J. Sweetlove).
[1] Contributed equally to this work.

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Ultimately, in order to properly analyze the change in a metabolic network, it is necessary to quantify the change in flux (Fernie et al., 2005).

Flux can be determined by following the fate of isotopically-labelled metabolic precursors supplied to the cells/tissue/organism of interest. There is an established literature dealing with calculation of metabolic flux from such isotope distributions (Hellerstein and Murphy, 2004; Ratcliffe and Shachar-Hill, 2006; Sauer, 2006; Schwender et al., 2004). The approach requires that the system reaches both isotopic and metabolic steady state. Under such conditions, fractional enrichment of label in specific isotopomers reports on specific fluxes. A solution for fluxes through the network under consideration can be attained by fitting fluxes to the isotopomer labelling data with the requirement for flux-balancing as a constraint (Wiechert et al., 2001). However, if one wants to analyze a system that is not at isotopic or metabolic steady state, one is faced with a considerable challenge. Because the flux-balancing constraint no longer applies, global fitting of flux parameters to the data is not likely to generate a unique solution except for very simple, essentially linear metabolic pathways (Morgan and Rhodes, 2002). The specific case of instationary labelling, but steady state pool sizes is amenable to global modelling (Noh et al., 2006; Stephanopoulos et al., 1998), but solution of fluxes during truly dynamic metabolic phases (i.e. neither labelling or pool size are steady state) requires alternative mathematical approaches.

In this paper, we establish a method for deriving non-steady-state fluxes based on the mass-balance of identifiable forms of molecules (Sims and Folkes, 1964). Following the introduction of a ^{13}C-labelled precursor, a time-series of samples are analyzed by gas chromatography–mass spectrometry to give information both on change in metabolite pool size and the percentage labelling of a series of mass isotopomers of each metabolite. We propose a method for constructing over-determined systems for non-steady-states based on this information. A demonstration of the determination of non-steady-state fluxes of threonine biosynthesis is presented based on a previously published GC–MS analysis of the distribution of ^{13}C amongst metabolites of Arabidopsis cells during oxidative stress (Baxter et al., 2007). Organic acid metabolism is also considered to highlight some common problems and limitations that can complicate accurate estimation of fluxes.

2. Theory and computation of non-steady-state fluxes

Using mass spectrometry, mass isotopomers ($m+0$, $m+1, m+2 \ldots m+n$, where n is the number of carbon atoms in the metabolite) of a ^{13}C-labelled metabolite may be measured. Although fluxes in and out of an intracellular metabolite pool do not balance in situations under which steady state is not reached, mass-balance is universally held for all mass isotopomers and is defined as follows: (change in the concentration of a mass isotopomer in a metabolite pool) = (total influx of the mass isotopomer into the pool) – (total efflux of the mass isotopomer out of the pool). In the following section, we demonstrate how to estimate non-steady-state fluxes based on the mass-balance of mass isotopomers.

2.1. Mass-balance of mass isotopomers of each metabolite involving reactions without carbon skeleton rearrangements

Mass spectrometric analysis allows the estimation of total metabolite pool size and the relative abundance of each mass isotopomer for the metabolite in question. The percentage of ^{13}C-label in each mass isotopomer is given from the fractional abundance of each mass isotopomer relative to total pool size. These two parameters (total pool size and percentage of label in each mass isotopomer) allow the description of the mass-balance of mass isotopomers. To illustrate the approach we consider a simple linear pathway in which there is unidirectional flux from a 4-carbon substrate (S) to a 4-carbon product (P) via two intermediate metabolites, (A) and (B). The fluxes (J) are annotated as below.

$$S \xrightarrow{J_{SA}} A \xrightarrow{J_{AB}} B \xrightarrow{J_{BP}} P.$$

In this pathway, we assume that the carbon skeleton is conserved. S, A, B and P all have five mass isotopomers ($m+0, m+1 \ldots m+4$). The mass-balance of mass isotopomers of A and B in this pathway can be represented as

$$\begin{aligned} \frac{\mathrm{d}f_{A_i}[A]}{\mathrm{d}t} &= f_{S_i}J_{SA} - f_{A_i}J_{AB}, \\ \frac{\mathrm{d}f_{B_i}[B]}{\mathrm{d}t} &= f_{A_i}J_{AB} - f_{B_i}J_{BP}, \end{aligned} \qquad i = 0, 1 \ldots 4, \tag{1}$$

where $[A]$ and $[B]$ represent the concentration of metabolite A and B; A_i and B_i ($i = 0, 1 \ldots 4$) represent the ith mass isotopomer of A and B, respectively; f_{S_i}, f_{A_i} and f_{B_i} ($i = 0, 1 \ldots 4$) are the percentage labelling of the ith mass isotopomer of S, A and B, respectively; and J_{SA}, J_{AB}, J_{BP} represent the concentration-dependent fluxes from S to A, from A to B and from B to P, respectively.

Similarly, the mass-balance of total metabolite pool size can be described by Eq. (2):

$$\begin{aligned} \frac{\mathrm{d}[A]}{\mathrm{d}t} &= J_{SA} - J_{AB}, \\ \frac{\mathrm{d}[B]}{\mathrm{d}t} &= J_{AB} - J_{BP}. \end{aligned} \tag{2}$$

In the case of reversible reactions, mass isotopomer mass-balances can also be described using this approach. When the reaction between A and B is reversible, for example, the mass-balance of mass isotopomers of A and B becomes

$$\begin{aligned} \frac{\mathrm{d}f_{A_i}[A]}{\mathrm{d}t} &= f_{S_i}J_{SA} - f_{A_i}J_{AB} + f_{B_i}J_{BA}, \\ \frac{\mathrm{d}f_{B_i}[B]}{\mathrm{d}t} &= f_{A_i}J_{AB} - f_{B_i}J_{BP} - f_{B_i}J_{BA}, \end{aligned} \tag{3}$$

where J_{BA} is the reverse flux from B to A.

Therefore this approach is applicable for the solution of all reaction schemes in which the carbon skeleton is conserved.

2.2. Mass-balance of mass isotopomers of each metabolite involving reactions with carbon skeleton arrangements

When a reaction involves carbon skeleton rearrangement, the change in the concentration of a mass isotopomer in a metabolite pool is also described by total influx of the mass isotopomer into the pool minus its total efflux from the pool. However, in order to accurately describe the influx and efflux the carbon transition must be accounted for. To illustrate this, we consider a linear pathway in which a 5-carbon molecule is converted to a 4-carbon molecule (i.e. the carbon skeleton is not conserved). The pathway is

$$X(5-\text{carbon}) \overset{J_{XY}}{\rightarrow} Y(4-\text{carbon}) \overset{J_{YZ}}{\rightarrow} Z(4-\text{carbon}),$$

If distinct positional isotopomer information can be acquired, the exact transition rule can be established (Schmidt et al., 2000). However, if only the mass isotopomers are available mass-balance requires that certain assumptions be made. For example, in the $m+1$ of 5-carbon X, if we assume that each of the 5-carbons has the same probability of being labelled, then the $m+1$ of 5-carbon X has a probability of 0.2 to become the $m+0$ of 4-carbon Y, and a probability of 0.8 to become the $m+1$ of 4-carbon Y. Following this assumption the mass-balance of mass isotopomers for Y can be expressed as

$$\begin{aligned}
\frac{\mathrm{d}f_{Y_0}[Y]}{\mathrm{d}t} &= \left(f_{X_0}+\frac{1}{5}f_{X_1}\right)J_{XY} - f_{Y_0}J_{YZ},\\
\frac{\mathrm{d}f_{Y_1}[Y]}{\mathrm{d}t} &= \left(\frac{4}{5}f_{X_1}+\frac{2}{5}f_{X_2}\right)J_{XY} - f_{Y_1}J_{YZ},\\
\frac{\mathrm{d}f_{Y_2}[Y]}{\mathrm{d}t} &= \left(\frac{3}{5}f_{X_2}+\frac{3}{5}f_{X_3}\right)J_{XY} - f_{Y_2}J_{YZ},\\
\frac{\mathrm{d}f_{Y_3}[Y]}{\mathrm{d}t} &= \left(\frac{3}{5}f_{X_3}+\frac{4}{5}f_{X_4}\right)J_{XY} - f_{Y_3}J_{YZ},\\
\frac{\mathrm{d}f_{Y_4}[Y]}{\mathrm{d}t} &= \left(\frac{1}{5}f_{X_4}+f_{X_5}\right)J_{XY} - f_{Y_4}J_{YZ}.
\end{aligned} \tag{4}$$

Based on the same principle, the mass-balance of mass isotopomers for all metabolites that involve carbon skeleton rearrangements can be described. Therefore, this approach is applicable for the solution of mass isotopomer for all metabolic transitions, including those with carbon skeleton rearrangements.

3. Flux estimation using mass-balance of mass isotopomers

Under non-steady-state conditions both metabolite pool size and all mass isotopomers distributions are time-dependent. Since fluxes depend on concentrations of substrates, products and effectors it follows that they are time-dependent also. Under non-steady-state conditions, the data for mass isotopomers are usually recorded for a certain time interval (say 1 h). During this time interval, flux is time-dependent. In order to estimate a flux using mass-balance equations of mass isotopomers, we define an average flux for the time interval and assume the average flux is independent of time within the time interval. For the mass-balance of mass isotopomers described by Eq. (1), we have three fluxes (J_{SA}, J_{AB}, and J_{BP} and 10 mass-balance equations, so the system is over-determined for estimating the fluxes for the pool of metabolite A and B. Similarly, the flux estimation can be extended to include other metabolite pools. For example, if metabolites A and B involve other reactions, the number of fluxes and mass-balance equations increases simultaneously and the number of degrees of freedom can be determined accordingly. Therefore, over-determined systems for computing fluxes can be constructed as long as the number of estimated fluxes is smaller than the number of the mass-balance equations of mass isotopomers.

Mathematically, fluxes can be estimated in one of two ways. First, mass-balance equations can be directly integrated using the data of mass isotopomers from $t=0$. For different values of J_{SA}, J_{AB}, and J_{BP}, the least-square errors between experimental data and the calculated data at $t=1$, can be calculated for all mass isotopomers. In this instance the fluxes displaying the minimal least square errors are assumed to be the most accurate. Under non-steady-state conditions the values of fluxes are not constrained. Therefore, the search for the minimal least-square errors may require lengthy computation time. Alternatively, mass-balance equations of mass isotopomers can be discretised over the time interval. Thus, Eq. (1) becomes:

$$\begin{aligned}
\frac{(f_{A_i}[A])^{k+1}-(f_{A_i}[A])^{k}}{\Delta t} &= \frac{(f_{S_i})^{k}+(f_{S_i})^{k+1}}{2}J_{SA}\\
&\quad - \frac{(f_{A_i})^{k}+(f_{A_i})^{k+1}}{2}J_{AB},\\
\frac{(f_{B_i}[B])^{k+1}-(f_{B_i}[B])^{k}}{\Delta t} &= \frac{(f_{A_i})^{k}+(f_{A_i})^{k+1}}{2}J_{AB}\\
&\quad - \frac{(f_{B_i})^{k}+(f_{B_i})^{k+1}}{2}J_{BP}
\end{aligned} \qquad (i=0,1\ldots 4), \tag{5}$$

where k and $k+1$ represent two time points subsequent to supply of labelled precursor, separated by a time interval of Δt. If one assumes that the labelling of molecules changes in a linear fashion between the time interval, then linear regression methods can be applied for flux estimation.

Eq. (5) can be rewritten as

$$\begin{aligned}
z_i &= x_i J_{SA} - y_i J_{AB},\\
w_i &= u_i J_{AB} - v_i J_{BP},
\end{aligned} \qquad (i=0,1\ldots 4), \tag{6}$$

with

$$\begin{aligned}
z_i &= \frac{(f_{A_i}[A])^{k+1}-(f_{A_i}[A])^{k}}{\Delta t}, \quad x_i = \frac{(f_{S_i})^{k}+(f_{S_i})^{k+1}}{2},\\
y_i &= \frac{(f_{A_i})^{k}+(f_{A_i})^{k+1}}{2}, \quad w_i = \frac{(f_{B_i}[B])^{k+1}-(f_{B_i}[B])^{k}}{\Delta t},\\
u_i &= \frac{(f_{A_i})^{k}+(f_{A_i})^{k+1}}{2}, \quad v_i = \frac{(f_{B_i})^{k}+(f_{B_i})^{k+1}}{2}.
\end{aligned}$$

In Eq. (6), x_i, y_i, z_i and u_i, $v_i w_i$ $(i = 0, 1 \ldots 4)$ can be calculated based on mass isotopomer data obtained at two time points. Therefore, J_{SA}, J_{AB} and J_{BP} can be calculated by linear regression. We employed GENSTAT software (VSN International Ltd., Hemel Hempsted, UK) to perform linear regression and statistical analyses.

4. Calculation of fluxes following imposition of oxidative stress in arabidopsis cells

To demonstrate the application of the approach outlined here, we have analysed a published dataset consisting of a time-series of samples analysed by GC–MS (Baxter et al., 2007). The experiment consisted of the introduction of menadione to a heterotrophic Arabidopsis cell suspension culture to induce oxidative stress. At the same time, glucose in the medium was replaced with [U–^{13}C] glucose. Enrichment of the medium with [U–^{13}C] glucose has no effect on metabolic network flux patterns (Kruger et al., 2007). Samples were taken every hour for 6 h and GC–MS analysis provided information both on the change in metabolite pool size and ^{13}C-labelling. The set of relevant metabolite pool size and mass isotopomer data is presented in Supplementary Table 1.

For fluxes to be soluble, it is necessary to have pool size and labelling information not only for the metabolite pool under consideration, but also for all metabolites which input into that pool. The data in Baxter et al. (2007) is derived from a standard GC–MS protocol designed to give an untargeted, broad analysis of a wide range of primary metabolites. As a consequence there are relatively few areas of the metabolic network where there is sufficient density of information for fluxes to be estimated reliably. As examples, we have chosen two metabolic pathways where there is sufficient information: threonine biosynthesis and the tricarboxylic acid (TCA) cycle.

4.1. Threonine biosynthesis

Threonine is synthesised from aspartate in a simple linear pathway (Fig. 1). The dataset in Baxter et al. (2007) contains pool size and labelling information for threonine. In order to estimate the unidirectional flux of threonine synthesis, information about the input metabolite into the threonine pool is also needed. No data were available for phospho-homoserine, the immediate precursor of threonine, but there was information for homoserine, one step preceding. Using time-dependent changes in pool size and labelling of mass isotopomers of homoserine and threonine (Supplementary Table 1) we were able to estimate the flux of homoserine to threonine. This reaction does not involve carbon skeleton rearrangements, so a straightforward relationship between mass isotopomers of homoserine and threonine exists. The homoserine to serine flux over a 6 h period in control cells and cells treated with menadione to induce oxidative stress is shown in Fig. 2. There was a

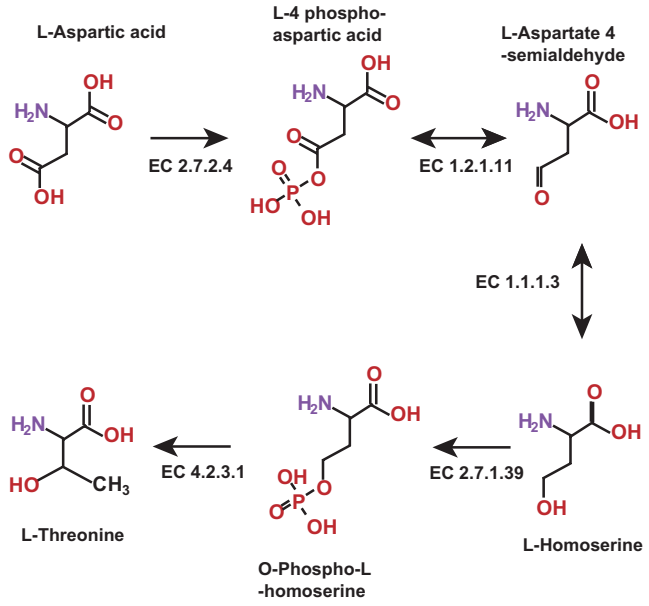

Fig. 1. Metabolic pathway of threonine biosynthesis. Atomic structures were generated using ARM software (Arita et al., 2006).

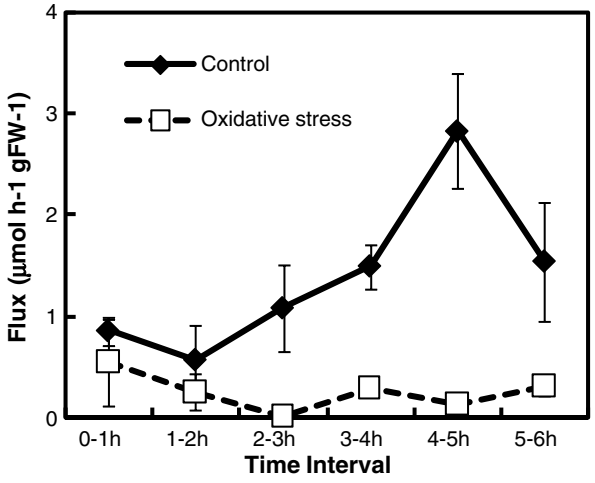

Fig. 2. Rate of threonine biosynthesis during oxidative stress.The unidirectional flux from homoserine to threonine at 6 time intervals (0–1 h, 1–2 h, 2–3 h, 3–4 h, 4–5 h, 5–6 h) in control cells and cells treated with menadione (oxidative stress) is shown. All data are the mean of 4 independent replicates ±s.e.m.

considerable change in the flux in the control cells over the 6 h time period. The initial flux was relatively low (approximately 1 µmol h^{-1} g FW^{-1}), but then rose steadily to a peak of approximately 3 µmol h^{-1} g FW^{-1} after 5 h. This most probably indicates an initial inhibition of threonine synthesis due to handling of the cells followed by a gradual recovery. In contrast, in cells treated with menadione, the threonine biosynthesis flux was low initially and remained so throughout the 6 h time period under consideration. In other words, oxidative stress imposes an additional restriction on threonine biosynthesis which is not overcome within the 6 h period. This confirms the assertion

made by Baxter et al. (2007) that amino acid biosynthesis is decreased during oxidative stress.

4.2. Tricarboxylic acid cycle

Pool size and labelling information was available for all organic acids of the TCA cycle with the exception of succinyl CoA and oxaloacetate (Baxter et al., 2007). In principle the same approach as outlined for threonine biosynthesis can be used to estimate fluxes through the TCA cycle. However, the TCA cycle presents certain issues that complicate the process. The first is that the carbon skeleton is not conserved throughout the cycle. Loss of CO_2 during the isocitrate to 2-oxoglutarate and 2-oxoglutarate to succinyl CoA conversions means that there is not a straightforward relationship between mass isotopomers of these metabolites. For example, there is a simple relationship between the $m+1$ mass isotopmer of isocitrate and the $m+0$ mass isotopomer of 2-oxoglutarate only if the $m+1$ mass isotopomer of isocitrate consists solely of isocitrate labelled at the 6-carbon. In our system, uniformly labelled glucose was supplied. If this leads to uniformly labelled isocitrate, then one can define relationships between specific isocitrate and 2-oxoglutarate mass isotopomers by assuming that each atom makes an equal contribution to each mass isotopomer (see previous section "Mass-balance of mass isotopomers of each metabolite involving reactions with carbon skeleton arrangements" for more details).

The extent to which this assumption holds true during dynamic labelling depends on the exact route of carbon flow. For example, if one considers the first labelled carbon entering the cycle as uniformly labelled acetyl CoA: if this labelled acetyl CoA reacts with unlabelled oxaloacetate made on the previous turn of the cycle, the resulting citrate will be non-uniformly labelled at the 4- and 5-carbons only. However, if the cycle operates in a non-cyclic, anaplerotic mode, with oxaloacetate being derived from phophoenolpyruvate via phophoenolpyruvate carboxylase, the resulting citrate will be labelled evenly at all atoms with the possible exception of the 1-carbon which is derived from CO_2 during the carboxylation of PEP to form oxaloacetate. In certain specialized tissues, cyclic flux mode makes a rather insignificant contribution (Schwender et al., 2004; Schwender et al., 2006). Non-cyclic operation of the TCA cycle may also be more generally prevalent. This view is supported by the observation that non-cyclic flux modes of the TCA cycle confer greatest dynamic stability (Steuer et al., in press). Thus, in certain circumstances, the assumption of an equal contribution of different atoms to different mass isotopomers of the TCA cycle may be valid. Ideally, this assumption would be checked by an empirical analysis of positional isotopomers.

A second complicating factor in the analysis of labelling of organic acids is the presence of large pools of these metabolites in the vacuole (Farre et al., 2001). When one is calculating the flux from citrate to isocitrate, for example, one is making a relationship between the change in labelling of the isocitrate pool and the change in labelling of the citrate pool that feeds into it. However, the change in labelling of these two pools may not necessarily be linked, because change in labelling can occur by movement of labelled mitochondrial organic acids into the vacuole. Because the vacuolar pools are so large, they label more slowly than the mitochondrial and cytosolic pools (Gout et al., 1993). Moreover, in some cases (such as citrate), metabolites are transported into the vacuole and retained there. Such metabolites are effectively metabolically inert. This creates a disconnection between the labelling of citrate and isocitrate which confounds flux calculation.

5. Discussion

In many biological systems a true metabolic and isotopic steady-state is never reached. For example, the metabolic network of the plant leaf is subject to diurnal and circadian oscillations (Blasing et al., 2005). To extend the reach of metabolic flux analysis based on isotope labeling, it will therefore be necessary to develop approaches that allow fluxes to be estimated during non-steady-state phases of isotope labeling and metabolite abundance. Under such conditions, fluxes in and out of metabolite pools do not necessarily balance. Nevertheless, mass-balance is universally held and the difference between influx and efflux will correspond to the change in metabolite abundance. Given an input of an isotopically-labeled precursor into the system, one can write differential equations that describe the mass-balance of influx and efflux of isotopically-labeled carbon into a given metabolite pool. These equations can be integrated to find flux values that generate metabolite labeling patterns that best fit the experimental data. However, because at non-steady-state there is no constraint on possible flux values, the computation time to generate minimal least-square errors will be excessive. As an alternative, we have developed a linearization approach in which the mass-balance equations are discretized over the experimental time interval and fluxes estimated by linear regression analysis. The approach requires that the labeling of molecules change in a linear fashion over the experimental time interval. In the Arabidopsis cell suspension system used here, a significant number of molecules do label in a linear fashion over a 6 h time period (Baxter et al., 2007). However, in other cases, the requirement for linearity places a constraint on the experimental time interval and it is likely that the linearity assumption will only be universally valid for very small time intervals (e.g. seconds). The reliable measurement of change in labeling over such small time intervals will require analytical approaches that are both highly accurate and sensitive and thus mass spectrometry is likely to be the most appropriate platform for such non-steady-state analyses.

To generate reliable flux estimations it is necessary to over-determine the flux. The most straightforward way of achieving this is to construct mass-balances for fractional enrichment of specific positional isotopomers rather than

the overall fractional enrichment of a metabolite. Labeling of multiple atoms of a metabolite allows multiple mass-balances to be written for influx and efflux of label into that pool and hence over-determination of flux. NMR can be used to quantify positional isotopomers. However, as discussed above, the relatively low sensitivity of NMR means that it may not be the most suitable technique dynamic labeling experiments. Alternatively, one can gain positional information by looking at the labeling of ion fragments that form during mass spectrometry. However, depending on the molecule and derivatization method, multiple ions containing different segments of the carbon backbone may not always be present. We therefore wanted to explore whether it was possible to use simple mass isotopomers to over-determine fluxes. Mass isotopomers generated by labeling of different numbers of carbon atoms in a molecule are readily quantifiable by mass spectrometry. Using threonine biosynthesis as an example, we have demonstrated that, for a reaction in which the carbon skeleton is conserved, it is possible to over-determine fluxes using mass-balance of mass isotopomers. We were able to estimate the average unidirectional flux from homoserine to threonine at 1 h time intervals and thus resolve the time-dependence of the rate of threonine synthesis. Moreover, because there is no requirement for metabolic or isotopic steady state, we were able to monitor the flux in the first 6 h following oxidative stress, demonstrating that threonine synthesis is strongly suppressed during oxidative stress and confirming previous qualitative assessments of amino acid metabolism during oxidative stress (Baxter et al., 2007). Mass isotopomers can also be useful even in the event of carbon skeleton rearrangements, provided that the metabolites under consideration are uniformly labeled. We provide a mathematical formulation to deal with this scenario. Uniform labeling of metabolites is most likely to be satisfied when a uniformly labeled precursor is supplied that is close to the reactions to be monitored and during the initial phase of labeling.

A significant complicating factor when interpreting time-dependent labeling patterns in plant cells, is the presence of multiple pools of the same metabolite that vary considerably in concentration. This problem is particularly pronounced in the case of organic acids, which can accumulate in the vacuole to concentrations that are an order of magnitude greater than that in the cytosol (Farre et al., 2001). This can lead to biphasic labeling patterns whereby the small pool labels rapidly and the large pool labels slowly. In the case of organic acids there is overlap in the labeling phases of the cytosolic and vacuolar pools (Gout et al., 1993) leading to superimposition in the apparent labeling rates. Moreover, the slow vacuolar labeling phase may not be useful for flux determination of subsequent steps if, as is the case for citrate, transport into the vacuole represents a metabolic dead-end (Gout et al., 1993). This emphasizes the need to consider compartmentation effects in dynamic labeling experiments. One approach is to focus on the initial phase of labeling (seconds/minutes) so that only fast labeling pools are considered. However, if this is not practical, or if a longer time period is relevant (for example to examine the correlation between flux change and transcriptomic change) then direct analysis of pool size and labeling of compartmented metabolites will be necessary (Fernie et al., 2005). In principle, this can be achieved by non-aqueous fractionation (Stitt et al., 1989), but the method has yet to be used for this purpose and some metabolic compartments (such as the mitochondrion) remain out of reach.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.04.026.

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