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TECHNIQUES FOR MOLECULAR ANALYSIS

Measuring multiple fluxes through plant metabolic networks

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Summary

Fluxes through metabolic networks are crucial for cell function, and a knowledge of these fluxes is essential for understanding and manipulating metabolic phenotypes. Labeling provides the key to flux measurement, and in network flux analysis the measurement of multiple fluxes allows a flux map to be superimposed on the metabolic network. The principles and practice of two complementary methods, dynamic and steady-state labeling, are described, emphasizing best practice and illustrating their contribution to network flux analysis with examples taken from the plant and microbial literature. The principal analytical methods for the detection of stable isotopes are also described, as well as the procedures for obtaining flux maps from labeling data. A series of boxes summarizing the key concepts of network flux analysis is provided for convenience.

Keywords: flux map, isotopomers, mass spectrometry, metabolic flux analysis, nuclear magnetic resonance spectroscopy, stable isotopes.

Introduction

Metabolic flux

Metabolic flux is the flow of matter through the metabolic network (Box 1; Figure 1). Each flux reflects the function of a specific pathway within the network, and, as all biological activity is contingent on metabolic activity, it is these fluxes that deliver the phenotype of an organism (Ratcliffe and Shachar-Hill, 2005). Measurements of flux are essential for understanding the control and regulation of metabolic networks (Fell, 1997), and an interesting consequence of the control structure of pathways is that significant changes in flux are sometimes associated with only modest adjustments in metabolite concentration (Fell, 2005). This suggests that metabolomic analysis of metabolite composition (Bino *et al.*, 2004) may be insufficient for a complete understanding of the metabolic phenotype, and that flux measurements could provide a useful complementary parameter for the system-wide characterization of metabolic networks (Cornish-Bowden and Cárdenas, 2000; Ratcliffe and Shachar-Hill, 2005).

Flux measurements

Flux measurement is a well-established tool in metabolic analysis. In plants, it is most commonly used for the characterization of the net flux along particular pathways, for example, the rate of starch synthesis or the rate of CO_2 fixation. Flux measurements are made by measuring changes in substrate and product concentrations, or by determining the flow of label from a labeled precursor. The principles of such measurements, and the pitfalls that can be encountered when applying these methods to plants, have been clearly described elsewhere (ap Rees and Hill, 1994).

Although measurement of individual fluxes is useful (Fernie *et al.*, 2005), the metabolic phenotype of a cell is the net result of multiple fluxes through the metabolic network. It follows that it would be advantageous to have methods that would allow the measurement of many fluxes simultaneously. Thus, in contrast to conventional flux analysis, with its emphasis on measuring a small number of net fluxes, the aim of network flux analysis is to measure

Box 1. Glossary of network and labeling terms

Bondomer. A set of isotopomers that contain a ^{13}C–^{13}C bond at a particular place within a molecule. Bondomers are commonly observed in experiments with uniformly labeled substrates, such as [U-$^{13}C_6$]glucose, and they are easily detected by ^{13}C nuclear magnetic resonance (NMR) because of the characteristic splitting of the signals in the spectra.

Cumomer. A set of one or more isotopomers that contain a particular labeled fragment. Cumomers are specified using a notation in which 1 indicates a labeled carbon atom, and X indicates an atom that is either labeled or unlabeled (see Figure B1).

Flux balancing analysis. Analysis of the steady-state fluxes in a metabolic network based on stoichiometry and the measured input and output fluxes.

Flux control coefficient. A measure of the influence of a specific enzyme (E) on the flux (J) through a step or pathway; defined as $(dJ/J)/(dE/E)$.

Flux ratio. Ratio of two metabolic fluxes, usually measured at a point of convergence or divergence in a network.

Fluxome. A system-wide set of fluxes for the reactions that occur in an organism; network flux analysis only measures a fraction of the complete fluxome.

Fractional enrichment. The extent to which the naturally abundant ^{12}C has been replaced by ^{13}C at a particular carbon atom in a metabolite (see Figure B1).

Global optimization. The process of finding an optimum fit between the parameters of a model and the measured data. For example, in steady-state network flux analysis the parameters are generally fluxes and the measurements are isotopic distributions.

Isotopic steady state. The point reached in a labeling experiment when the labeling of each metabolite in a network is constant.

Isotopolog(ue). Often used in flux analysis as a synonym of isotopomer, although more properly defined as one of a set of molecules with the same chemical but different isotopic composition.

Isotopomer. Strictly, one of a set of molecules with the same chemical and isotopic composition, but differing in the positional arrangement of the isotopes. However, isotopomer is also used to describe molecules with the same chemical structure that differ in isotopic composition. This usage is common in flux analysis and such molecules are more correctly called positional isotopomers. Isotopomers are specified using a notation in which 0 and 1 indicate ^{12}C and ^{13}C, respectively. A compound with n carbon atoms has 2^n positional isotopomers (see Figure B1).

Mass isotopomer. A set of positional isotopomers with the same mass. A compound with n carbon atoms has $n + 1$ mass isotopomers (see Figure B1).

Metabolic control analysis. A framework for analyzing the contribution of individual enzymes and metabolites to flux control in a steady state. The analysis can be performed theoretically, if the kinetic properties of the enzymes and transporters in the network are known, or empirically, by manipulating the levels of enzymes and metabolites, and measuring changes in flux.

Metabolic engineering. Genetic manipulation of an organism, with the aim of altering specific metabolic fluxes.

Metabolic flux (*J*). The flow of material through a step or pathway; measured in moles per unit time, for example micromoles per hour per gram fresh weight. For a reversible reaction it is often convenient to report the net forward flux (J_f–J_b) and an underlying exchange flux (J_b) rather than the individual forward (J_f) and back (J_b) fluxes.

Metabolic pathway. A route linking two or more metabolites in a network.

Metabolic steady state. A state in which all the fluxes in a network are invariant, so that the concentrations of the metabolic intermediates are constant.

Network flux analysis. Procedure for generating a map showing multiple fluxes in a metabolic network. The term is usually applied to a steady-state analysis of the redistribution of label from a ^{13}C-labeled substrate.

Sensitivity analysis. Assessment of how well the fluxes obtained by network flux analysis are determined by the labeling measurements. Thus, while it may be possible to obtain a good fit to the data for a given set of parameters, there may be a large degree of uncertainty in the parameters either because some of them have little influence on the labeling pattern or because the quality of the measurements is inadequate to constrain the fit correctly.

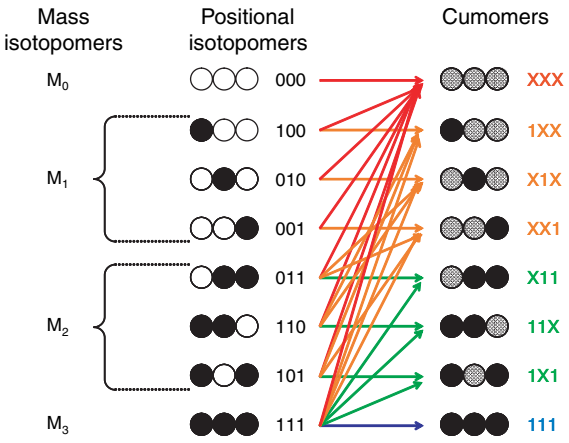

Figure B1. Mass isotopomers, positional isotopomers and cumomers for a three-carbon compound. Key: open circles, ^{12}C; closed circles, ^{13}C; hatched circles, ^{12}C or ^{13}C. Mass isotopomers and cumomers correspond to particular combinations of positional isotopomers. The fractional enrichment at position *i* is determined by the abundance of the positional isotopomers with label at that carbon atom.

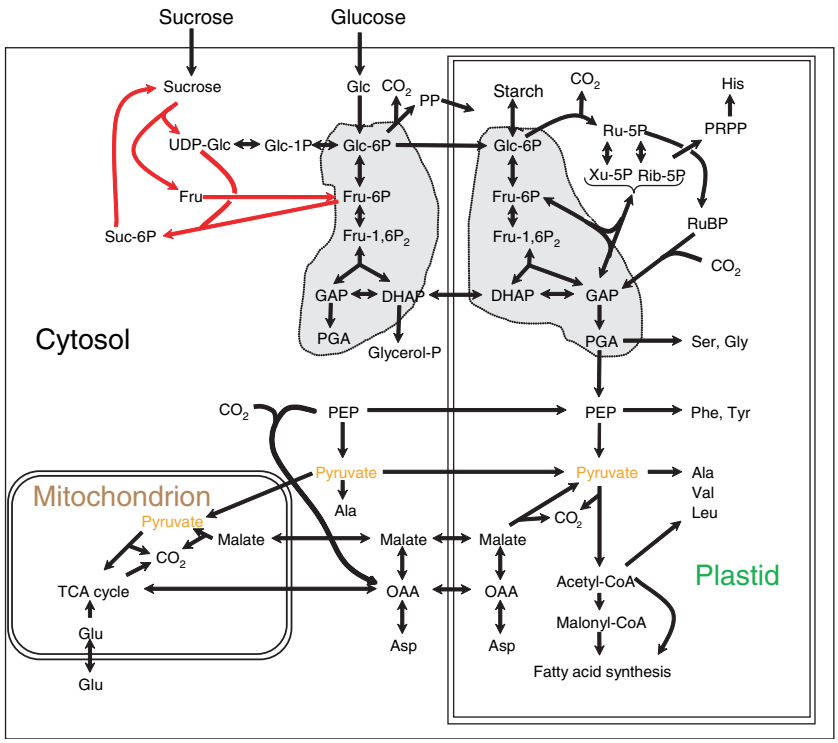

Figure 1. The network of central carbon metabolism in developing *Brassica napus* seeds, based on the literature.
Features of the network that make it a challenging target for flux analysis include: multiple subcellular compartments (plastid, mitochondrion, and cytosol, but also the central vacuole), often containing pools of the same metabolites (e.g. pyruvate, shown in orange); duplication of reactions, and even subnetwork elements in different compartments (e.g. the duplication of glycolysis/gluconeogenesis, shown in gray); reversible reactions and reaction sequences (e.g. between glucose 6-phosphate and the triose phosphates); and substrate cycles (e.g. the sucrose cycling reactions, shown in red).

as many fluxes as possible and to create a flux map that can be superimposed on the metabolic network. The large set of fluxes in a flux map is sometimes described as the fluxome (Krömer *et al.*, 2004), although in practice even the most detailed analysis is only capable of defining a fraction of the fluxes in the complete metabolic network.

Initial attempts to develop such methods for microbes focused on the technique of flux balancing, a method in which intracellular fluxes are inferred from measurements of metabolic inputs and outputs on the basis of an assumed network stoichiometry (Bonarius *et al.*, 1997; Converti and Perego, 2002). However, the complexity of the plant metabolic network, particularly the existence of storage pools, duplication of steps in more than one subcellular compartment, and opportunities for substrate cycling (Figure 1), compromises the application of flux balancing to plant cells, and necessitates the use of more powerful techniques (Roscher *et al.*, 2000).

These more powerful techniques are based on introducing a labeled precursor into the network, and then measuring the redistribution of the label into other metabolites. In one approach, dynamic labeling, metabolic fluxes are obtained by analyzing the time-course for the redistribution of the label, while in the second approach, steady-state labeling, the fluxes are determined by measuring the redistribution of the label after the system has reached an isotopic steady state. The kinetic principles behind these methods have been known for many years (Boxes 2 and 3; Borowitz *et al.*, 1977; Katz and Rognstad, 1967; Sims and Folkes, 1964), but the effectiveness with which they can be applied has been greatly increased by advances in analytical methodology and developments in computational analysis. Steady-state analysis in particular has seen substantial development and refinement following its widespread adoption as an alternative to flux balancing in microbes (Schmidt *et al.*, 1999; Wiechert, 2001; Wiechert *et al.*, 2001), and this has triggered a range of applications for network flux analysis in plants (Kruger *et al.*, 2003; Schwender *et al.*, 2004b).

Dynamic labeling has not seen such rapid progress as steady-state labeling (Wiechert and Nöh, 2005), and it still suffers from inherent limitations in complex networks (Roscher *et al.*, 2000). However, it has several positive features that allow it to complement the steady-state approach, and it is particularly effective for the analysis of secondary metabolism (Boatright *et al.*, 2004). In general, the resulting flux maps are not as large-scale as those that can now be generated by steady-state methods for central carbon metabolism, but this is offset by two important advantages. First, the application of dynamic labeling is not restricted by the need to achieve an isotopic and metabolic steady state – an important consideration given the flexibility of plant metabolic networks under normal physiological conditions (Morgan and Rhodes, 2002). Secondly, dynamic labeling experiments can also be used to construct mechanistic models of metabolism with predictive value (McNeil *et al.*, 2000b; Morgan and Rhodes, 2002). This is important because steady state flux maps are deduced from labeling experiments without reference to the kinetic properties of the enzymes in the

network, and thus provide an empirical model of the metabolic phenotype rather than a predictive one.

Benefits of network flux analysis

The overall incentive for pursuing network flux analysis is the contribution that it can make to the definition of metabolic phenotypes (Ratcliffe and Shachar-Hill, 2005). Moreover, post-genomic research, with its emphasis on transcriptomes, proteomes and metabolomes, has not yet increased understanding of metabolic networks to the point where rational plant metabolic engineering is a routine option (Kruger and Ratcliffe, 2006). In contrast, steady-state flux analysis in microbial systems has proved to be an invaluable tool for the dissection of metabolic networks (Dauner *et al.*, 2001; Petersen *et al.*, 2000), and for the analysis of their control and regulation. This has led directly to the development of rational engineering strategies and some notable successes in the manipulation of pathway flux (de Graaf *et al.*, 2001; Koffas *et al.*, 2003; Petersen *et al.*, 2001).

More specifically, network flux analysis delivers major benefits in pathway delineation and flux mapping. Information on new pathways is gained both qualitatively, by interpreting the redistribution of the label, and quantitatively, by establishing the flux distribution through alternative pathways. For example, the role of Rubisco in CO_2 recovery during triglyceride synthesis in developing embryos of *Brassica napus* and its quantitative significance were confirmed by analyzing the redistribution of label from [1-^{13}C]alanine to phenylalanine and tyrosine in a steady-state labeling experiment (Schwender *et al.*, 2004a). Similarly, quantitative analysis of fluxes through the pathways of benzenoid metabolism in petunia (*Petunia hybrida*) petals established the relative importance of two parallel, but biochemically inequivalent, pathways in the synthesis of benzenoid compounds (Boatright *et al.*, 2004).

The flux values generated by network flux analysis are also of fundamental interest in relation to the control and regulation of the network. Flux is an important parameter in metabolic control analysis (ap Rees and Hill, 1994), being a prerequisite for the calculation of flux control coefficients (Box 1). Moreover, in favorable instances, dynamic labeling experiments can be used to create mechanistic models of metabolism that allow the control architecture to be explored on the basis of *in vivo* measurements of the kinetic parameters of the participating enzymes. Such models have immediate implications for rational metabolic engineering (McNeil *et al.*, 2000b).

Accordingly, this article provides a guide to flux mapping, with the aim of presenting the procedures that are currently used to determine multiple fluxes through plant metabolic networks. After a section on general considerations regarding experimental design, there is a section on the isotope detection methods, principally mass spectrometry (MS) and NMR spectroscopy, which lie at the heart of network flux analysis. These sections are followed by a detailed discussion of the dynamic and steady-state labeling methods that are used for flux mapping, in each case providing a critical account of both data acquisition and data interpretation. Representative applications of both methods are illustrated using examples from the plant and microbial literature – space precludes any discussion of the related applications in animal metabolism (Hellerstein, 2004; Marin *et al.*, 2004; Mason and Rothman, 2004).

General considerations

Network flux analysis – limitations as an experimental tool

First, it should be recognized that the measurement of multiple fluxes requires a significant commitment of time and effort. Thus, while techniques are emerging that offer the prospect of relatively high-throughput analysis in microbial systems (Fischer and Sauer, 2003; Sauer, 2004; Zamboni and Sauer, 2004), network flux analysis in plants is not high throughput at its current level of development.

Secondly, there are certain practical limitations that may constrain the investigation. For example, most studies are performed on cell suspensions and excised tissues rather than whole plants because of labeling constraints, and any investigation is contingent on the availability of suitably labeled precursors for the pathways of interest. Subsequently, it is necessary to acquire a detailed knowledge of the redistribution of the label and difficulties in the extraction of particular intermediates may limit the interpretation of the data.

Thirdly, mapping multiple fluxes through a network generally requires access to advanced analytical and computational methods that are not commonly available in plant laboratories. Thus, significant progress usually depends on forging strong links between people with different skills.

Finally, the successful implementation of network flux analysis is dependent on substantial prior knowledge about the structure of the network and its components. As this information may well be incomplete, either because of unknown links in the network or, in the case of mechanistic descriptions, because of a shortage of *in vivo* data for the enzymes and transporters, it is usual to limit the scale of the analysis to a manageable subnetwork, typically the core reactions of central metabolism or discrete areas of secondary metabolism.

Dynamic labeling versus steady-state labeling

There are two distinct labeling strategies for measuring multiple fluxes through metabolic networks. In dynamic

labeling studies (Box 2) the aim is to measure the time-course for the flow of label through the network; whereas in steady-state labeling (Box 3) the aim is to characterize the steady state that is reached when a labeled precursor is supplied continuously. Steady-state labeling is only applicable if the system can be shown to reach an isotopic and metabolic steady state during the experiment (Roscher *et al.*, 2000; Wiechert and Nöh, 2005). These requirements are quite stringent and preliminary experiments are usually necessary to establish their validity. In contrast, dynamic labeling is more widely applicable, although preliminary experiments are again useful to ensure that both the fastest and the slowest steps of interest are captured in the time-course.

Low-molecular-weight intermediates generally reach an isotopic steady state over time-scales of minutes to hours. For example, substantial numbers of metabolites, but not all, reached isotopic steady state in about 5 h in potato (*Solanum tuberosum*) tuber discs (Roessner-Tunali *et al.*, 2004); and detailed analysis of excised maize (*Zea mays*) root tips showed that an isotopic steady state was established for a set of key metabolites over a 12-h time-scale (Dieuaide-Noubhani *et al.*, 1995). For macromolecules, particularly storage and structural pools, the question of whether the system reaches an isotopic steady state is more problematic (Kruger *et al.*, 2003; Rontein *et al.*, 2002; Roscher *et al.*, 2000). In the case of starch in heterotrophic plant tissues, the endogenous pool can be reduced before labeling with a short period of starvation (Dieuaide-Noubhani *et al.*, 1995), while in the case of protein or storage lipid it may be necessary to rely on dilution of the original unlabeled pool over an extended period of growth (Schwender *et al.*, 2003). Thus, labeling periods of days or even weeks have been used in studies of tomato (*Lycopersicon esculentum*) cell cultures (Rontein *et al.*, 2002), tobacco (*Nicotiana tabacum*) seedlings (Ettenhuber *et al.*, 2005) and cultured oilseed embryos (Schwender *et al.*, 2003; Sriram *et al.*, 2004). Ultimately, if the turnover of a component is low, then the observed labeling will reflect the metabolic history of the pool over the labeling period, rather than the isotopic and metabolic state of the system at the time of the measurement.

Assuming that the existence of an isotopic and metabolic steady state can be demonstrated, then the choice between the two labeling strategies is likely to be determined by the nature of the investigation. Dynamic labeling is best suited to small-scale networks with limited opportunities for cyclic fluxes (Roscher *et al.*, 2000). The analysis becomes progressively more complex as the label moves further away from the entry point and into branches of the network with different kinetic properties. Thus, dynamic labeling tends to be particularly suitable for flux analysis at the periphery of a metabolic network, and hence for the analysis of secondary metabolism (Boatright *et al.*, 2004; Matsuda *et al.*, 2003).

In contrast, steady-state analysis is much better suited to the analysis of complex networks, as the method depends on assigning the observed labeling of a metabolite to competing fluxes from sources with different positional enrichments (Box 3). When applicable, and with an appropriate choice of label, steady-state analysis can cope more readily with reversible steps, cyclic fluxes and the increased complexity arising from subcellular compartmentation, making it the method of choice for generating flux maps of central pathways of plant metabolism (Kruger *et al.*, 2003; Schwender *et al.*, 2004b). However, this conclusion is limited to heterotrophic metabolism because the assimilation of labeled CO_2 via photosynthesis leads to an uninformative steady state in which all metabolites are uniformly labeled (Roscher *et al.*, 2000). Hence autotrophic metabolism can only be studied by dynamic labeling methods.

A further experimental option in dynamic labeling experiments relates to the provision of the label. In steady-state experiments the label has to be supplied continuously, and a similar procedure is often adopted in dynamic labeling experiments. However, it is also possible to feed the tissue with a finite quantity of label – a pulse or bolus (Box 2). This approach, which was used in a quantitative analysis of the pathways of choline biosynthesis in tobacco using ^{33}P-labeled precursors (McNeil *et al.*, 2000a), has the advantage that the specific activities of the intermediates will first increase and then decrease, providing further constraints on the fitting procedure. Note that the analysis is simplified if the experimental protocol allows introduction of the pulse of label without significant change in the substrate concentration, as under these conditions the label will enter the metabolic network without perturbing metabolite levels.

Stable isotopes versus radioisotopes

Steady-state labeling is usually best done with ^{13}C-labeled precursors. The preference for ^{13}C reflects both the difficulty of obtaining positional labeling information with ^{14}C and the ease with which such information can be obtained for ^{13}C by MS and NMR methods. Measurements of the redistribution of the label at steady state are sufficient to generate flux ratios for different branches of the network (Box 3). These measurements are then converted into absolute fluxes, for example by using ^{14}C-labeled precursors to measure one or more input or output fluxes. Thus, if ^{13}C-labeled glucose is used for the steady-state analysis, ^{14}C-labeled glucose could be used to measure the rate of glucose uptake and the rate of production of various metabolic end products (Dieuaide-Noubhani *et al.*, 1995). The position of the labeled atoms in the precursor needs to be chosen carefully. Labeling with different isotopomers, for example [1-^{13}C]- and [2-^{13}C]glucose, will lead to significant differences in the steady-state labeling patterns of the metabolic intermediates and end products in the network. This positional (isotopomeric) information (Box 1) is of central importance for the subsequent analysis (Box 3;

Box 2. Dynamic labeling

Suppose a labeled substrate (S), with a fractional enrichment f_S, is introduced into a linear pathway:

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

The redistribution of the label is governed by a set of mass balance equations, and by the concentration dependence of the fluxes (J_{ij}).

Thus, for intermediate A, the time dependence of the concentration is given by:

$$\frac{d[A]}{dt} = J_{SA} - J_{AB}$$

and the time dependence of the labeling is given by:

$$\frac{df_A[A]}{dt} = f_S J_{SA} - f_A J_{AB}$$

The concentration dependence of each flux depends on the underlying catalytic mechanism, and might, for example, be given by the equation for irreversible Michaelis–Menten kinetics:

$$J_{SA} = \frac{V_{max}[S]}{K_m + [S]}$$

The full set of equations for S, A, B and P can be used to simulate the response of the system to any change in substrate supply. As an example, the figures show the changes in concentrations (Figure B2) and fluxes (Figure B3) during an experiment in which a substrate is supplied at constant concentration for a fixed period.

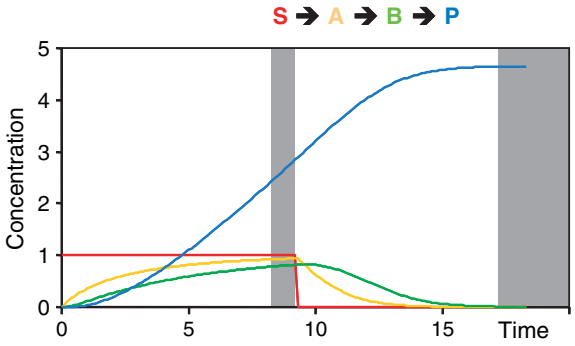

Figure B2. The effect of introducing a bolus of substrate at time zero, and then removing it some time later. The concentrations of the intermediates (A and B) increase and approach steady-state levels in the first gray zone. Removing the substrate leads to a decline in [A] and [B], but the product continues to increase until the conversion of S to P is complete.

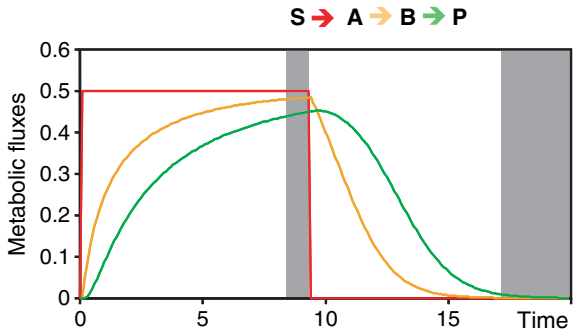

Figure B3. The time dependence of the three metabolic fluxes during the simulation in Figure B2. Fluxes were calculated using irreversible Michaelis–Menten kinetics, and the slow approach of J_{AB} and J_{BP} to J_{SA} in the steady state reflects the build-up of [A] and [B] shown in Figure B2.

If the unlabeled substrate is replaced by the same concentration of labeled substrate after intermediates A and B have reached a metabolic steady state, then the entry of label into the pathway can be simulated using the same kinetic parameters. The result of such a dynamic labeling experiment is shown in Figure B4.

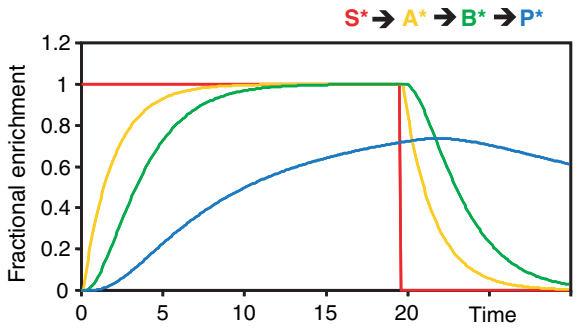

Figure B4. Fractional enrichments during a dynamic labeling experiment. f_A and f_B approach an isotopic steady state, and a fractional enrichment of 1, relatively quickly, whereas f_P approaches the isotopic steady state asymptotically as a result of the existence of an unlabeled pool of P at time zero and the continued synthesis of P from unlabeled A and B.

Replacing the labeled substrate with unlabeled substrate reverses the changes observed in the first part of Figure B4, and the observed time-course is once again a function of the mass balance equations and kinetic parameters that define the system.

While simulating time-courses for the redistribution of label in a defined network is straightforward, network flux analysis aims to reverse this process, extracting fluxes and kinetic parameters by fitting experimental data to the model of the network. This process is much more challenging, as discussed in the text.

Box 3. Steady-state labeling

The principles of steady-state analysis can be illustrated by considering the redistribution of label in the simple network shown in Figure B5.

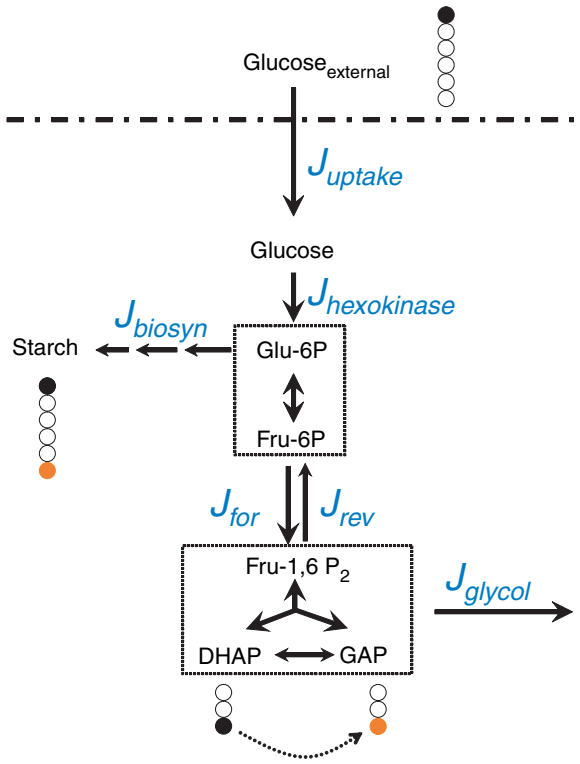

Figure B5. A metabolic subnetwork in which externally supplied [1-^{13}C]glucose is converted into hexose phosphates, and thence into starch (irreversibly) and triose phosphates (reversibly). Fluxes are shown in blue, and the redistribution of label from C1 to C6 is shown in orange. Several features of steady-state metabolic flux analysis are illustrated: (a) the pooling of metabolites whose interconversion is rapid and reversible (here the hexose phosphate pool, and the triose phosphate/Fru-1,6P_2 pool); (b) the inclusion of growth- or storage-related fluxes (here the synthesis of starch, and glucose uptake); and (c) the analysis of labeling in a product (starch) to determine the labeling of a precursor.

The fractional enrichments at C1 and C6 in the hexose phosphate pool depend on the influx and efflux of labeled hexose units. At isotopic steady state, the influx and efflux rates of label at a particular carbon atom must be equal. Thus for C1:

$$(J_{for} + J_{biosyn}) f^{C1}_{HexP} = J_{hexokinase} f^{C1}_{Glucose} + J_{rev} f^{C1}_{Fru-16P2}$$

where $f^{position}_{molecule}$ is the fractional enrichment at a particular carbon atom in the specified molecule. Similarly for C6:

$$(J_{for} + J_{biosyn}) f^{C6}_{HexP} = J_{hexokinase} f^{C6}_{Glucose} + J_{rev} f^{C6}_{Fru-16P2}$$

At isotopic steady state, the labeling of the internal glucose pool is the same as that of the external pool, so assuming 100% enrichment in carbon 1 of the external glucose, and ignoring the natural abundance of ^{13}C, $f^{C1}_{Glucose} = 1$ and $f^{C6}_{Glucose} = 0$. Using these values, and assuming rapid equilibration between DHAP and GAP, so that $f^{C1}_{Fru-16P2} = f^{C6}_{Fru-16P2} = 0.5$, allows the steady-state equations for the labeling of C1 and C6 to be rewritten as:

$$(J_{for} + J_{biosyn}) f^{C1}_{HexP} = J_{hexokinase} + 0.5 J_{rev}$$

$$(J_{for} + J_{biosyn}) f^{C6}_{HexP} = 0.5 J_{rev}$$

Dividing one equation by the other:

$$f^{C1}_{HexP} / f^{C6}_{HexP} = 1 + (2 J_{hexokinase} / J_{rev})$$

This equation shows that the distribution of label between C1 and C6 in the hexose phosphate pool is determined by the ratio of the fluxes that bring in the label. At isotopic steady state, f^{C1}_{HexP} and f^{C6}_{HexP} can be determined from the labeling of C1 and C6 in the glucosyl units of starch, providing a convenient method for the measurement of the flux ratio $J_{hexokinase}/J_{rev}$.

Note that, although the network contains six fluxes, the requirement for a metabolic steady state imposes constraints on their values to ensure that the levels of the metabolic intermediates are constant. Thus:

$$J_{uptake} = J_{hexokinase}$$

$$J_{hexokinase} + J_{rev} = J_{biosyn} + J_{for}$$

$$J_{for} = J_{rev} + J_{glycol}$$

These equations reduce the number of independent fluxes in the system to just three. The ratio of two of these fluxes, $J_{hexokinase}/J_{rev}$, can be determined from $f^{C1}_{HexP}/f^{C6}_{HexP}$, and so two more measurements are required to complete the flux map. These might be obtained from measuring any of the following: the rate of starch accumulation (J_{biosyn}); the rate of glucose uptake (J_{uptake}); or the rate of glycolysis (J_{glycol}). If all three measurements are made, then the flux map is said to be over-determined, i.e. there are more measurements than the minimum required to determine the fluxes, and this provides a useful check on the consistency of the data and the validity of the flux map.

Finally, the key to analyzing more complicated networks is to measure isotopomer abundances rather than fractional enrichments. The principle of the analysis remains unchanged – the levels of all metabolic intermediates remain constant in a metabolic steady state, and the abundance of each isotopomer remains constant in an isotopic steady state – but the scale of the calculation is greatly increased and it requires dedicated software to compute the best-fit fluxes.

Roscher *et al.*, 2000), and in fact combining the results of experiments with different precursors can be an effective way of increasing the definition of the flux map (Schwender *et al.*, 2004b).

Although stable isotopes, for example ^{2}H (Matsuda *et al.*, 2003), ^{13}C (Roessner-Tunali *et al.*, 2004) and ^{15}N (Tesch *et al.*, 1999), may also be used for dynamic labeling experiments, radioisotopes are often preferred. High sensitivity favors the use of radioisotopes, and they are particularly convenient if there is no need for positional labeling information in the analysis. As with steady-state analysis, it is often useful to conduct experiments with several precursors to maximize coverage of the network. Representative examples of this approach include the use of: multiple ^{14}C-labeled substrates to measure fluxes of carbohydrate metabolism in banana fruit (*Musa cavendishii*; Hill and ap Rees, 1994); multiple ^{35}S-labeled precursors to analyze the synthesis of dimethylsulfoniopropionate in *Spartina alterniflora* (Kocsis *et al.*, 1998); and ^{14}C- and ^{33}P-labeled compounds to analyze choline synthesis in tobacco (McNeil *et al.*, 2000a).

Sample preparation for isotopic analysis

Although NMR can be used to analyze some stable isotope labeling experiments *in vivo*, network flux analysis is largely based on the analysis of cell-free extracts. Extraction procedures are likely to follow standard protocols, but need to be optimized as appropriate. Absolute values of pool sizes are required for the analysis of dynamic labeling experiments, and so procedures with poor and variable recoveries should be avoided. In contrast, steady-state analysis depends on measuring labeling patterns, and variability in the extraction efficiency between different metabolites or replicates is less problematic. As usual with extraction procedures, it is important to ensure that the extracted material is stable and not subject to further degradation through residual enzyme activities.

The construction of a comprehensive and accurate flux map depends on obtaining as much information as possible about the redistribution of the label. Accordingly, many different extraction and sample handling procedures are used, reflecting the chemical diversity of the target analytes, and the specific requirements of the techniques that are used to detect and quantify the label. The analysis of macromolecules and metabolites can be equally informative, and a powerful steady-state method for analyzing central carbon metabolism uses the labeling of proteins to report on the labeling of metabolic intermediates (Szyperski, 1995, 1998). The protein is hydrolyzed, either chemically or enzymically, and the resulting amino acids can be analyzed after derivatization by GC–MS (Schwender *et al.*, 2003) or without further separation by NMR (Maaheimo *et al.*, 2001; Szyperski, 1995)). Nucleic acid (Pasternack *et al.*, 1996), carbohydrate, particularly starch (Dieuaide-Noubhani *et al.*, 1995), and lipid (Schwender *et al.*, 2003) fractions may also need to be separated and analyzed. Extraction of low-molecular-weight metabolites is also important, particularly in dynamic labeling experiments, which are generally performed over faster time-scales than the turnover of the macromolecular pools, but also in some steady-state experiments, where the information from a small number of readily extracted intermediates may be sufficient to answer a specific question (Edwards *et al.*, 1998; Fernie *et al.*, 2001).

Following extraction, the handling of the sample prior to analysis is determined by the choice of analytical method. Thus, extensive fractionation is essential for analyzing the redistribution of radioisotopes (Hill and ap Rees, 1994), and derivatization to increase volatility, often using different reagents for different compound classes, is required prior to GC–MS analysis of stable isotopes (Roessner *et al.*, 2000). The fractionation requirements are least demanding for NMR analysis of stable isotopes, because NMR is well suited for the analysis of complex mixtures (Fan, 1996; Krishnan *et al.*, 2005). However, even with NMR, fractionation can be advantageous to minimize the number of overlapping signals and thus maximize the available information on the labeling of such metabolites as glucose (Ettenhuber *et al.*, 2005; Rontein *et al.*, 2002).

Isotope detection

Radioisotopes are analyzed by liquid scintillation counting. Differences in counting efficiency can be corrected by spiking the different fractions with a standard, and samples can usually be re-analyzed after a period in storage without difficulty.

Stable isotopes are analyzed by MS or NMR (Figure 2). These versatile techniques can be implemented in many ways, so choices have to be made prior to measurement, and, in contrast to radioisotopes, there is a much greater emphasis on extracting the positional information that describes the distribution of the label within the detected molecule. It is possible to use stable isotopes in dynamic labeling experiments simply as reporters for the labeling of a particular pool. For example, the $[^{2}H_{5}]$phenyl moiety has been used for this purpose in studies of phenylpropanoid metabolism (Boatright *et al.*, 2004; Matsuda *et al.*, 2003). However, in steady-state experiments, and potentially also in dynamic labeling experiments, the redistribution of label within individual molecules is critical for the analysis. Thus, stable isotope analysis usually focuses on the identification of the isotopomeric composition of the analyte (Box 1; Figure 2).

MS methods

MS methods are used extensively in network flux analysis (Wittmann, 2002) and the sample may be fractionated by either gas or liquid chromatography (GC and LC,

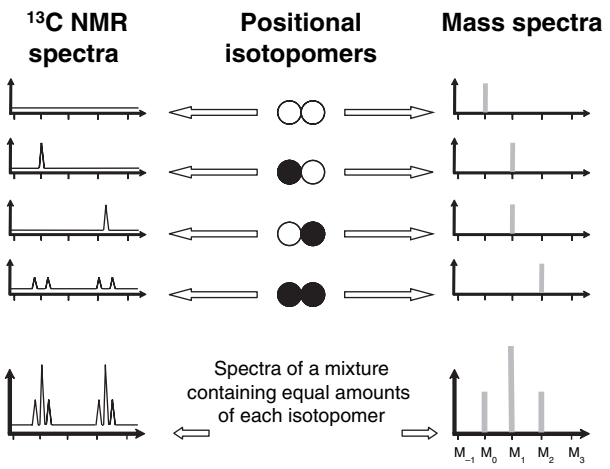

Figure 2. Diagram showing the relationship between the ^{13}C positional isotopomers of a two-carbon metabolite and the corresponding one-dimensional ^{13}C NMR and mass spectra.
The carbon atoms in the four possible positional isotopomers are depicted as either open circles (^{12}C) or closed circles (^{13}C). Only ^{13}C atoms give NMR signals, so the unlabeled isotopomers are undetectable in a ^{13}C NMR spectrum. The NMR signals from the two carbon atoms typically occur at different frequencies, and the signals are split when adjacent carbon atoms are both labeled. This allows single- and double-labeling to be quantified from the NMR spectrum. In contrast, the mass spectrum includes peaks for each of the mass isomers, containing in this case zero, one or two ^{13}C atoms. The mass spectrum does not distinguish between positional isotopomers with the same mass, unless the molecule gives rise to fragments that contain different parts of the molecule (Figure 3).

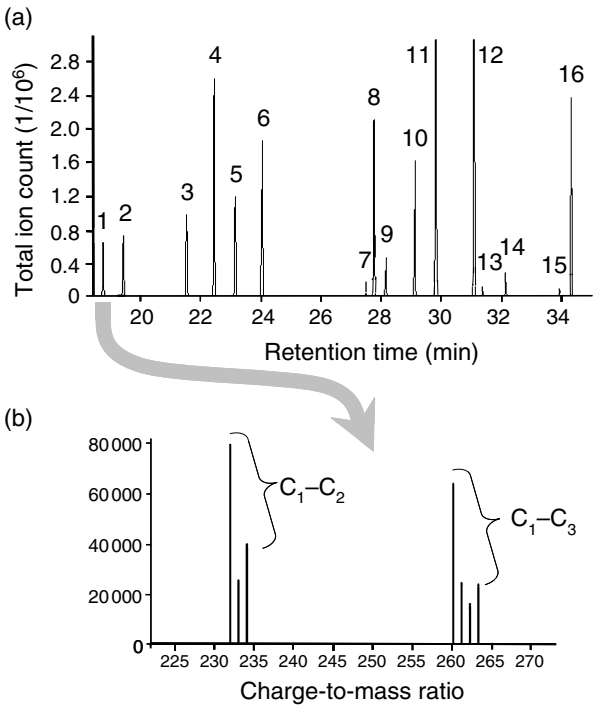

Figure 3. Mass spectroscopy of protein amino acids from developing soybean (*Glycine max*) seeds grown in the presence of [U-$^{13}C_6$]glucose.
Total protein was extracted and hydrolyzed with HCl, followed by cation exchange chromatography to isolate the amino acids. The amino acids were derivatized to make them volatile and analyzed by GC–MS. (a) Chromatograph showing the total ion current recorded by the mass detector as a function of elution time from the column. The peaks correspond to the derivatives of 16 amino acids. (b) The mass spectrum of alanine (peak 1 in the chromatograph) showing two clusters of ions, one representing ions containing all three carbons of alanine, and the other fragment ions lacking C3. Each cluster represents a set of mass isotopomers (Figure 2) with (left to right) zero, one, two or three ^{13}C atoms.

respectively). LC–MS can have advantages in terms of simplified sample handling, with no need for derivitization, increased metabolic range and reduced run time, as demonstrated in recent analyses of amino acids (Jander *et al.*, 2004) and plant volatiles (Boatright *et al.*, 2004). However, ease of access and operation, the existence of established protocols for the analysis of most compound classes, and the availability of compound libraries for many metabolites (Box 4; Schauer *et al.*, 2005) may well favor the use of GC–MS.

GC–MS involves the separation of volatile compounds by gas chromatography, then ionization by electron impact or chemical ionization, and characterization of the resulting ions on the basis of the mass (m) to charge (z) ratio (m/z; Figure 3). As the ions usually carry a single positive charge, each ion is detected with an m/z value corresponding to the molecular weight in atomic mass units. Typically the spectrum will contain lines corresponding to fragments of the detected molecule, as well as the molecule itself, and the observed m/z values will reflect both the derivatization performed prior to analysis and the isotopic composition of the molecule. A three-carbon fragment will therefore give up to four lines, with masses m, $m+1$, $m+2$ and $m+3$, corresponding to the presence of zero, one, two or three atoms of ^{13}C. These lines correspond to the mass isotopomers of the fragment (Figure 3).

A GC–MS analysis might take about an hour, with the mass detector set to cover molecular weights in the range 50–800, and the output will consist of a total ion chromatogram (TIC) that shows the retention time for each peak, and a mass spectrum corresponding to each time-point along the chromatogram (Figure 3). Assuming that the compounds of interest are present and well separated, and that the quality of the mass spectra is sufficient for quantitative analysis, the first step is to assign the spectra. This is achieved by computer-assisted comparison of each spectrum with a suitable library, such as the libraries that have been developed for the identification of biological molecules in association with the burgeoning field of metabolomics (Box 4; Schauer *et al.*, 2005).

At this point, it may be necessary to optimize one or more aspects of the overall process of extraction, purification, derivatization, gas chromatography or MS detection to ensure effective analysis of the compounds of interest. For example, if the intention is to analyze the labeled amino acids in a protein hydrolysate, then it should be noted that

some amino acids tend to be lost during acid hydrolysis (Gln and Asn), that others can be lost during ion exchange (Cys and Met), that amino acid derivatization is unsatisfactory in the presence of substantial carbohydrate, and that some amino acids are much less easy to detect by GC–MS (e.g. Arg). Some effort is therefore required at the outset of a new set of analyses, but it is usually possible to develop a robust and reproducible protocol that will generate large amounts of MS data.

After assignment, the next step is quantitative analysis (Wittmann, 2002). This might simply involve determining the fractional enrichment of a target analyte that has incorporated a stable isotope marker, or the measurement of a set of mass isotopomers generated in a steady-state labeling experiment. The first step is to correct the mass spectrum for the effects of the derivatization on the isotopomer distribution of the target analyte, the point being that the groups added during derivatization invariably exist as more than one isotopomer. It is then usually necessary to correct the isotopomer distribution of the labeled analyte and its fragments for the contribution from the natural abundance of ^{13}C (Fernandez *et al.*, 1996). This is essential for generating net enrichments, but note that some algorithms, for example 13C-FLUX^{TM}, accept uncorrected mass isotopomer data. Finally, for some applications it is necessary to deduce the relative abundance of the positional isotopomers of a ^{13}C-labeled metabolite from a consideration of the mass distributions of the intact molecule, and one or more fragments of either the molecule itself (Figure 3) or other metabolically related analytes (Christensen and Nielsen, 1999).

NMR methods

Several stable isotopes that are used for network flux analysis, principally ^{2}H, ^{13}C and ^{15}N, have magnetic properties, and are readily detected by NMR. The spectra provide quantitative information about both the chemical identity of the labeled molecule and the distribution of the label within the molecule. In fact, NMR is the most direct way of measuring the fractional enrichment of specific carbon atoms in a ^{13}C-labeled molecule, and it also provides information on the relative abundance of positional isotopomers (Figure 4).

Descriptions of the full range of NMR experiments applicable to plant metabolite detection are available elsewhere (Fan, 1996; Ratcliffe and Shachar-Hill, 2005; Ratcliffe *et al.*, 2001). Solution-state spectroscopy of extracts is usually the best option for network flux analysis, although *in vivo* spectroscopy can sometimes be useful for analyzing the redistribution of a label non-destructively (Ratcliffe and Shachar-Hill, 2001; Tesch *et al.*, 1999). NMR spectra of tissue extracts can be recorded with sufficient resolution and sensitivity on spectrometers operating with a ^{1}H frequency at or above 300 MHz, with a preference for the highest

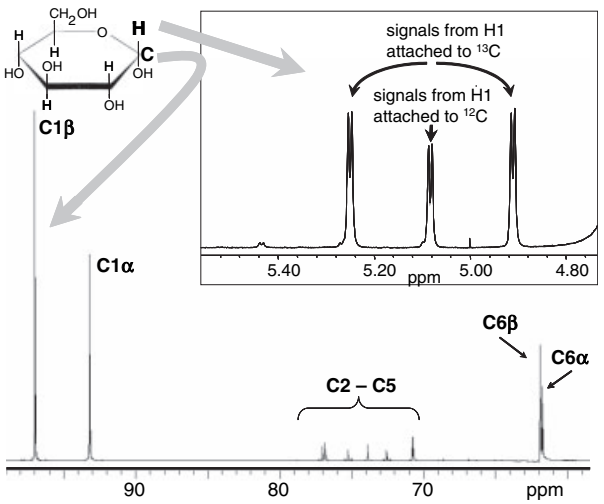

Figure 4. One-dimensional NMR spectra of hydrolysed starch from developing maize (*Zea mays*) kernels grown on hexose labeled with ^{13}C in the C1 position.
The ^{13}C NMR spectrum contains two sets of signals, corresponding to the α and β anomers of glucose, and the peak intensities reflect the extent of labeling at each carbon atom. The small region of the ^{1}H NMR in the inset shows the signals from the hydrogen attached to the C1 position in the α anomer. The inner signal is from molecules that have ^{12}C at C1, whereas the outer signals are from molecules that have ^{13}C at C1. The relative intensity of these signals allows the direct determination of $f^{C1}_{Glucose}$.

available frequency. The most commonly used NMR-detectable stable isotope for network flux analysis is ^{13}C, and the relevant NMR experiments, all of which can be implemented routinely on modern spectrometers, fall into three categories.

First, direct observation of the ^{13}C NMR spectrum (Figure 4) allows identification of labeled metabolites and the position of labeling. In general, each chemically inequivalent ^{13}C carbon atom gives a separate signal (in practice some signals overlap and in an extreme case two chemically inequivalent atoms might give indistinguishable signals) and this signal has a fine structure that reports on the ^{13}C labeling of the neighboring carbon atoms in the molecule. Consequently, each molecule in the sample has a characteristic fingerprint, allowing both simultaneous identification of different metabolites in a mixture without prior separation, and the determination of the label distribution within each metabolite. Thus, in contrast to MS, it is not necessary to analyze molecular fragments to obtain information on positional labeling, and NMR provides the most direct method of measuring the relative abundance of the isotopomers that are usually used for steady-state analysis.

Secondly, direct observation of the ^{1}H NMR spectrum (Figure 4) provides a method for determining the fractional enrichment of particular carbon atoms. The ^{1}H NMR signal from a hydrogen atom attached to ^{13}C is split into a pair of signals, and comparison of the intensities of the split and unsplit peaks gives a direct measure of the enrichment of the carbon atom. In practice, the ^{1}H NMR spectra of

Box 4. Online resources

Enzymes and pathways

http://www.brenda.uni-koeln.de/
Extensive database of enzymes and their properties.

http://www.genome.jp/kegg/pathway.html
Comprehensive metabolic pathway database with links to genes, proteins and metabolites.

http://metacyc.org/
Comprehensive metabolic pathway database with links to genes, proteins and metabolites.

http://www.metabolome.jp/
A pathway database that aims to facilitate the interpretation of labeling experiments by providing information on the fate of each atom in a metabolite. It includes a useful set of links to related resources.

Metabolic modeling – general sites

http://www.biochem.med.umich.edu/brupalf/software.htm
Listing of free and commercial software sites for analyzing metabolic kinetics.

http://sbml.org/index.psp
Systems Biology Markup Language is increasingly used for kinetic simulation of biochemical pathways and networks**. The site also has many links for kinetic simulation.

Metabolic modeling – selected sites

http://www.hort.purdue.edu/cfpesp/models/models.htm
Extensive kinetic simulation website includes detailed tutorials, online interactive models† and downloadable Visual Basic‡ models*.

http://www.gepasi.org (Gepasi)
Widely used software package† for kinetic metabolic analysis*. It simulates time-courses, performs metabolic control analysis, and fits models to kinetic data. There are good tutorials and documentation. Gepasi is useful for teaching.

http://slow.kgi.edu/ (JDesigner/Jarnac)
Another widely used software package† for metabolic modeling*. It simulates time-courses and performs metabolic control analysis.

http://www.bii.a-star.edu.sg/research/sbg/cellware/index.asp (Cellware)
Metabolic modeling software package†*. It simulates time-courses, performs metabolic control analysis and fits models to kinetic data**. Documentation is good.

http://labs.systemsbiology.net/bolouri/software/Dizzy/ (Dizzy)
Modeling software for simulating metabolic kinetics stochastically*. It can import models from other model databases or build your own. The software runs in Java‡ and is fairly well documented.

Model libraries

http://jjj.biochem.sun.ac.za/
Library of kinetic metabolic models from published studies. Simulations can be performed online† or models can be downloaded.

http://www.biomodels.net/
New database of kinetic models for downloading. It is under development, but likely to become extensive*.

Stoichiometric and steady-state flux analysis

http://www.mpi-magdeburg.mpg.de/de/research/projects/1010/1014/1020/mfaeng/intro.html (Flux Analyzer)
Tools for analyzing metabolic networks based on reaction stoichiometries including elementary modes and flux balance analysis*. The tools run under the Matlab‡ software suite.

http://www.simtec.mb.uni-siegen.de/434.0.html (13C-FLUX)
Powerful software package for simulating and analyzing steady-state ^{13}C labeling experiments** including statistical analyses of output. The software runs in the Linux environment‡. 13C-FLUX is documented in papers and a tutorial.

http://www.svizsystem.com/metabiologica.htm/
Tool for simulating and analyzing steady-state labeling experiments*.

http://gcrg.ucsd.edu/
Explanation and tools for flux balancing analysis and other stoichiometric analyses of metabolic networks. Demonstration software* is available for download.

Spectroscopy

http://www.spectroscopynow.com/
Extensive website on MS, NMR and other spectroscopies, providing general information and links to websites for education, databases, hardware, software, supplies etc.

http://www.aist.go.jp/RIODB/SDBS/cgi-bin/cre_index.cgi
Searchable database of NMR, MS and other spectra for over 30 000 compounds, including most core metabolites.

http://csbdb.mpimp-golm.mpg.de/index.html
Includes the Golm metabolome database with access to MS libraries.

http://www.mpimp-golm.mpg.de/mms-library/index-e.html
GC–MS libraries of plant metabolites. It is downloadable for use on mass spectrometers or offline‡.

*Requires modest computer expertise (downloading, installing, etc.). **Requires more computer expertise (programming, interfacing programs etc.). †Self-contained software. ‡Requires other software.

Metabolic networks reading list

Blanch, H.W. and Clark, D.S. (1996) *Biochemical Engineering*. New York: Marcel Dekker.
Fell, D. (1997) *Understanding the Control of Metabolism*. London: Portland Press.
Heinrich, R. and Schuster, S. (1996) *The Regulation of Cellular Systems*. New York: Chapman & Hall.
Stephanopoulos, G.N., Aristidou, A.A. and Nielsen, J. (1998) *Metabolic Engineering: Principles and Methodologies*. San Diego: Academic Press.
Voit, E.O. (2000) *Computational Analysis of Biochemical Systems*. Cambridge: Cambridge University Press.

unfractionated extracts are so crowded with overlapping peaks that it is only possible to apply this method with confidence to signals that lie on the edge of the main spectral region. However, some important metabolites, such as glucose and alanine, give ^{1}H NMR signals that can be analyzed easily in ^{1}H NMR spectra, and fractionation of the sample allows further signals to be measured reliably (Rontein *et al.*, 2002). Note that, once the fractional enrichment of one carbon atom has been determined, the enrichments of the other carbon atoms in the molecule can be determined from the ^{13}C NMR intensities.

Thirdly, increasing use is now made of NMR experiments in which the interactions between the ^{1}H and ^{13}C isotopes are exploited to generate correlated two-dimensional spectra with the ^{1}H and ^{13}C NMR signals dispersed along two axes (Figure 5). Two-dimensional NMR methods increase resolution (decreasing overlap in the ^{1}H spectrum), and increase the detection sensitivity for ^{13}C. They also establish connections between the ^{1}H and ^{13}C signals, which is useful for metabolite identification. Although more difficult to optimize and calibrate, two-dimensional data spectroscopy has many advantages for stable isotope analysis, particularly for interpreting spectra from unfractionated mixtures of metabolites (Fan, 1996). Several related two-dimensional NMR experiments are commonly used for the analysis of ^{13}C-labeled metabolites, including heteronuclear [^{13}C, ^{1}H] COSY (Schmidt *et al.*, 1999; Szyperski, 1995), INADEQUATE (Glawischnig *et al.*, 2002), HMQC (Yang *et al.*, 2002) and HSQC (Sriram *et al.*, 2004). These, and other techniques, have all been used for network flux analysis, and while their implementation almost invariably requires assistance from an NMR specialist, the wealth of labeling information that can be obtained is invaluable.

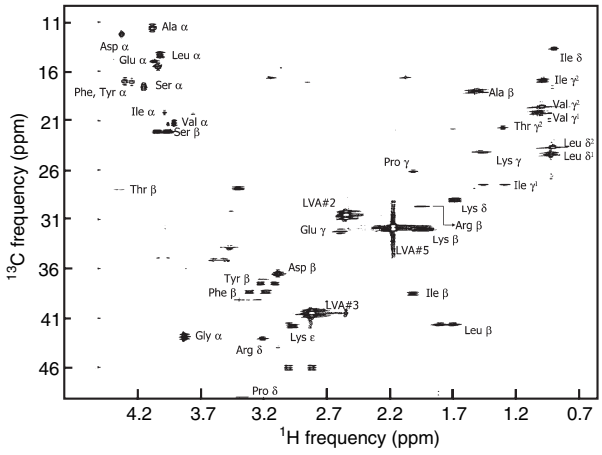

Figure 5. A two-dimensional ^{1}H-^{13}C HSQC NMR spectrum of hydrolyzed protein from developing soybean (*Glycine max*) seeds grown on 10% [U-$^{13}C_6$]sucrose.
The signals arise from H atoms bonded to ^{13}C atoms, and the intensities are proportional to the fractional enrichment at the different carbon positions of the amino acids and levulinic acid (LVA, produced from glycosyl moieties attached to the proteins). The figure is adapted from Sriram *et al.* (2004) with the permission of the American Society of Plant Biologists.

Dynamic labeling methodology

Data acquisition

Labeling time-courses are constructed from multiple measurements of pool size and specific activity for intermediates and products in the metabolic subnetwork of interest. It is essential to ensure that these measurements are distributed over the full time-course for the redistribution of the label, and that sufficient measurements are made to define the time-courses with a precision that will allow meaningful fitting of the data to a kinetic model (Box 2). While it may sometimes be useful to acquire data directly, for example *in vivo* ^{15}N NMR was used to follow the incorporation of [^{15}N]ammonium into glutamine and glutamate in a flux analysis of the assimilatory pathways of nitrogen metabolism in the bacterium *Corynebacterium glutamicum* (Tesch *et al.*, 1999), it is more usual to construct the time-course by analyzing the extracts of tissue samples taken during the redistribution of the label.

As regards the choice of a labeled precursor, it is usual to choose a metabolite that is as close as possible to the subnetwork of interest, both to minimize dilution effects and kinetic damping and to reduce the complexity of the kinetic model that is required to explain the data. This strategy does not preclude attempts to probe multiple fluxes by monitoring a label that is widely redistributed; for example, [$^2H_5$]phenylalanine has been used to characterize no fewer than 38 fluxes in the production of benzenoid compounds in petunia petals (Boatright *et al.*, 2004) and [$^{13}C_6$]/[$^{14}C_6$]glucose has been used to probe the fluxes of central carbon metabolism in tobacco callus (Fernie *et al.*, 2001) and potato tubers (Roessner-Tunali *et al.*, 2004). However, it is more common to implement more targeted labeling schemes, such as the use of ^{35}S-labeled substrates to characterize the synthesis of dimethylsulfoniopropionate (Kocsis *et al.*, 1998), ^{14}C- and ^{33}P-labeled substrates to establish the pathway of choline synthesis (McNeil *et al.*, 2000a), and [$^2H_5$]phenylalanine to analyze fluxes through a phenypropanoid pathway in potato tuber tissue (Matsuda *et al.*, 2003, 2005). Another factor that needs to be considered is the possible loss of the label from the subnetwork to be analyzed through exchange processes – H and N isotopes can be vulnerable to this hazard, for example through aminotransferase activity – and this problem should be avoided or minimized by careful choice of the input label.

Irrespective of the precursor that is used for the experiment, analysis of the redistribution of the label will almost invariably involve extraction and fractionation of multiple tissue samples prior to the determination of pool sizes and specific activities. No attempt is made to describe these methods here, but care should be taken to choose the most appropriate protocols and to perform the necessary checks on the recovery efficiency.

Data interpretation

Conversion of the raw data from a dynamic labeling experiment into a set of fluxes describing the redistribution of the label is a three-step process.

First it is necessary to set up the mathematical model that describes the operation of the metabolic network (Box 2). The model is a set of coupled differential equations that embody prior knowledge and/or hypotheses about the subnetwork that was explored in the experiment. As a minimum, this prior knowledge must include the architecture of the network, essentially the known or predicted links between metabolites, and information about the compartmentation of the system. In some cases it may be appropriate to extend the basic model by specifying the mechanistic link between pool sizes and fluxes. For example, in a model of glycine betaine synthesis in tobacco, flux rates were assumed to respond to substrate pool size according to the Michaelis–Menten equation, and the majority of the associated K_m and V_{max} values were treated as variable parameters within the model (McNeil *et al.*, 2000b). A generalized mathematical modeling strategy for dynamic labeling data has recently been published (Voit *et al.*, 2004) and the more pragmatic approaches described in recent plant studies (Matsuda *et al.*, 2003; McNeil *et al.*, 2000a,b) can be viewed as special cases of this more general formulation.

Secondly, it will often be necessary to make simplifying assumptions to render the equation system tractable. For example, if the model is over-elaborate, with a large number of variable parameters, then the data from the time-course may provide insufficient constraints to generate a useful fit; in other words there may be too many apparent solutions. Thus, initial models should be conservative, with the recognition that they can always be extended as the quality and range of the measurements increase. Simplifying assumptions may include considering a set of metabolites to constitute a single pool, defining particular steps as irreversible, and assuming constant pool sizes or fluxes (Figure 6). Clearly, it is undesirable to make such assumptions if there is experimental evidence to the contrary. For example, in a flux analysis in potato tubers, the observed variation in the specific labeling of the precursor pools was reduced to an average value to facilitate the calculation of the fluxes, and the positional labeling information that must have been present in the mass spectra was ignored in favor of using a mean fractional enrichment (Roessner-Tunali *et al.*, 2004). The authors of this study adopted this approach for pragmatic reasons, but these simplifications inevitably undermine the reliability of the resulting fluxes, as the authors acknowledge (Roessner-Tunali *et al.*, 2004). Similarly, if a mechanistic model is proposed, then it makes sense: (i) to use the correct relationship between the substrate concentration and flux rate when known, and (ii) to avoid treating kinetic parameters as variables if there is good evidence for particular values *in vivo* (McNeil *et al.*, 2000b).

Finally, it is necessary to fit the mathematical representation of the metabolic network to the data. This is accomplished by a process of iteration, in which the parameters in the model are varied until the best fit is achieved between the time-course data and the predictions of the model. For the simplest networks, such as that explored by [$^2H_5$]phenylalanine labeling in potato tuber discs (Matsuda *et al.*, 2003; Figure 6), it may be possible to derive an analytical solution for the kinetic equations, and then use non-linear regression to optimize the agreement between the predicted and experimental time-courses. In a system of intermediate complexity, rate constants for hexose metabolism were extracted from a time-course analysis of [U-^{14}C]glucose labeling after solving a set of five differential equations, and then fitting the data by iteration of the rate constants (Fernie *et al.*, 2001). For more complicated networks, the scale of the network makes the task much more difficult, and the

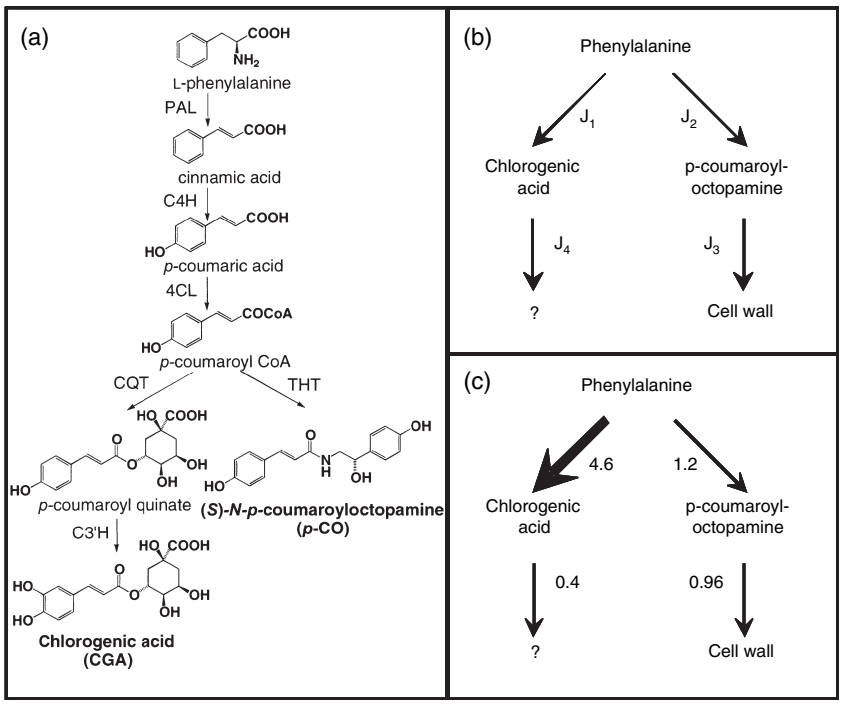

Figure 6. Three representations of the phenylpropanoid pathway in potato (*Solanum tuberosum*) tuber discs.
(a) Metabolic conversions leading from phenylalanine to chlorogenic acid and *p*-coumaroyloctopamine showing structures and enzymes. (b) A model of the subnetwork in which four fluxes (J_1–J_4) determine the metabolic flow. The intermediates between L-phenylalanine and *p*-coumaroyl CoA are present at very low levels and they can be ignored in the kinetic analysis of the redistribution of label. (c) Flux map derived from a dynamic labeling experiment using [$^2H_5$]phenylalanine. The figure is adapted from Matsuda *et al.* (2003) with the permission of Oxford University Press.

automation of effective methods for estimating the parameters that govern the observed time-courses is a challenging problem (Mendes, 1993; Moles *et al.*, 2003). Software packages for simulating and fitting time-courses are widely available (Box 4), but these essential tools differ considerably in their usability (Pettinen *et al.*, 2005). In general, the aim is to simulate the time-course while systematically varying the parameters in the model, and in one well-documented approach time-courses are calculated directly from the differential equations in an iterative fashion (Boatright *et al.*, 2004; Kocsis *et al.*, 1998; McNeil *et al.*, 2000a,b). The quality of the fit can be assessed numerically for each set of parameters and, although this approach is time-consuming, it is likely to provide a more reliable set of fluxes than can be obtained by imposing average values on the specific activities of the intermediates.

The output of the fitting process will be a set of optimized values for the variable parameters in the model, and in most cases the immediately interesting parameters will be the calculated fluxes, as these will establish the quantitative significance of the various routes through the network, effectively delineating the pathways for particular metabolic transformations under the conditions of the labeling experiment.

Representative applications

The main value of dynamic labeling experiments lies in their contribution to understanding the operational structure of metabolic networks, whether through the delineation of pathways or through an analysis of their control structure.

In fact, pathway delineation at the level of identifying new pathways in an incompletely defined network does not necessarily require a full kinetic analysis. The redistribution of stable isotope labels from specific positions in a precursor to a product is frequently diagnostic of the pathway between them, and NMR in particular is used routinely to obtain evidence of this kind. However, while flux analysis is not necessarily required to uncover the existence of a particular pathway, it becomes essential if there are multiple pathways between a substrate and product. Thus, flux analysis, by quantifying the contribution of alternative routes through the metabolic network, can establish the existence or absence of a preferred pathway (Bacher *et al.*, 1999; Glawischnig *et al.*, 2000).

This general principle is well illustrated by the recent study of benzenoid metabolism in petunia petal tissue (Boatright *et al.*, 2004). Dynamic labeling allowed the measurement of 38 fluxes in a complex network (Figure 7) and revealed the existence of two alternative pathways for the formation of benzenoid compounds. The presence of two pathways – one CoA-dependent with benzylbenzoate as an intermediate and the other CoA-independent with benzaldehyde as an intermediate – was deduced from the time-dependent labeling of multiple metabolite pools using [$^2H_5$]phenylalanine as the precursor. This in turn led to the biochemical characterization of an enzyme for the conversion of benzyl alcohol to benzylbenzoate. Thus, substantial new knowledge about the metabolic network was obtained by constructing a model that could account for the time dependence of the redistribution of the label.

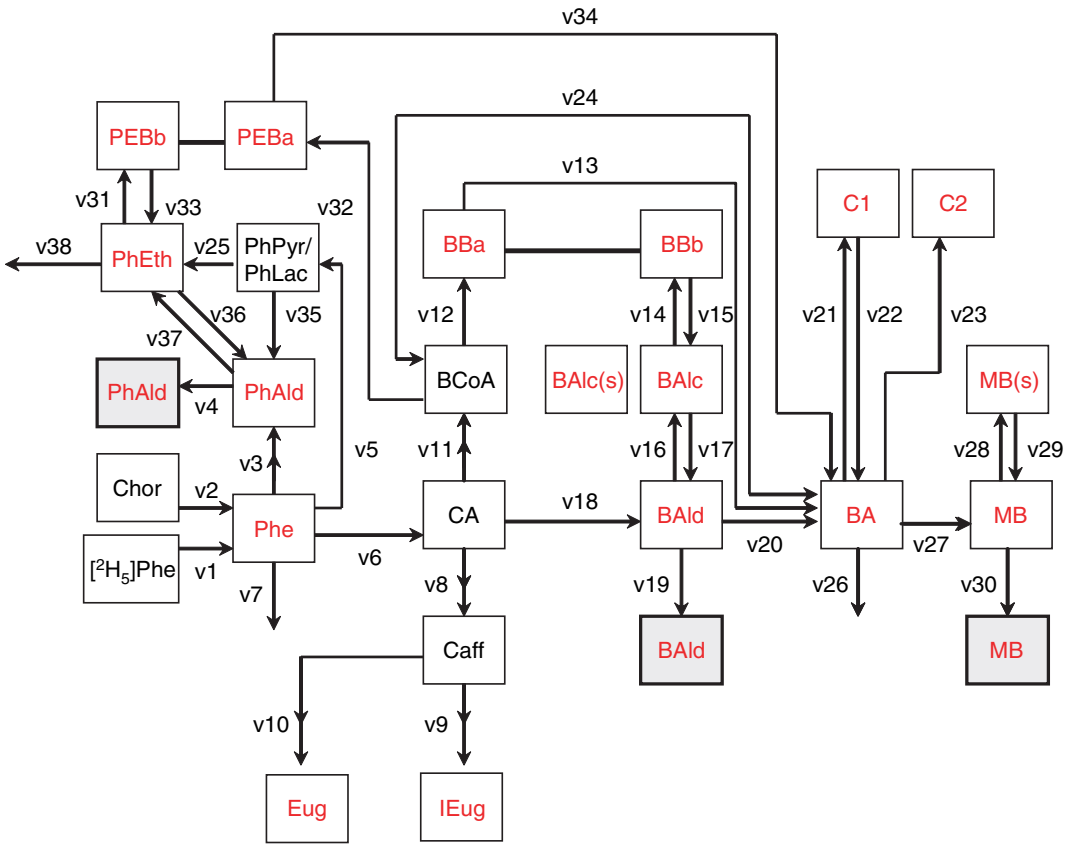

Figure 7. Flux map used to analyze benzenoid and phenypropanoid metabolism in petunia (*Petunia hybrida*) petal tissue.
Label was introduced as [$^2H_5$]phenylalanine, and the pool sizes and labeling of the metabolites shown in red were measured over a 4-h time-course. Numerical fitting of the data to the model defined 38 fluxes (v1–v38) and allowed the major routes through the network to be determined. Gray shading indicates a metabolite that was volatized, and the pools labeled BAlc(s) and MB(s) are storage pools. The figure is adapted from Boatright *et al.* (2004) with the permission of the American Society of Plant Biologists.

The complexity of the network in Figure 7 is daunting, and for a smaller scale investigation it is useful to turn to an analysis of the phenylpropanoid pathway in potato tuber discs (Matsuda *et al.*, 2003). Here it was possible to model the redistribution of label from the same precursor using just four fluxes, corresponding to the synthesis and degradation of the two principal hydroxycinnamic acid conjugates formed in cut tuber tissue. The limited redistribution of the label in this system favored a relatively straightforward analysis (Figure 6), and the study provides an exemplary description of the implementation of a dynamic labeling strategy for flux analysis.

Flux measurement is an prerequisite for metabolic control analysis (ap Rees and Hill, 1994) and in principle it should be possible to determine multiple flux control coefficients by using dynamic labeling and mathematical modeling to extract multiple fluxes from suitable lines of transgenic material. A comparative study of wild-type and GDH-negative strains of *C. glutamicum* shows how multiple fluxes change in response to a change in enzyme activity (Tesch *et al.*, 1999), but the approach does not seem to have been exploited to characterize transgenic lines that would be suitable for metabolic control analysis.

However, an alternative approach to the control structure of a pathway, again based on dynamic labeling experiments, has been demonstrated in tobacco. Here, modeling of time-courses was first used to establish the predominant pathway of choline synthesis (McNeil *et al.*, 2000a). Subsequently, further labeling experiments provided sufficient data to construct a mechanistic model, permitting estimation of the kinetic properties of the enzymes and transporters involved in choline metabolism *in vivo* (Figure 8; McNeil *et al.*, 2000b). This second model then provided direct insight into the factors limiting glycine betaine synthesis in tobacco plants engineered to express choline monooxygenase in the chloroplast. In particular, the mechanistic model showed that choline transport into the chloroplast is a major constraint on the effectiveness of the metabolic engineering strategy, and that correcting this limitation would be necessary for physiologically significant glycine betaine synthesis in transgenic plants.

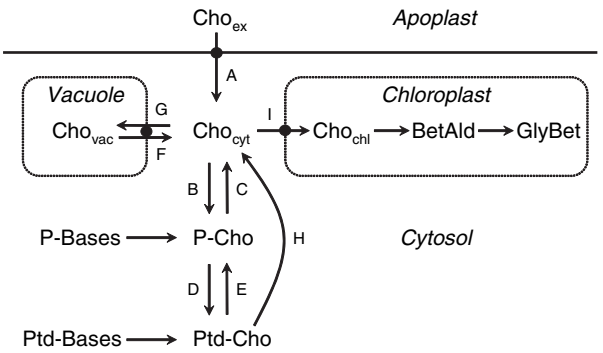

Figure 8. Model used to analyze the synthesis of glycine betaine from choline in transgenic tobacco (*Nicotiana tabacum*).
Leaf discs were incubated with [^{14}C]choline, and labeling time-courses were measured for total choline (Cho), phosphocholine (P-Cho), phosphatidylcholine (Ptd-Cho), and glycine betaine (GlyBet). V_{max} and K_m values for fluxes B, C, D, E, G and H, and the first-order rate constants for fluxes A, F and I, were obtained by optimizing the fit between the simulated kinetics and the labeling time-courses. BetAld, betaine aldehyde; P-Bases, phosphobases; Ptd-Bases, phosphatidylbases. The figure is adapted from McNeil *et al.* (2000b) with the permission of the American Society of Plant Biologists.

Steady-state labeling methodology

Data acquisition

In steady-state experiments the labeled precursor is supplied continuously, and the redistribution of the label is measured after the system reaches an isotopic and metabolic steady state. Experimental verification that these conditions are satisfied is crucial, and evidence should be presented whenever steady-state methods are used. Only when a suitable end-point has been identified through time-course observations is it legitimate to proceed to a steady-state analysis.

The steady-state approach is particularly suitable for the analysis of complex networks, such as the central pathways of carbon metabolism. Choosing a precursor for introducing the label into the network is usually straightforward; for example, there are many studies on plant and microbial systems that use glucose, but the labeling of the precursor needs to be chosen carefully. In one approach, the system is incubated with a mixture of uniformly labeled and unlabeled substrate (Ettenhuber *et al.*, 2005; Glawischnig *et al.*, 2001, 2002; Maaheimo *et al.*, 2001; Sriram *et al.*, 2004; Szyperski, 1995), while in an alternative approach the system is incubated with a specific isotopomer in which some, but not all, positions are labeled. The complexity of the network may make the optimal choice of isotopomer non-trivial (Roscher *et al.*, 2000), and modeling the outcome in advance is the best way to ensure that precursors are chosen that will lead to an informative steady state (Schwender *et al.*, 2004b; Wiechert *et al.*, 2001). Ultimately, because the reliability of the calculated flux map is determined by the constraints on label redistribution within the network, conducting parallel experiments with different labeled precursors is the best way to ensure optimal determination of the fluxes.

Similarly, while the analysis of the steady-state labeling of just a handful of easily extractable metabolites may be sufficient to generate a flux map, complementary measurements of as many labeled components as possible, including macromolecules, will provide further constraints and increase confidence in the measured fluxes. Thus, while it may be sufficient to construct flux maps of central carbon metabolism in microbial systems by analyzing the redistribution of uniformly labeled glucose into protein (Szyperski, 1995, 1998), it is not yet clear that such an approach on its own will be sufficient to obtain reliable fluxes through the more complicated network of central carbon metabolism in plants. The approach is certainly technically feasible (Glawischnig *et al.*, 2001; Sriram *et al.*, 2004) but there is a strong case for using complementary labeling strategies to test the significance of the results obtained so far.

Information on the subcellular compartmentation of the metabolic network is obtained by analyzing the labeling of metabolites and end-products that are synthesized uniquely in one compartment (Ratcliffe and Shachar-Hill, 2005; Schwender *et al.*, 2004b). For example, the labeling of starch reports on the plastidic hexose phosphate pool, and the labeling of the carboxyl end of long-chain (C_{20}, C_{22}) fatty acids reports on the cytosolic acetyl CoA pool. This approach is powerful, because it yields subcellular information with only minimal fractionation of the extract. However, given incomplete knowledge of the network, caution is required and assumptions about the subcellular location of particular steps may need to be revisited in the light of new information. Thus, new data on cytosolic ADP-glucose synthesis may force a re-evaluation of the compartmentation of the metabolic route to starch in photosynthesizing leaves (Baroja-Fernández *et al.*, 2004, 2005; Neuhaus *et al.*, 2005), emphasizing the point that fundamentally important features of the primary metabolic network are still being discovered. Recently it has been proposed that subcellular labeling information could be obtained by non-aqueous fractionation of labeled plant material before extraction (Fernie *et al.*, 2005), and this would provide a useful method for testing assumptions about the subcellular compartmentation of the network. Finally, note that rapid exchange of intermediates between compartments may mean that apparently separate pools act as a functional unit (see below; Schwender *et al.*, 2003).

Ideally the label distribution in the steady state should be characterized by both MS and NMR. Each method can provide sufficient isotopomeric information for network flux analysis on its own (Christensen and Nielsen, 1999; Schmidt *et al.*, 1999), but neither approach has an overwhelming advantage, making it is advantageous to use data from both methods (Wiechert, 2001; Yang *et al.*, 2002). Even when MS

and NMR give entirely equivalent information, having independent measurements increases confidence in the eventual flux map, but in fact the information from the two techniques is often complementary (Box 4), creating a powerful argument for using the two techniques in tandem. For example, a detailed analysis of the validity of using MS for network flux analysis in *C. glutamicum* (Klapa *et al.*, 2003) concluded that MS was better than NMR for analyzing the anaplerotic flux distribution, but that NMR was better than MS for elucidating the relative contribution of the parallel pathways of lysine synthesis. To date, most steady-state network flux analyses in plants have used NMR, with only one major application of MS (Schwender and Ohlrogge, 2002; Schwender *et al.*, 2003, 2004b), but there is a strong case for following the microbial lead and using both techniques wherever possible.

MS and NMR characterize the labeling of specific molecules in terms of (i) the relative abundance of mass isotopomers, and (ii) the relative abundance of isotopomers and cumomers, including fractional enrichments at specific atomic positions (Box 1; Figure 2). In the analysis of experiments with uniformly labeled substrates, there is also the computationally efficient option of focusing on carbon–carbon bonds that remain intact during metabolism, and using NMR to characterize the redistribution of the label in terms of bondomers and cumulative bondomers (Box 1; Sriram and Shanks, 2004; van Winden *et al.*, 2002). The labeling information is sufficient to generate flux ratios through different parts of the network, but to obtain a map of absolute fluxes it is also necessary to measure one or more input or output fluxes to calibrate the ratios. Thus, it is usual to measure substrate uptake and flux into end-products and/or biomass in parallel with the steady-state stable isotope analysis.

Data interpretation

As with dynamic labeling, data interpretation hinges on setting up a mathematical representation of the network in steady state (Box 3), and then adjusting the model parameters to fit the data. The model parameters are the forward and reverse fluxes that link the branch points in the network, usually represented as the equivalent exchange fluxes and net fluxes, and the final output is a flux map for the whole network. The analysis depends on applying flux balancing to the labeling of individual carbon atoms, and, while simple in principle, this process is much more time-consuming than data acquisition, emphasizing the importance of obtaining a high-quality dataset at the outset.

Early applications of steady-state flux analysis to plant metabolism focused on obtaining an exact solution for a set of steady-state equations using the requisite number of positional fractional enrichments as inputs (Dieuaide-Noubhani *et al.*, 1995; Edwards *et al.*, 1998; Fernie *et al.*, 2001; Rontein *et al.*, 2002; Roscher *et al.*, 2000). Although mathematically straightforward, this procedure has the disadvantage that it provides no indication as to the validity of the resulting flux map, because a solution can be found for any set of observed fractional enrichments. With this approach, therefore, the best way to test the map is to measure the positional fractional enrichments obtained with two different precursors, for example [1-^{13}C]glucose and [2-^{13}C]glucose. More recently, as documented elsewhere (Kruger *et al.*, 2003), and drawing on developments in microbial network flux analysis (Schmidt *et al.*, 1999; Wiechert, 2001; Wiechert *et al.*, 2001), the emphasis has shifted to a full isotopomeric (cumomer) analysis in which a numerical fitting procedure is used to find the flux distribution that provides the best fit to the experimental data. In this scheme, the system is over-determined, allowing a statistical assessment of the quality of the fit and the confidence range of each fitted parameter.

Irrespective of the basis on which the analysis is carried out, most investigators have tended to set up their own routines for solving steady-state equations or for fitting data. This complicates the comparison of flux maps obtained on different systems by different groups, particularly if the more powerful approach of numerical fitting is used, and it also acts as a serious deterrent for new entrants into the field. Some of the software that has been developed is freely available (Box 4), for example 13C-FLUX^{TM} (Wiechert *et al.*, 2001), NMR2Flux (Sriram *et al.*, 2004) and 4F (Ettenhuber *et al.*, 2005), and this may ultimately lead to a standardized platform for steady-state network flux analysis. 13C-FLUX^{TM} is a flexible program that has been used extensively on microbial systems, and it has recently been adopted by several plant groups, including our own. The mathematics on which it is based, and the program itself, are documented (Wiechert *et al.*, 2001), and as well as accepting both NMR and MS data, 13C-FLUX^{TM} also includes advanced fitting, simulation and statistical routines. The recently published NMR2Flux program operates on similar principles to 13C-FLUX^{TM}, while 4F is a more specialized, rules-based program that calculates the underlying fluxes responsible for the isotopic equilibration of a uniformly labeled precursor, currently [$^{13}C_6$]glucose, when supplied in the presence of an excess of its unlabeled form (Ettenhuber *et al.*, 2005).

One final point concerns the complexity of the model. It is always best to start with a simple representation of the network when performing a full isotopomeric analysis with numerical fitting, and then to develop this as the extent and quality of the labeling data increase. Increasing the scope of the data collection, for example by using more than one labeled precursor and/or by extending the subsequent analysis of the redistribution of the label, allows more complicated models to be compared with the initial one. If it then turns out that the more complicated model is no more satisfactory than the simple one, even though it is a more

realistic representation of the molecular components that are thought to be present, then the possibility has to be considered that the network is functionally less complicated in flux terms than it actually appears.

Thus, to take a specific example, the decision as to whether to include one or two pentose phosphate pathways in models of heterotrophic carbon metabolism is pulled in one direction by the molecular evidence that at least some of the enzymes are present in both cytosol and plastid (Kruger and von Schaewen, 2003), and in the other direction by the difficulty in evaluating the relative merits of models with either one or two complete or incomplete pathways. For example, there is molecular evidence in oilseed rape embryos for the presence of the oxidative steps of the pathway in both the cytosol and the plastid. However, network flux analysis showed that a satisfactory fit could be obtained with a single pentose phosphate pathway, pointing to rapid exchange of intermediates between the plastid and cytosol. Thus, irrespective of the subcellular distribution of the enzymes, it appears that the compartmented pentose phosphate pathway operates as a single functional entity in oilseed rape embryos (Schwender *et al.*, 2003).

Representative applications

Steady-state flux analysis generates detailed flux maps of central carbon metabolism (Krömer *et al.*, 2004; Kruger *et al.*, 2003). These maps highlight several features that are characteristic of metabolic networks, but are otherwise difficult to study, including bidirectional fluxes, substrate cycles (Portais and Delort, 2002), and subcellular compartmentation (Figure 1; Ratcliffe and Shachar-Hill, 2005). Each flux map provides a description of a fundamental cellular activity under the physiological conditions of the experiment and as such provides a definition of the metabolic phenotype of the organism.

Measuring multiple fluxes simultaneously provides new insights into the operation of plant metabolic networks. For example, the first large-scale application of steady-state analysis concluded that 70% of ATP turnover in maize root tips could be attributed to the synthesis and degradation of sucrose (Dieuaide-Noubhani *et al.*, 1995). A similar process, responsible for 60% of ATP turnover, was found in a related study of cultured tomato cells during exponential growth (Rontein *et al.*, 2002). While the recent discovery of a glucose 6-phosphatase activity in maize root tips (Alonso *et al.*, 2005) suggests that the sink for ATP may actually be a cycle between glucose and glucose 6-phosphate rather than sucrose cycling, the point should not be lost that looking at the overall operation of the network in this way can lead to interesting conclusions about network efficiency. Thus, network analysis of developing oilseed embryos has defined the metabolic route from carbohydrate to storage lipid in unprecedented detail (Figure 9; Schwender *et al.*, 2004b), revealing important information on the metabolic origin of the NADPH required for biosynthesis (Schwender *et al.*, 2003) and leading to the discovery of a novel metabolic route operating in the production of seed oil (Schwender *et al.*, 2004a).

The phenotypic value of flux maps is well established in the analysis of microbial metabolism (Krömer *et al.*, 2004; Marx *et al.*, 1999; Sauer *et al.*, 1999) and similar applications can be expected in plants. The current plant focus on developing the methodology, without biological replication in some instances, should change as the field moves away

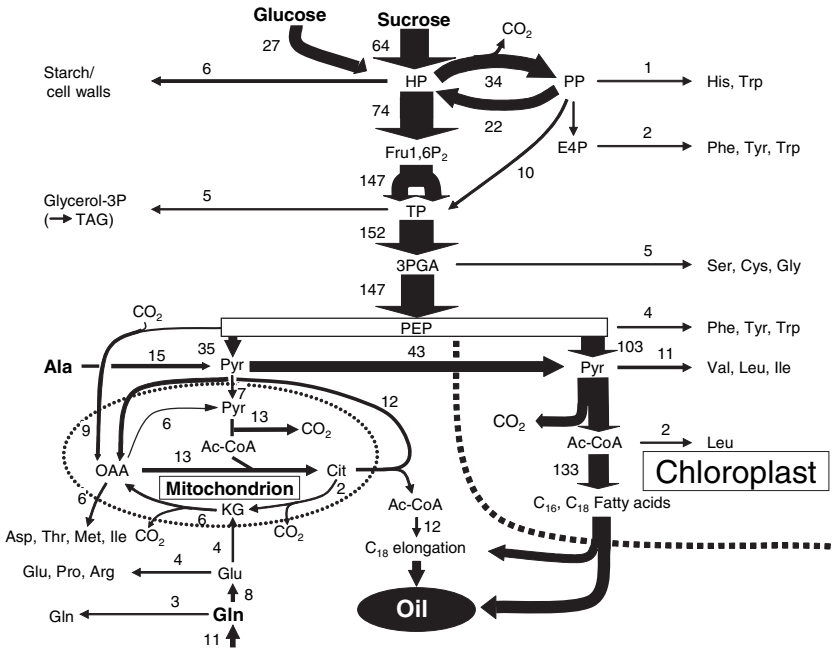

Figure 9. Flux map of central carbon metabolism in developing *Brassica napus* seeds based on a combination of steady-state ^{13}C labeling experiments and biomass accumulation measurements (Schwender *et al.*, 2003, 2004b). Developing seeds were cultured in labeled medium for up to 14 days, with low-molecular-weight metabolites reaching isotopic steady state within 3 days, and oil and protein a week later. Net fluxes (nmol h^{-1} per embryo) are represented as arrows with a thickness proportional to the magnitude of the flux. The labeling measurements are consistent with several simplifications of the network in Figure 1, including: treating the hexose phosphates as one pool, and similarly the triose phosphates and the pentose phosphates; and combining cytosolic and plastidic metabolism between hexose phosphate and phospho*enol*pyruvate (PEP). The overall pattern of fluxes is dominated by synthesis of oil, protein and carbohydrate. Note that this flux map does not include the activity of a recently discovered route involving Rubisco (Schwender *et al.*, 2004a).

from proof of concept mode. In particular, there is likely to be greater emphasis on the response of flux maps to physiological and genetic manipulation in future work. Examples of physiological studies already exist, including an analysis of the effect of hypoxia on the flux catalyzed by malic enzyme in maize root tips (Edwards *et al.*, 1998), and a description of the response of central metabolism to progression through a cell culture cycle (Rontein *et al.*, 2002). An analysis of the cycling between triose phosphate and hexose phosphate in transgenic tobacco lines, which demonstrated that pyrophosphate: fructose-6-phosphate 1-phosphotransferase is sensitive to physiologically relevant fluctuations of its effector *in vivo* (Fernie *et al.*, 2001), is currently the only example of network flux analysis on transgenic plant material. However, it is clear from microbial work that flux maps can be invaluable tools for rational metabolic engineering (de Graaf *et al.*, 2001; Petersen *et al.*, 2001) and establishing the potential of a similar approach in plants must be a high priority as the methodology becomes established.

Although most steady-state flux maps describe the conversion of precursors into products, it is also possible to construct maps that focus exclusively on the recycling of particular metabolites (Ettenhuber *et al.*, 2005). In the first application of this approach, glucose recycling was analyzed in tobacco seedlings, and it was concluded that the redistribution of label supplied as [$^{13}C_6$]glucose into a much larger number of glucose isotopomers could be attributed to the net effect of six metabolic loops. In contrast to the flux maps described above, the measured fluxes through these loops refer specifically to the conversion of glucose to glucose, which may or may not be the main function of any particular segment in the loop. For example, one loop involved metabolism of glucose down to the level of the Krebs cycle followed by gluconeogenesis (Ettenhuber *et al.*, 2005). Clearly, this provides only limited information on flux through the Krebs cycle, because carbohydrate synthesis is unlikely to be the principal function of the cycle under most conditions. However, despite this limitation, the fluxes through these metabolic loops are diagnostic of the overall phenotype, and as such are likely to be useful in probing the metabolic response of the network to perturbation. A notable advantage of this recently proposed approach is that the scale of the numerical analysis is greatly reduced by focusing on the recycling of a single substrate.

Conclusion

Successful dynamic labeling studies depend on selecting appropriate precursors, and then constructing detailed time-courses for the levels and specific enrichments of as many intermediates and end-products as possible. The method is particularly suitable for exploring the pathways of secondary metabolism, and in favorable cases the analysis can form the basis for a mechanistic model of the network.

Steady-state experiments depend first and foremost on experimental verification of the necessary isotopic and metabolic steady state. Success then depends on: (i) careful choice of labeled precursors, based on simulation of the metabolic network, to provide reliable fluxes for the whole network; (ii) analysis of a range of molecules for each precursor, including metabolic intermediates, macromolecules, and reporters for specific subcellular locations; (iii) numerical fitting of the data to an over-determined model, in preference to an exact solution of a set of flux equations, particularly in the case of large-scale analyses; (iv) biological replication; (v) a conservative model; and (vi) flexible software, including statistical tools, for exploring the full range of possible solutions.

In conclusion, dynamic and steady-state labeling experiments provide powerful methods for measuring multiple fluxes through plant metabolic networks. The resulting flux maps provide a fundamental description of network function and they have the potential to be invaluable in characterizing the effects of physiological and genetic manipulation.

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