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Understanding flux in plant metabolic networks
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The revolutionary growth in our ability to identify the 'parts list' of cellular infrastructure in plants in detail, and to alter it with precision, challenges us to develop methods to quantify how these parts function. For components of metabolism, this means mapping fluxes at the level of metabolic networks. Advances in experimental, analytical and software tools for metabolic flux analysis now allow maps of the fluxes through central metabolism to be obtained from the results of stable-isotope-labeling experiments. Such maps have led to notable successes in understanding and engineering metabolic function in microorganisms. Recent studies in plants are giving insight into particular fluxes, such as those of the pentose phosphate pathway, and into general phenomena, such as substrate- or futile-cycles and compartmentation. The importance of experimental design and statistical analysis have been illustrated, and analyses of fluxes in heterotrophic plant tissues have been carried out recently.

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Abbreviations

GC	gas chromatography
G6PDH	glucose-6-phosphate dehydrogenase
MFA	metabolic flux analysis
MS	mass spectrometry
NMR	nuclear magnetic resonance
OAA	oxaloacetate
OPPP	oxidative pentose phosphate pathway
PEP	phosphoenolpyruvate
TAG	triacylglycerol
TCA	tricarboxylic acid cycle

Introduction
A living organism must perform the continuous conversion of material and energy, which is accomplished through thousands of metabolic reactions and transport processes. Recent decades have seen enormous success in the use of reductionist approaches for the study of plant metabolism. Biochemical and genetic tools in particular have been used to delineate pathways and to elucidate mechanisms at the molecular level. With the arrival of

high-throughput methods for identifying and quantifying DNA, mRNA, proteins and metabolites, we are now privileged to have access to system-wide information on the cellular infrastructure of metabolism. These data focus attention on the complexity of plant metabolic networks and challenge us to develop synthetic approaches to reveal their function.

Data-driven methods of informatics are being developed to reduce, compare and probe 'omic'-scale information [1,2], and are proving useful in generating hypotheses about gene function, physiological regulation and molecular interactions. As informatics and theory-driven *in-silico* biology methods [3] grow, so will the need for methods to test these hypotheses by directly measuring the functioning of plant metabolism. Thus, the complete analysis of how a cell functions will include not only the description of its molecular parts but also an understanding of flux distribution in complex and dynamic metabolic networks. Furthermore, because metabolic flux and its regulation operate at the level of networks rather than of isolated linear pathways, we need sophisticated experimental and theoretical tools to allow us to analyze metabolic networks.

In this review, we highlight recent advances in the analysis of the central carbon metabolism network in plants that have been made using stable-isotope labeling under steady-state conditions. Metabolic flux analysis (MFA) by this approach has been important in allowing us to understand and engineer the metabolism of microorganisms in recent years, and we think it will be of increasing importance in plant research in the future. Other methods of MFA in plants, such as kinetic tracer techniques and the analysis of small-scale metabolic networks, have recently been well reviewed elsewhere [4•,5•].

Principles and practice of stable-isotope metabolic flux analysis
A metabolic pathway can be defined as a sequence of feasible and observable biochemical reaction steps, but metabolic flux is the rate at which material is processed through that pathway [6]. Mathematical descriptions of flux distributions in metabolic networks of microorganisms and mammalian cells that were based on extracellular flux measurements [7,8] were developed before the widespread use of stable-isotopic-labeling methods. The mass–balance approach requires measurements of the rates of substrate uptake, rates of secretion of metabolites into the growth medium, and the rates of biomass formation. Together with the definition of a network of biochemical reactions, the formulation of mass–balance

equations for metabolite pools allows a map of net metabolic fluxes to be derived. In contrast to kinetic models, this analysis requires only information on reaction stoichiometry, it does not require any knowledge of metabolite pool sizes or kinetic enzyme properties.

However, cyclic or parallel competing pathways and reversible fluxes (i.e. exchange fluxes), all of which are prominent in plant metabolism, cannot be quantified by this original MFA approach [6,8]. This limitation can often be overcome by the use of substrates that are labeled with stable isotopes. ^{13}C flux through two parallel pathways — for example, glycolysis and the oxidative pentose phosphate pathway (OPPP) — can usually be distinguished if the two pathways produce a difference in the labeling of a common product. The basic MFA model is then extended by label measurements, and the parallel fluxes can be quantified using steady-state isotopomer balance equations. (Given a molecule with n atoms, an isotopomer [isotope isomer] defines one of 2^n possible positional combinations of ^{12}C and ^{13}C nuclei in the molecule.)

The elements of ^{13}C-MFA experiments: steady state, measurements and isotopomers

In a typical steady-state ^{13}C-MFA experiment, a cell culture or isolated tissue is provided with a ^{13}C-labeled carbon source. The label is allowed to distribute through the metabolic network for long enough to reach a stationary distribution in the different metabolite pools (i.e. an isotopic steady state). Labeled intracellular metabolites are then extracted, and the ^{13}C label is measured by nuclear magnetic resonance (NMR) [9] or by gas chromatography (GC)–mass spectrometry (MS) methods [10,11]. In addition to external flux measurements (i.e. measurements of metabolite uptake and excretion), the labeling of metabolites is determined by NMR, which reveals either ^{13}C enrichment at particular carbon positions or bond labeling [9], and/or by MS, which reveals fractional ^{13}C enrichments in different molecules and molecular fragments. The labeling distribution in the network can be modeled using the notation of isotopomers.

An example of the power of stable-isotope MFA: genetic engineering of *Corynebacterium*

Determining which enzyme catalyses key metabolic fluxes *in vivo* by conventional labeling without modeling can be difficult, and *in-vitro* measurement can be misleading. By overcoming these difficulties, MFA can play a key role in rational metabolic engineering. Lysine-overproducing strains of *Corynebacterium glutamicum* are used for industrial lysine production, and a significant part of the development of ^{13}C-MFA methods has been done using this organism (e.g. [12–15]). One approach to maximizing lysine yield is to increase carbon flux at the start of the lysine pathway by increasing the flux of the carbox-

ylation of phosphoenolpyruvate (PEP) or pyruvate into oxaloacetate (OAA). Originally PEP carboxylase was thought to be the main enzyme of anaplerotic formation of OAA in *C. glutamicum*, but the use of ^{13}C-MFA revealed that pyruvate carboxylase, although it is hard to detect in enzyme assays, catalyses 90% of the carboxylation flux *in vivo* [14]. Conversely, the PEP carboxylase activity that is detectable *in vitro* seems to be strongly inhibited under *in-vivo* conditions [16]. The identification of pyruvate carboxylase as the main carboxylation step *in vivo* by ^{13}C-MFA led to the successful improvement of lysine production in *C. glutamicum* by overexpression of pyruvate carboxylase together with aspartate kinase [17]. In addition, Petersen *et al.* [14,16] showed that PEP carboxykinase catalyzes large fluxes of interconversion of OAA and PEP in lysine-overproducing *Corynebacterium*, constituting a futile cycle. Accordingly, genetic knockouts for PEP carboxykinase increased the lysine yield [14,16]. This example of the use of ^{13}C-MFA to untangle redundant network connections, leading to successful genetic engineering, serves to demonstrate the usefulness of this approach and its potential in plants.

Experimental systems used for ^{13}C-MFA in plants

A number of studies have used batch cultures in which plant cell suspensions are grown for several days on a medium that contains ^{13}C- or ^{14}C-labeled glucose as carbon source [18–21,22$^{\bullet\bullet}$]. Fast-growing excised maize root tips have also been used to study central carbon metabolism by keeping them for 12–18 hours in a medium containing ^{13}C-labeled glucose [23–25]. In two significant studies, the most abundant labeled free intracellular metabolites (i.e. sugars and amino acids) were analyzed by NMR or MS, and large flux maps of central carbon metabolism were derived [22$^{\bullet\bullet}$,23]. Owing to the metabolic changes in batch cultures that shift from exponential growth to a stationary stage and in tissues following excision, the MFA is performed on metabolites that have a turn-over time of several hours or less. Only then does the label in the metabolites represent a metabolic steady state. The resulting labeling information can only be an approximation for cell cultures and maize root-tip cultures because carbohydrates and proteins are turned over continually in these systems, causing slower changes in the labeling of other metabolites [22$^{\bullet\bullet}$,23].

The use of developing seeds in culture can potentially overcome the limitations of using batch-cultured cells or excised plant tissues for ^{13}C-MFA. Several studies looking at starch and sucrose biosynthesis in seeds have been performed, mainly addressing sucrose and triose cycling [18,26,27]. In other studies [28,29$^{\bullet}$], uniformly ^{13}C-labeled glucose was used to label maize kernels, and label was analyzed in both glucose and amino acids that were derived from accumulated starch and protein, respectively. These studies, although not quantifying flux, demonstrate in a seed culture system how labeling

in the amino acids of seed protein can be used to infer the labeling of many intracellular metabolites by a 'retro-biosynthetic' approach [9]. This gives more information than is usually gained from the measurement of free metabolites in cell cultures because of issues of low abundance, compartmentation and lability that compli-cate the direct analysis of labeling in many metabolic intermediates. These studies also demonstrate the use-fulness of uniformly ^{13}C-labeled glucose as a substrate.

Any isotope-labeling experiment can potentially perturb metabolism, especially if the concentrations of substrates provided are not the same as those *in planta*. To address such concerns, Schwender and colleagues [30••,31••] developed a culture system for embryos of *Brassica napus* to study metabolism during the main phase of oil and protein accumulation. To approximate the development and physiology of embryos *in planta*, the culture medium contained carbohydrates and amino acids at the concen-trations found in the liquid endosperm of developing seeds. The cultured embryos remain in an embryonic mode, grow at the same rate as *in planta*, produce essen-tially the same storage products as embryos *in planta* and, after 14 days, are fully capable of germination [30••]. The labeling pattern in free amino acids after 3 days of culture is almost the same as that found in the amino acids of protein after growth for 10 days (J Schwender, Y Shachar-Hill, J Ohlrogge, unpublished). This, and observations on other storage compounds, shows that the embryos are very close to isotopic steady state during the culture period. Thus, embryo culture systems should yield flux maps (see Figure 1) that represent the storage-accumula-tion phase of developing embryos *in planta*.

Addressing metabolic questions in plants using ^{13}C-MFA
Flux through the OPPP
The OPPP operates in heterotrophic tissues of plants, where it is thought to supply NADPH and carbon ske-letons for biosynthetic processes. *In vivo*, the activity of the first enzyme of the pathway, glucose-6-phosphate dehydrogenase, is probably modulated by the redox bal-ance of the NADPH/NADP pool [32]. Thus, OPPP flux *in vivo* may not be accurately assessed by extractable enzyme activity. Classical methods of measuring OPPP flux *in vivo* involving the release of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose, as well as some methods using ^{13}C labeling, are prone to potentially serious errors [33–35]. Among several precautions, the reversibility of transketolase and transaldolase must be quantified as exchange fluxes in order to account properly for the net OPPP flux *in vivo*.

Futile cycles
A futile cycle is a metabolic cycle for which the net balance consists solely of the dissipation of free energy, typically from the hydrolysis of ATP [36••]. Such cycles 'waste'

ATP and do not result in net carbon flux. Futile cycles are found in the central metabolism of mammals and micro-organisms [36••], as well as in plants [21,22••,23,37]. Examples are the cyclic synthesis and degradation of sucrose (sucrose cycling), the interconversion of triose-phosphate and fructose-6-phosphate (hexose-triose cycling) and the interconversion of PEP and oxaloacetate.

It has been reported that futile cycles account for a dramatic portion of the ATP consumption of plant cells [22••,23], and different possibilities have been proposed to explain why cells maintain these energy-consuming cycles [22••,36••]. To understand the biological role of futile cycles and to develop quantitative models of cell metabolism, these cyclic fluxes must be quantified. Fernie *et al.* [21] quantified triose cycling in heterotrophic tobacco tissue that was transformed with 6-phospho-fructo-2-kinase. No changes in the net fluxes of central carbon metabolism were observed but triose cycling was increased as a result of increased levels of fructose-2,6-bisphosphate. This study demonstrates the usefulness of ^{13}C-MFA for analyzing the metabolic effects of genetic transformation and for testing hypotheses about flux regulation *in vivo*.

Insights gained from mapping larger sections of central metabolism
Dieuaide-Noubhani *et al.* [23] created the first large-scale metabolic flux map for a plant by determining some 20 fluxes in three cellular compartments. This was achieved by labeling excised maize root tips and modeling the resultant labeling patterns. They found that sucrose turn-over constitutes the largest flux, consuming about 60% of the ATP produced by mitochondrial respiration. They also found that about one third of the flux from triose-phosphate into the tricarboxylic acid cycle (TCA) cycle is via PEP carboxylase and two thirds is via pyruvate kinase. Rontein *et al.* [22••] compared the flux distribution in central carbon metabolism at different stages of the cell cycle in batch cultures of tomato cells. In the transition from exponential growth to a pre-stationary phase, several key fluxes (i.e. glycolysis, the OPPP and the flux through pyruvate dehydrogenase) remained remarkably constant relative to the total glucose influx. Futile cycles (i.e. sucrose- and triose-phosphate cycling) also remained relatively unchanged and were proposed to be important for the stability of the central carbon metabolic network. At the same time, fluxes into the major anabolic pathways were highly variable.

We studied developing embryos of *Brassica napus* during oil and protein accumulation [30••,31••]. Figure 1 shows a metabolic flux map for the synthesis of oil, protein and carbohydrate products in the developing embryo. The figure focuses on net fluxes into products and is based on a MFA analysis that involved the modeling of ^{13}C and ^{15}N labeling in oil, carbohydrate and protein. Mass balancing

Figure 1
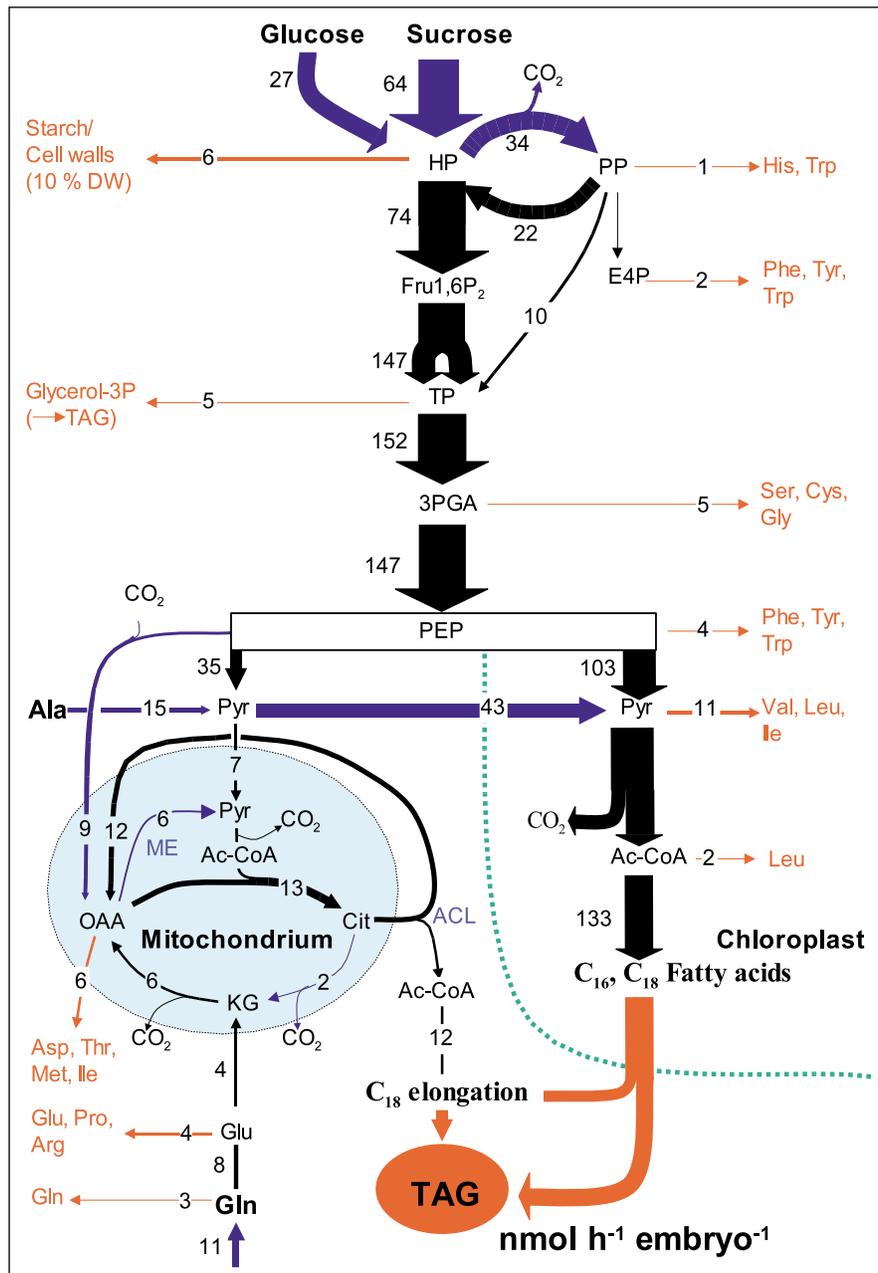
Preliminary flux map for the central carbon metabolism of *B. napus* embryos during oil and protein accumulation. The map summarizes both the results from Schwender and colleagues [30••,31••] and unpublished findings (J Schwender, J Ohlrogge, Y Shachar-Hill, unpublished). Only net fluxes are shown; the width of arrows indicates the sizes of the net carbon fluxes, the numbers are nmol h^{-1} embryo^{-1} at about 25 days after flowering. Fluxes into biomass (red arrows) were derived by considering the three main biomass components, seed oil, protein and starch, using the stoichiometries of their biosyntheses. Additional fluxes (blue arrows) are derived by ^{13}C- and ^{15}N-labeling experiments and flux-parameter fitting. Fluxes whose sizes were determined by the values of the biomass and modeling-derived fluxes (i.e. dependent fluxes) are represented by black arrows. Ac-CoA, acetyl co-enzyme A; ACL, ATP citrate lyase; Ala, alanine; Arg, arginine; Asp, asparagine; Cit, citrate; Cys, cysteine; DW, dry weight; E4P, erythrose-4-phosphate; Fru1,6P2, fructose 1,6-bisphosphate; Gln, glutamine; Glu, glutamate; Gly, glycine; His, histidine; HP, hexose-phosphate; Ile, isoleucine; KG, ketoglutarate; Leu, leucine; ME, malic enzyme; Met, methionine; PGA, 3-phosphoglycerate; Phe, phenylalanine; PP, pentose-phosphate; Pro, proline; Pyr, pyruvate; Ser, serine; Thr, threonine; TP, triose-phosphate; Trp, tryptophan; Tyr, tyrosine; Val, valine.

of products and substrates was used to provide further flux constraints.

In *B. napus* embryos, 50% or more of total carbon flux is directed into storage triacylglycerol (TAG) and 25–30% into protein. The series of MFA experiments summarized in Figure 1 have provided a number of insights. First, mitochondrial carbon metabolism is oriented primarily towards providing precursors for amino-acid synthesis and cytosolic for fatty-acid elongation rather than cyclic TCA flux. Consequently, more than 80% of carbon dioxide produced by the embryo is from plastid pyruvate dehydrogenase rather than from oxidative decarboxylation in the mitochondria. Second, approximately half of mitochondrial pyruvate is derived from malic enzyme and half from pyruvate kinase. Mitochondrial malic enzyme (ME) and cytosolic ATP citrate lyase (ACL) are involved in the generation of cytosolic acetyl-CoA. Third, on the basis of label in sucrose, starch and glycerol from TAG, hexose phosphates and triose phosphates have similar labeling patterns in both the cytosol and plastid compartments, suggesting that these metabolites are rapidly exchanged by plastid envelope transporters [31**]. Therefore, the distribution of glycolytic flux between these two compartments was not resolved. Fourth, pyruvate is clearly not in equilibrium between cytosol and plastid, and pyruvate derived from the cytosol contributes less than one third of carbon to plastid fatty-acid synthesis. Finally, the OPPP cannot supply most of the NADPH needed for fatty-acid synthesis, which points to the need for an expanded analysis to account for the total balance of co-factors. In addition to illustrating the power of MFA for quantitatively describing the fluxes through much of central metabolism, metabolic flux maps demonstrate the importance of considering networks as a whole rather than fragments. Only then can one make sense of subcellular processes and account for global balances of co-substrates such as ATP and NAD(P)(H).

Methodological advances

The importance of MFA methods that are based on stable-isotopic labeling for engineering microorganisms, and the insights already yielded by their application to plants, make it likely that the next few years will see the increased application of ^{13}C-MFA to problems in plant metabolism and its engineering. As this happens, it will be useful to consider the advances made recently in methods for ^{13}C-MFA and how they are used.

Experimental overdetermination

Most ^{13}C-MFA studies with plants have involved selecting a number of labeling measurements that allow the construction of an equation system with an exact analytical solution. To obtain more reliable flux information, most studies in microorganisms use overdetermination of networks with labeling data. This means that more labeling information is collected than the minimum required

to define the fluxes. This leads to overdetermined equation systems in which flux is estimated by a numerical fitting procedure. The quality of the fit can be judged by a chi-squared test [8,38–40]. Overdetermination gives more confidence in the flux measurements because the error in them can be decreased, and more importantly, because the additional label data make the flux model more trustworthy. This is because the greater the number of independent isotopomer balance relations that can be built around a metabolite pool, the more likely it is that previously unknown details of the model network will be revealed. This can also result in the discovery of network connections that were not thought to operate, and for which the flux model has to be adapted.

Several options are available for achieving this increase in the number and quality of measurement data. Combining NMR and GC–MS measurement data can increase the information obtained on the labeled intermediates [31**,39,41**]. In addition, using more than one labeled substrate can give increased flux resolution. This can involve using either the same substrate (usually glucose) with isotopic labeling in different positions [31**] or different substrates, as shown by Petersen *et al.* [14] who used ^{13}C-labeled lactate in addition to ^{13}C-labeled glucose. Multiple [13]C- and ^{15}N-substrates were used to derive the map shown in Figure 1 in which glucose, sucrose, glutamine and alanine are the natural carbon sources *in planta* and each was provided to embryos in different ^{13}C-labeled forms.

### Software tools

When larger networks are considered, general purpose mathematical software has been used to solve the complex equation systems [22**,23]. A number of dedicated software packages have recently been developed for ^{13}C-MFA that allow flux parameters to be determined from labeling patterns by numerical fitting procedures (software by Schmidt *et al.* [42], MetaboLogic [43] and ^{13}C-FLUX [44]). ^{13}C-FLUX allows the definition and simulation of large metabolic networks with the automatic generation and handling of typically more than 1000 isotopomer balance equations. The program provides information on the sensitivity with which different parameters are determined and performs statistical analyses that are based on the variability of all experimental data [44].

The benefits of optimizing experimental design using modern software tools

The confidence and precision with which metabolic fluxes can be quantified depends very much on the choice of labeled substrates, the choice and accuracy of label measurements, and the assumptions made in constructing a model of the metabolic network. Dedicated software packages are also useful in allowing the investigator to compare different potential experimental configurations

Figure 2
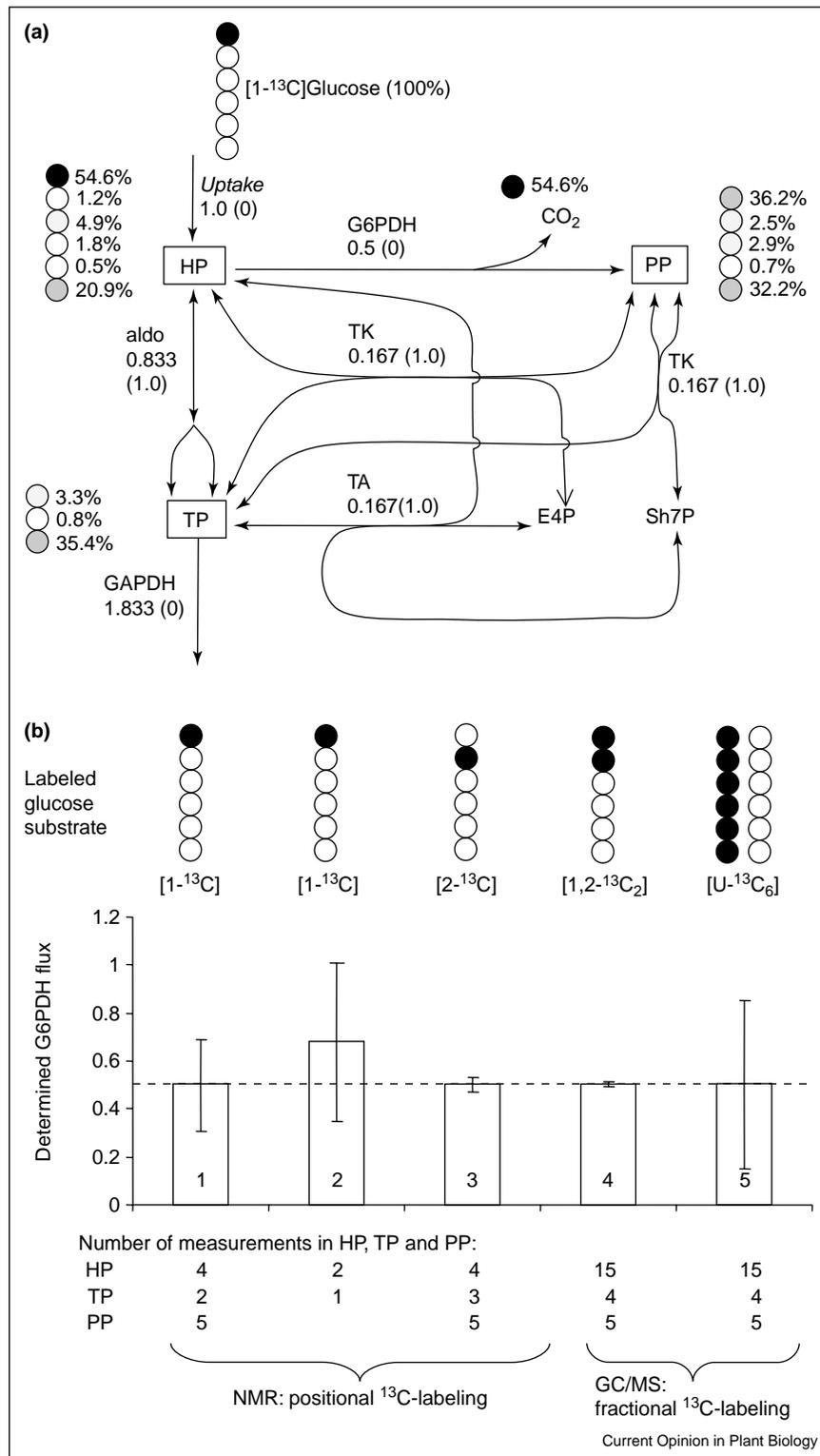
Flux estimation in the glycolysis/OPPP network. **(a)** The redistribution of ^{13}C-label from [1-^{13}C]glucose occurs in different intermediates of the network because of reversible reactions. A hypothetical network topology was used with the uptake of glucose set to 1 and that of G6PDH set to 0.5. Exchange fluxes from reversible reactions (in parentheses) are set to 1.0. Other values are derived by the model. The direction of net flux is indicated by solid arrowheads. Natural ^{13}C abundance is not considered. **(b)** The effects of ^{13}C-substrate selection, modeling decisions and measurement method (NMR or GC–MS) on the outcome of a ^{13}C-labeling experiment. The fitted G6PDH flux with a 90% confidence interval is shown for five different experimental configurations. The calculated labeling measurements were similar to the measurements used in studies

(including different definitions of the metabolic network, different labeled substrates, and the use of MS and/or NMR measurements) before carrying out the labeling and modeling experiments [44]. These aspects are illustrated by considering the analysis of the glycolysis/OPPP sub-network (Figure 2), which is a longstanding and frequent target of MFA analyses but which poses serious challenges to MFA [22[••],23[••],30[••],31[••],41[••]].

Using the network and flux values shown in Figure 2a, the theoretical labeling pattern was calculated for the glycolysis/OPPP sub-network in different metabolite pools. The impact of using different substrates or modeling assumptions is demonstrated in Figure 2b. Data from NMR and MS (with noise and standard errors) were calculated for each substrate choice, and the flux parameters were then re-determined by parameter fitting with a least-square-fitting approach using the ^{13}CFlux modeling software [44]. For the NMR experiments, using [1-^{13}C]glucose gives a much greater uncertainty for the flux through glucose-6-phosphate dehydrogenase (G6PDH) than using [2-^{13}C]glucose (Figure 2b), confirming the analysis by Roscher *et al*. [34]. When label from a [1,2-^{13}C2]glucose substrate is measured by MS, the G6PDH flux is also very well determined (see also [31[••]]). [1-^{13}C]glucose labeling was also analyzed by modeling transketolase and transaldolase as irreversible reactions (Figure 2b), an assumption sometimes made in the literature. No fit was found that would pass a statistical chi-squared test, and the simplified model could only fit to a reduced set of measurements (C-1 and C-6 of hexose-phosphate and C-3 of triose-phosphate). Furthermore, G6PDH flux was substantially overestimated by this model (Figure 2). Such *in silico* explorations, which have been made possible by the availability of modern software tools, demonstrate the importance of experimental design, of statistical treatments, of maximizing the experimental measurements made and of taking reversibility and futile cycling into account when using MFA to determine net fluxes.

Conclusions

Those using ^{13}C-MFA methods face several challenges. First, the complexity of a metabolic network, which is defined by the number of nodes and connections in the network, must be matched by sufficient accurate labeling data and extracellular flux measurements to allow the flux values to be determined [6]. Even under these conditions, some fluxes may still be statistically weakly determined. Second, flux mapping begins with a description of which

metabolic reactions exist in a given network, and the results of ^{13}C-MFA can only be as accurate as the model that describes the system [35]. For example, most studies that have quantified OPPP flux by ^{13}C-MFA assumed a network topology in which pentose-phosphate is converted to hexose-phosphate and triose-phosphate via transketolase and transaldoase, which may not represent reality. Instead of the three different reactions that are usually assumed to be catalyzed by transketolase and transaldolase, these enzymes can in fact catalyze nine different reversible carbon-transfer reactions between pentose phosphates, glyceraldehyde-3-phosphate, fructose-6-phosphate and sedoheptulose-7-phosphate. Van Winden *et al*. [35] concluded that not considering all of the possible reactions in a model network can lead to wrong estimates for G6PDH flux. Furthermore, assumptions such as the lumping together of pools that are in rapid equilibrium have to be well-founded on knowledge of the system. For plants, we have an imperfect knowledge of the subcellular compartmentation of reactions and the occurrence of tissue- or organism-specific enzymes. Third, it is important to maintain metabolic and isotopic steady state during the labeling experiment. As described above, this is often not easy to achieve and to document in plant systems. Fourth, when mapping the fluxes in purely autotrophic metabolism, steady-state labeling patterns are uninformative if labeled CO_2 is used because all carbon metabolites will become uniformly labeled.

For these reasons, stable-isotope MFA is currently most useful for quantifying fluxes in the metabolic networks of heterotrophic tissues of well-studied plant systems. Developing seeds not only meet these criteria but, as the major product of agriculture, are also a plant system whose metabolic engineering attracts intense interest. Significant new insights have been gained into the operation of metabolic networks in plants using developing seeds. There is much more to be learned from the use of stable-isotope MFA about genetic, developmental and environmental effects on metabolic fluxes. The developments and achievements in microorganisms to date also suggest that MFA will provide both important insights for studies of plant functional genomics and guidance for more rational approaches to metabolic engineering.

(Figure 2 Legend Continued) using NMR [22[••],23] and GC–MS [31[••]]. Realistic measurement errors were derived from these studies and applied to the calculated values when fitting flux parameters. The consistent values for G6PDH flux were found using the ^{13}CFLUX evolutionary parameter-fitting algorithm [8] and different starting values for the free fluxes. Statistical analysis of the experiments yielded the 90% confidence regions for the flux parameters, which are shown as error bars [45]. aldo, aldolase with phosphofructokinase; E4P, erythrose-4-phosphate; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HP, hexose-phosphate; PP, pentose-phosphate; Sh7P, sedoheptulose-7-phosphate; TA, transaldolase; TK, transketolase; TP, triose-phosphate.

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The authors present one of the most comprehensive steady-state flux models in plants. Steady state labeling with [1-^{13}C]glucose was performed during three different stages of the growth cycle of tomato cells in suspension cultures. The decrease in nutrients in the growth medium and the increases in biomass (i.e. fresh weight and dry weight) and in free cellular sugars, organic acids, protein, starch and cell wall polysaccharides were measured during culture. The labeling in sucrose, starch, glutamate and alanine (as determined by NMR) was used to derive flux ratios in central carbon metabolism. Upon the transition from exponential growth to a pre-stationary phase (which occurs via an arrest of cell division) several key fluxes (i.e. those for glycolysis and the OPPP, as well as flux through the pyruvate dehydrogenase complex) remain remarkably constant relative to the total glucose influx. In addition, futile cycles (i.e. sucrose- and triose-phosphate cycling) remained relatively unchanged and are considered to be important for the stability of the central carbon metabolism network. At the same time, fluxes into the major anabolic pathways were highly variable.

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Maize kernels were grown with uniformly ^{13}C-labeled glucose and sucrose. NMR analysis of the glucosyl units of starch indicates that the cyclic metabolism of glucose by the reactions of glycolysis and

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The authors establish the conditions necessary for steady-state stable-isotope labeling of developing embryos of *Brassica napus*. To grow embryos in culture conditions that mimicked growth *in planta*, the main carbon (i.e. sucrose and glucose) and nitrogen (i.e. amino acids) sources were included in the growth medium at the concentrations found in the endosperm liquid. The contribution of the different carbon sources to biomass formation during embryo culture was measured by stable-isotope labeling. Different criteria for establishing *quasi* metabolic and isotopic steady-states during the labeling experiment are tested and discussed. A low contribution of the OPPP to hexose breakdown was revealed by the labeling data. In addition, it was found that cytosolic acetyl-CoA is generated from TCA-cycle intermediates whereas plastidic acetyl-CoA derives mainly from glycolysis.

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Developing embryos of *B. napus* synthesize seed oil from sucrose and hexoses. Growing embryos were cultured with [U-$^{13}C_6$]glucose, [1-^{13}C]glucose, [6-^{13}C]glucose, [U-$^{13}C_{12}$]sucrose and/or [1,2-$^{13}C_2$]glucose, and the labeling patterns in amino acids, lipids, sucrose and starch were measured by GC—MS and NMR. The data were used to verify a reaction network of central carbon metabolism that is distributed between the cytosol and plastid. Computer simulation of the steady-state distribution of isotopomers in intermediates of the glycolysis/OPPP network was used to fit metabolic flux parameters to the experimental data. The glycolysis/OPPP network has to be overdetermined by different labeling experiments in order to measure the key fluxes reliably.

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Synechocystis was grown under heterotrophic and mixotrophic conditions on ^{13}C-labeled glucose. Labeling was detected in biomass hydrolysates by NMR and GC–MS. Steady-state flux distribution was estimated by fitting the flux parameters to the label measurements. A flux model that includes glycolysis, the OPPP and the TCA cycle was used for the heterotrophic experiment, and revealed that the OPPP is a major pathway for glucose breakdown. The model was modified for the mixotrophic culture by using the Calvin cycle instead of the OPPP. This study demonstrates both the use of ^{13}C-MFA of a photosynthetic organism and the acquisition of labeling information by the use of both NMR and GC–MS for the analysis of labeled biomass.

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