

REVIEW PAPER

Metabolic cartography: experimental quantification of metabolic fluxes from isotopic labelling studies

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Abstract

For the past decade, flux maps have provided researchers with an in-depth perspective on plant metabolism. As a rapidly developing field, significant headway has been made recently in computation, experimentation, and overall understanding of metabolic flux analysis. These advances are particularly applicable to the study of plant metabolism. New dynamic computational methods such as non-stationary metabolic flux analysis are finding their place in the toolbox of metabolic engineering, allowing more organisms to be studied and decreasing the time necessary for experimentation, thereby opening new avenues by which to explore the vast diversity of plant metabolism. Also, improved methods of metabolite detection and measurement have been developed, enabling increasingly greater resolution of flux measurements and the analysis of a greater number of the multitude of plant metabolic pathways. Methods to deconvolute organelle-specific metabolism are employed with increasing effectiveness, elucidating the compartmental specificity inherent in plant metabolism. Advances in metabolite measurements have also enabled new types of experiments, such as the calculation of metabolic fluxes based on ¹³CO₂ dynamic labelling data, and will continue to direct plant metabolic engineering. Newly calculated metabolic flux maps reveal surprising and useful information about plant metabolism, guiding future genetic engineering of crops to higher yields. Due to the significant level of complexity in plants, these methods in combination with other systems biology measurements are necessary to guide plant metabolic engineering in the future.

Key words: Compartmentation, isotopic labelling, metabolic flux analysis, metabolic modelling, oilseed metabolism.

Introduction

Maps provide perspective. They show the lay of the land and show which ways are possible, and often which ways are more efficient, from one point to another. A metabolic map is similar to a roadmap, detailing the myriad of pathways through which metabolism may occur. An effective and helpful metabolic map is one that enables researchers to determine quantitatively what is occurring within the organism, how alterations affect the map, and what changes might lead to more desirable traits.

Metabolic engineering is the improvement of cellular activities through the manipulation of cellular functions employing recombinant DNA technology (Bailey, 1991). The ability to directly manipulate an organism's genome

has had profound and wide-reaching impacts on every biological industry in the world today. It has enabled revolutions in agriculture (Stein *et al.*, 2008), pharmaceutical production (Chemler and Koffas, 2008), chemical synthesis (Carothers *et al.*, 2009), and many other fields. The complexity of a biological system makes it difficult for scientists to determine what the actual effect of a change was and why it did or did not result in the desired aims. Measuring the inputs and outputs of single cell type is relatively straightforward, but understanding what is occurring within a cell, particularly in plants, requires much greater knowledge and effort. Metabolic engineering tools such as metabolic pathway analysis or metabolic flux

analysis (MFA) make it possible to understand what is occurring within an organism's metabolism, and in the case of genetically modified organisms, allow researchers to determine which mechanisms are contributing to the success, or failure, of recombinant DNA interventions.

Central to the understanding of metabolism is the detection and quantification of metabolic fluxes (Stephanopoulos, 1999). Increased quantitative knowledge about metabolism has enabled efforts towards alterations in both organisms and their environments in order to optimize production. An example of improvement is increasing carbon efficiency, i.e. reducing the amount of carbon expelled as CO₂ in a synthesis process rather than being utilized in the creation of the desired product (Alonso *et al.*, 2007a). An important aspect of metabolic engineering is to determine which reactions within an organism's metabolism carry significant fluxes and which reactions have a major impact on the control of various routes in order to better guide the synthesis of more efficient or novel pathways.

The foundation of a metabolic map is the structure of the metabolic network. It is essential to determine beforehand the scope of the system and to ensure that adequate information is available for accurate construction. At a minimum, accurate stoichiometry is the basis for reactions. Additionally, depending on the approach, different information is required, such as energy cofactors for the reactions or carbon rearrangement patterns. Fortunately there are many tools available for the reconstruction of plant metabolic networks such as textbooks, biochemical literature, and, more recently, online sources such as KEGG, MetaCyc, and genome annotations. Some of these tools are becoming automated (Henry *et al.*, 2010), but there is usually a non-trivial amount of manual curation required.

Network topology and extracellular flux measurements are not sufficient to generate probable or actual flux distributions of a metabolic network. This is due to branch points, cycles, and parallel pathways, which result in many degrees of freedom even in simplified metabolic networks. In order to determine the probable or actual patterns of flux in a given system it is necessary to apply more information. By applying physicochemical constraints such as the conservation of each element, of energy, charges, and free energy changes to determine reaction directions, and keeping the levels of metabolic intermediates constant (metabolic steady state), it is possible to constrain the range of possible flux patterns. This range of fluxes, known as the solution space, is generally quite large. Therefore to make predictions about what the actual fluxes are likely to be, further assumptions or measurements are needed. The simplest way to meet this requirement is the application of an objective function. An objective function seeks to optimize a goal or set of goals, such as maximizing biomass production, maximizing chemical energy production, or another objective (Feist and Palsson, 2010). With this additional information, a reduced solution space of possible fluxes that more likely depict those chosen evolutionarily can be found for the model. This method is called

constraints-based or flux balance analysis (FBA), and has been employed for many microorganisms, including several phototrophic organisms on both small to mid-size scales (Shastri and Morgan, 2005; Grafahrend-Belau *et al.*, 2009) as well as on a full genome scale (Boyle and Morgan, 2009; Poolman *et al.*, 2009).

In the simplest cases (typically prokaryotic heterotrophs), measurements of substrate uptake rate, biomass composition, growth rate, and product excretion rates can be sufficient to constrain the model and yield flux maps with ranges for the major net metabolic fluxes. Frequently, though, even these measurements leave the model highly underdetermined, and estimates for internal fluxes – particularly reversible ones – can be potentially misleading (Chen *et al.*, 2011).

To further resolve the metabolic fluxes, techniques have been developed using isotopically labelled substrates to provide additional information about the metabolism. By employing isotopically labelled substrates and examining the amount of isotopic label incorporated into the downstream products to determine which metabolic pathways were employed, MFA provides a 'snapshot' of flux rates within the organism (Wiechert, 2001). These isotopically labelled products, which are isomeric with respect to the position and number of isotopically labelled atoms they contain, are referred to as 'isotopomers'. Because of the isotopic labelling of the substrate (usually the carbon), individual atoms can be traced through the organism's metabolism, greatly increasing the amount of information available about various reaction rates. This is particularly useful in distinguishing between pathways that use different reactions to arrive at the same final metabolic product. The specific labelling patterns observed correlate to the fluxes of the different pathways. While MFA is unable to predict what fluxes will result from a change in environment or genetic engineering, it provides a highly accurate picture of observable *in vivo* fluxes in a given organism. This ability makes it a useful tool in evaluating the effect, or lack of effect, of individual mutations at the metabolic level (Lonien and Schwender, 2009). It provides more guidance than general phenotypic evidence such as growth or transcriptome data by allowing detailed quantitative estimates of what is actually occurring at the functional metabolic level *in vivo*.

In contrast to FBA which relies on optimality assumptions to predict fluxes, ¹³C-MFA exploits the information obtained from measuring labelled metabolites to determine the fluxes in a given organism. This makes ¹³C-MFA significantly superior in quantitative and diagnostic cases. From this point on, all references to MFA will be specifically referring to ¹³C-MFA. Whereas FBA can provide a range within which the flux is expected to fall, MFA gives the statistical best fit values seen within the cell (Williams *et al.*, 2010; Hay and Schwender, 2011a; Young *et al.* 2011) including confidence intervals for each flux estimated. Also, unlike FBA, MFA is equally effective in genetically engineered organisms that may no longer operate at a physiological optimum and are therefore

unlikely to be following a single simple objective function. There are forms of FBA such as MOMA (minimization of metabolic adjustment) that are better for genetically engineering organisms than basic FBA (Segré *et al.*, 2002), but because FBA is predictive rather than diagnostic, it is not equally suited for genetically modified cases. Finally, due to the difference in label scrambling through different pathways, MFA may enable the determination of fluxes through parallel pathways and cycles as well as the exchange fluxes through individual reactions, which FBA cannot. This makes it possible to use MFA to confirm activity in pathways that were previously believed inactive (Schwender *et al.*, 2004), or inactivity in pathways believed to be active. MFA, though, requires much more computational power to resolve fluxes than most forms of FBA. Therefore, most MFA models have been restricted in scope, although recent advances have been made to extend them to a much greater scale (Ravikirthi *et al.*, 2011). Despite this limited scale, many discoveries and advances have been made using MFA, as apparent in an array of earlier reviews (Kruger and Ratcliffe, 2008; Libourel and Shachar-Hill, 2008; Schwender, 2008; Allen *et al.*, 2009a).

Analytical tools

Multiple tools have been employed to discern isotopic labelling patterns. Each of these has their own distinct advantages and disadvantages and all have seen significant advances over the recent years. Additionally, further experimental techniques, such as subcellular fractionation (Farré *et al.*, 2001) and the analysis of compartment-specific metabolic products (Allen *et al.*, 2007), are being developed to obtain a greater amount of information.

Nuclear magnetic resonance spectroscopy

One major tool for analysing the isotopic labelling patterns of metabolites is nuclear magnetic resonance (NMR) spectroscopy (Ratcliffe and Shachar-Hill, 2005), sometimes paired with liquid chromatography (LC) for metabolite separation (Exarchou *et al.*, 2005). The greatest advantage of NMR is that it can be used to measure labelling in different carbon positions within the metabolite. This provides the maximum amount of information available to solve the metabolic flux map. Additionally, using multidimensional NMR, it is possible to get high resolution with respect to the separation of signals from different metabolites. In favourable cases, such as protein hydrolysates, NMR can be used without chromatographic separation because of this high resolution. This significantly decreases the sample preparation work as well as the total time needed for each sample run. However, the complexity of plant metabolic networks is so great that it is often difficult to decipher meaningful results from plant extracts without additional upstream separation (Kim *et al.*, 2011).

The most significant disadvantage of NMR in MFA is the relatively low sensitivity of ^{13}C in NMR. This is the biggest detractor from widespread NMR usage because

frequently there is not a large amount of sample due to the prohibitive cost of labelled substrate and the small size of many metabolite pools. Advances in NMR spectroscopy, such as cryo- and small sample volume probes and multinuclear and multidimensional techniques, have worked to overcome this lack of sensitivity and potentially re-establish NMR as an effective tool for the measurement of intracellular metabolites (Fan and Lane, 2008). NMR results often also require significant training to interpret correctly, which has limited the number of those using it for MFA.

Mass spectrometry

A second major analytical method for the analysis of isotopically labelled metabolites is mass spectrometry (MS). MS can be paired with either gas or liquid chromatography or capillary electrophoresis to separate metabolites with overlapping mass to charge signals and MS can be performed in tandem for a more accurate analysis and increased resolution. Gas chromatography (GC) is advantageous because of its superior separation efficiency and resolution. This capability makes it attractive for quantifying non-abundant metabolites. However, gas chromatography requires that the analytes are capable of volatilization (Roessner *et al.*, 2000). Most metabolites of interest are not volatile and therefore must be derivatized before analysis using GC (Kanani *et al.*, 2008). This derivatization and subsequent volatilization commonly strips the phosphate group from sugar phosphates, causing them to be indistinguishable from the phosphate free sugars or metabolites in which the position of the phosphate group was the only difference. Additionally, heat labile compounds may be destroyed during derivatization and/or volatilization. Therefore, there are many metabolites for which GC/MS is not a suitable method of analysis. (Villas-Boas *et al.*, 2005).

LC coupled with MS using electrospray ionization, is a less harsh ionization method. Although LC does not resolve peaks as clearly as GC, it is capable of analysing some key metabolites such as sugar phosphates, nucleotide sugars, and others that are labile or present at low concentrations. Tandem MS methods can efficiently yield the necessary resolution to distinguish individual metabolites from each other. A number of papers in recent years detail methods such as ion pairing with reversed-phase chromatography or other separation methods to efficiently separate large numbers of even chemically very similar metabolites (Hayashi and Satoh, 2006; Luo *et al.*, 2007; Alonso *et al.*, 2010a). Additional work has also been done to optimize the combination of LC and tandem MS (Bajad *et al.*, 2006).

The other method used to separate metabolites for analysis is capillary electrophoresis. It is frequently paired with either regular MS or tandem MS for increased specificity and resolution. A number of methods have been developed to utilize the separation capabilities of capillary electrophoresis for a variety of organisms (Soga *et al.*, 2003; Edwards *et al.*, 2006), including plants (Sato *et al.*, 2004;

Harada *et al.*, 2008; Hasunuma *et al.*, 2010). Like LC, capillary electrophoresis is a gentler method of separation and is able to maintain the more labile metabolites in their native forms and coupled with tandem MS achieves a similar high resolution (Monton and Soga, 2007).

Mass spectrometry has many attractive features. The results of MS analysis are simple to collect and interpret. Additionally, using tandem MS it is possible to obtain very high sensitivity, detecting metabolites with nanomolar concentrations. In comparison to NMR methods, this higher sensitivity is the driving force behind the widespread use of MS for MFA. The downside to mass spectrometry is that it groups isotopomers together by mass, as shown in Fig. 1, decreasing the total amount of information collected due to a loss of knowledge relating to the positioning of the labelled carbon atoms. This shortcoming is not crippling, however, because even grouped in this manner the analysis of labelled metabolites can provide enough information to constrain the solution space and to overdetermine the fitting process for obtaining flux values.

Tandem MS or the full analysis of ion fragmentation in single quadrupole MS has the ability to recover some of the positional labelling data which is obscured by grouping isotopomers by mass. In this case, because metabolites have known and consistent fragmentation patterns, it is possible to not only observe the whole metabolite mass distribution, but also the mass distribution of key fragments (Allen *et al.*, 2007) making it possible to compare the two and decipher the labelling of individual metabolite fragments. The absence of derivatization and less energetic ionization methods reduce the fragmentation of metabolites and therefore the positional labelling information obtained in the simplest LC-MS methods, but tandem MS allows a comparison of the labelling of daughter ions with their parent ions to determine if labelled or unlabelled atoms were lost during the secondary ionization (Choi and Antoniewicz, 2011). While neither of these methods generally yields the level of detail of label positions that may be obtained from NMR, they each serve to increase the

amount of information gathered from MS analysis without greatly increasing the sample preparation or run time. In principle, a combination of NMR and MS methods is complementary (Ratcliffe and Shachar-Hill, 2006), and they have been used synergistically in MFA of plant systems (Alonso *et al.*, 2007a, 2010b); but in practice, MS methods are more frequently used alone, especially where intermediates are analysed and in studies of prokaryotic systems (Young *et al.*, 2011) or metabolic subnetworks in higher plants (Colón *et al.*, 2010) where a simpler network and/or model is being used.

Mathematical tools for MFA

MFA, like all types of metabolic mapping, is based on a predetermined set of metabolic pathways being analysed. Additionally, it is necessary to track the various positional changes of labelled atoms. This is frequently handled by atom mapping matrices, which are a concise mathematical format describing the physical rearrangement of atoms from the substrate(s) to the product(s) of a reaction (Zupke and Stephanopoulos, 1994). Upon this basic framework, several methods have been built to determine the actual fluxes within the metabolic pathway from the observed labelling patterns of downstream products or intermediate metabolites. The two formulations generally used today are cumomer analysis (Wiechert *et al.*, 1999) and elementary metabolite unit (EMU) analysis (Antoniewicz *et al.*, 2007a). These formats are popular because they reformulate the problem of tracking label into a system of linear equations. This significantly decreases the computational power necessary to solve them for a set of fluxes that best fits the labelling and other measurements. In addition to these overall formats, modifications can be made to the computational methods in order to accommodate deviations from the core assumptions (including metabolic and isotopic steady state) upon which the model is built.

Assumptions

As with all models, in MFA there are a few key assumptions. In order to accurately quantify fluxes, the experimental analysis cannot disturb the fluxes and the fluxes must remain constant long enough to be quantified. This produces the first two assumptions of MFA. The first assumption is that enzymatic reactions do not discriminate between isotopically labelled and unlabelled substrates. Detailed previous studies have shown that while there is a slight discrimination in enzymatic substrate selection due to the minor difference in bond energies between ^{12}C - ^{12}C and ^{12}C - ^{13}C bonds, it is small enough not to have any significant impact on the calculated fluxes (Kruger *et al.*, 2007a; Feng and Tang, 2011).

The second assumption is that fluxes within the metabolic pathways being studied are constant. This assumption is thought to be valid if the cultures are grown in balanced growth over several generations, meaning there is no change in the biomass composition with time. The most common

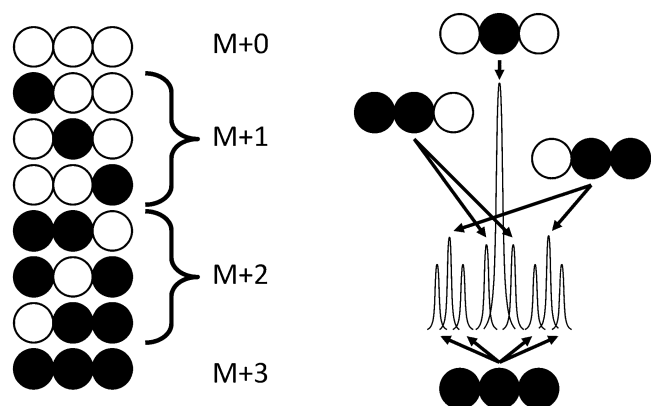


Fig. 1. Mass spectrometry analysis clusters isotopomers of the same molecular weight together, reducing the degree of information collected during analysis as compared to nuclear magnetic resonance readings.

method of confirming this is by analysis of metabolites demonstrating that an isotopic steady state is reached (Zamboni *et al.*, 2009), meaning that the concentrations and labelling patterns of the metabolites are not changing with time. Moreover, for an isotopic steady state to be reached the system must also be at a metabolic steady state. It should be noted that isotopic steady state is not necessary for analysis, but if not present requires significantly more data and a more advanced computational framework (Shastri and Morgan, 2007; Noack *et al.*, 2011).

The final assumption is that the metabolic model contains all fluxes with a significant impact on the labelling patterns of the measured metabolites. This assumption should be based in a solid understanding of the organism's biochemistry and is validated by checking the statistical fit of the resulting model calculations (Antoniewicz *et al.*, 2006). If the model fails to properly fit the data, it must be reformulated. This is aided by observing where major disparities occur in the original fit of the model, but, as with model formulation, all changes to the model should still be based on an understanding of the organism's metabolism.

Current modelling techniques

EMU analysis has grown in popularity since its introduction due to the significant reduction in computational power necessary to solve for fluxes. This is largely due to the omission of extraneous calculations found within other methods. Cumomer modelling, in contrast, resolves the full labelling pattern of all intermediates. However, if not all metabolites are measured then not all of them need to have their full labelling pattern resolved. Additionally, in experiments where MS is used to determine labelling, significant positional information is lost. This latter case is where EMU analysis excels the most, since discarding this information significantly reduces the size of the model that must be calculated. By focusing only on resolving the observable results of a metabolic network EMU analysis is more efficient. This has resulted in a roughly 10-fold reduction in the number of variables necessary for the final resolution of the model while obtaining the same results (Antoniewicz *et al.*, 2007a).

Further reduction of the computational resources required has been achieved through other methods, which can be coupled with any of these modelling frameworks. Flux coupling operates by observing which reactions are connected and the inherent restrictions they impose on each other. While flux coupling does not reduce the connected fluxes to a single entity, it mathematically relates them in a rigid format to reduce the amount of calculation to arrive at a solution (Burgard *et al.*, 2004; Suthers *et al.*, 2010). Another way in which computational load has been reduced is through the use of Dulmage-Mendelsohn decomposition. Although this form of reduction is less useful as the complexity of the network increases, it has been shown to be effective in smaller systems (Young *et al.*, 2008).

Non-steady-state MFA

In some cases, it is impractical to wait for an isotopic steady state to be reached or steady-state labelling is uninformative. In these cases, an alternative mathematical framework must be used in order to obtain flux values. In recent years, multiple modelling approaches have connected steady-state and dynamic approaches (Young *et al.*, 2008; Suthers *et al.*, 2010; Noack *et al.*, 2011). In cases where pool turnover is not significant enough to achieve steady state in a reasonable amount of time, a dilution parameter can be applied to take into account the proportion of the pool which has not turned over and remains from before the input of labelled substrate to the system. This parameter can be fixed as a single value for the whole cell, specific cellular compartments, or metabolite pools individually. A more in-depth discussion of the application of this dilution parameter can be found in Antoniewicz *et al.* (2007b). Additionally, methods have been developed for dynamic analysis of labelling. These methods are particularly useful in situations where isotopic steady state is unattainable or uninformative (Young *et al.*, 2008). In these instances, multiple time points must be taken and the framework used involves solving for both relative distribution of label within each metabolite and the metabolite concentrations. An in-depth discussion of the application of dynamic EMU analysis can be found in Young *et al.* (2008). In addition to providing a viable solution strategy in conditions where the isotopic steady-state criterion for MFA is not met, non-stationary measurements provide a much larger data set for analysis. This leads to a significant increase in the reliability of the flux estimates results by providing confidence ranges smaller than those possible solely through measurements of isotopic steady-state labelling patterns (Noack *et al.*, 2011).

For the study of organisms growing purely photoautotrophically such as plants, dynamic MFA is essential. Because the labelled substrate is by necessity $^{13}\text{CO}_2$, the steady-state labelling pattern will contain a uniform labelling pattern devoid of any information relating to fluxes. By feeding ^{13}C -labelled CO_2 and tracing the dynamic incorporation of the label into various intracellular metabolites, it is possible to determine the fluxes of a photoautotrophic organism, as postulated by Shastri and Morgan (2007) and demonstrated conclusively by Young *et al.* (2011). Until now nearly all plant tissues studied have been grown hetero- or mixotrophically in order to attain observable labelling patterns. With dynamic MFA, however, it is possible to measure the fluxes of autotrophic tissues as well.

Statistical analysis

Finally, it is important to have well-developed methods for the statistical analysis of a computed metabolic flux map, and recent years have seen greater use of appropriate statistical tools in MFA. In calculating the map, it is necessary to use the variance-weighted values of the experimental data to avoid bias in the resulting solution. Appropriate choice of weightings for label and other

measurements is not trivial in MFA since some measurements may be highly reproducible in experimental replicates but subject to systematic errors that exceed the experimentally observed variances. Such measurements may include biomass composition and some stable isotopic labelling data. In such cases, it may be appropriate to use larger variances than observed by replication in order to rely more equally on the large number of measurements and avoid potentially depending too much on a subset of data. Further statistical analysis can be employed to verify the accuracy of the metabolic map itself, checking to determine that the errors between measured and computed values follow an appropriate χ^2 distribution, as they should if the error is random rather than systematic (Klapa *et al.*, 2003). Systematic error is indicative of a problem with the model, either in the proper weighting of experimental values or else in the basic structure of the metabolic map. This could be caused by a missing reaction, or series of reactions, or else a reaction that is not feasible. In the case of eukaryotic organisms, this error can be caused by a lack of proper separation between reactions that occur in more than one cellular compartment. Additionally, it is important that appropriately obtained confidence intervals are reported with the fluxes. Instead of calculating the effect of the variance of each experimental measurement on the overall solution space this is done by testing the sensitivity of the minimized sum of squared residuals to each individual flux as detailed by Antoniewicz *et al.* (2006). Another approach to obtaining confidence intervals is to perform multiple fitting computations using input data values that have been randomly perturbed according to their experimentally observed variances (Monte Carlo methods) and reporting

a confidence interval for each flux value based on the range of values observed. However, Monte Carlo methods are computationally burdensome for large systems and the method proposed by Antoniewicz *et al.* (2006) has been shown to be equally effective.

Compartmentation: added complexity

Eukaryotic algae and plant cells are divided into intracellular organelles. Each of these organelles has unique and essential functions, but there are also frequently overlapping functions, metabolite pools, and reactions. This creates many issues when trying to distinguish which metabolic pathways are active. As previously discussed, the main method by which MFA distinguishes pathways is through different isotopic labelling patterns. If a reaction can occur in more than one organelle, then additional information is required to determine the amount of flux in each compartment.

Intracellular compartmentation

In the case of plants it is frequently found that metabolite pools exist in more than one location (Fig. 2) or that the subcellular location of one or more reactions is uncertain. Entire sections of metabolic pathways like glycolysis are duplicated between organelles, particularly the plastid and cytosol, with both being potentially active and carrying flux (Dennis and Blakeley, 2000). In such cases it is crucial to have methods that are able to determine the pathways in separate organelles from one another (Kruger and Ratcliffe, 2008). There are multiple ways of doing this. The first and

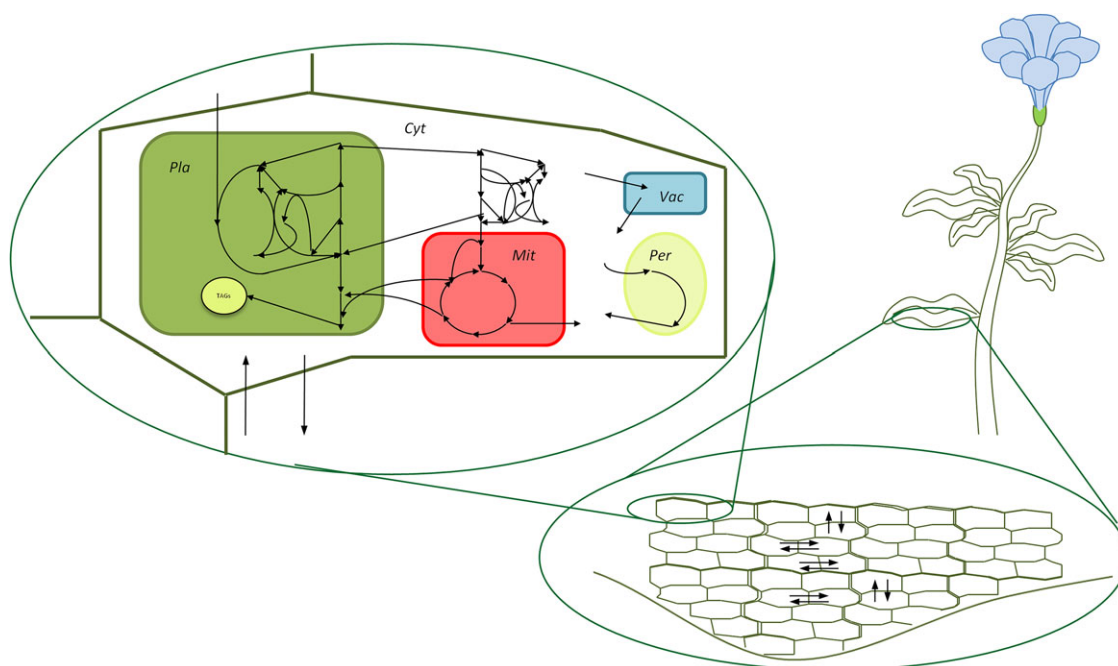


Fig. 2. Plant metabolism is highly compartmentalized on the intracellular, intercellular, and whole-organism scale. This high degree of complexity and interactivity makes accurate study of their metabolism an exacting science. Cyt, cytosol; Mit, mitochondria; Per, peroxisome; Pla, plastid; Vac, vacuole.

simplest way is to examine metabolites which are formed in only one of the compartments (Sriram *et al.*, 2004, 2007; Schwender *et al.*, 2006; Allen *et al.*, 2007; Lonien and Schwender, 2009). This method uses amino acids, lipids, and carbohydrates for which the compartment-specific formation is well established, although other metabolites could potentially be used. Unfortunately, even with the supplemental information provided by analysing compartment-specific metabolites, it may still be impossible to statistically distinguish different configurations of the metabolic map (Masakapalli *et al.*, 2010). Another method involves the fractionation of cellular material prior to metabolite analysis (Gerhardt *et al.*, 1983; Farré *et al.*, 2001; Krueger *et al.*, 2011). For time-course labelling experiments, in which metabolic intermediates rather than end products are analysed, the presence of multiple sub-cellular pools of the same metabolite, including those in vacuoles, makes such subcellular fractionation procedures desirable. Unfortunately, this method is experimentally challenging because of the difficulties of separating sub-cellular fractions while preventing metabolite diffusion and of reliably estimating the extent of any such redistribution. If cells can be successfully fractionated, the differences in labelling patterns between compartments should be discernable, and compartment-specific labelling patterns, and therefore flux rates, are in principle obtainable.

Tissue compartmentation

In addition to its intracellular complexity, plant metabolism includes the possibility of further separation of fluxes for the same reaction, as they can also differ between cells and tissues within the same organ (Fig. 2). This can lead to flux estimates based on labelling patterns that do not directly represent the metabolism of the cells being analysed (Ettenhuber *et al.*, 2005; Spielbauer *et al.*, 2006; Tcherkez and Hodges, 2009). In order to avoid this, there are multiple solutions. The first is to focus on cells that do not draw as heavily on other cells for their metabolism, such as leaf cells. This minimizes the impact of the cellular exchange of metabolites on the single-cell metabolic network. However, even in the case of leaf cells, where photosynthesis is highly active it has been shown that there is still significant usage of carbon from the rest of the plant (Gauthier *et al.*, 2010). Another approach is to include multiple cell types in the tissue being studied but to make measurements of metabolite flow from one tissue to another and of any contributions by one tissue to label rearrangements made in another (Alonso *et al.*, 2011).

In studying plant cell culture suspensions (Baxter *et al.*, 2007; Williams *et al.*, 2008; Masakapalli *et al.*, 2010; Williams *et al.*, 2010), it was possible to avoid the complications of multiple cell types. Similarly, seed embryos cultured in a constant environment can be assumed to be largely uniform in their metabolism during the extended period when their development is dominated by the accumulation of storage compounds (Allen *et al.*, 2009b; Lonien and Schwender, 2009; Alonso *et al.*, 2010b). Such

systems have the advantage of controllable substrate feeding to the cells, allowing inputs to the metabolism to be varied as well as facilitating the use of multiple labelling schemes to yield enlarged labelling datasets. Additionally, all the products are either stored in measurable pools separated from the ongoing metabolism or else they are excreted to the medium. In this way, a single cell type can be isolated for the study of its metabolism. The challenge of this method is to accurately reproduce the metabolism that would be found *in planta* under realistic physiological conditions. If the media formulation does not accurately mimic the substrate concentrations that the cells would observe *in vivo* then the metabolism may not either. Growing cells in suspension also alters the cell-cell signalling that would be found in a whole-plant system. These limitations mean that researchers must take special care when designing and performing these experiments to ensure that the results capture the intended real-world applications.

A window into maize metabolism

Maize is an important world crop and a long-standing model species for studies of plant growth and metabolism, and several groups have applied MFA tools to this species. Indeed the first major application of ^{13}C -MFA to measuring fluxes through the central metabolic network in higher plants was a study of maize root tips (Dieuaide-Noubhani *et al.*, 1995) in which steady-state labelling of soluble metabolites was used together with ^{14}C labelling measurements to derive net and exchange fluxes through the major pathways of central metabolism. This notable study demonstrated for the first time in plants that modelling steady-state positional labelling data could be used to obtain a large set of flux values, which had up to this time required multiple sets of experimental data, often occupying multiple studies over a period of years. Further applications of ^{13}C -MFA to maize roots have shed light on the role of sucrose synthase isoforms in carbohydrate metabolism and on the changes induced in central metabolism by oxygen limitation (Alonso *et al.*, 2007b,c).

Among the findings reported in this study was the apparent dissipation of most of the ATP generated by respiration through turnover and resynthesis of sucrose. This substrate or futile cycling had been reported previously, but ^{13}C -MFA studies in maize roots (Dieuaide-Noubhani *et al.*, 1995) and tomato cells (Rontein *et al.*, 2002) highlighted this as a potentially dramatic process. Subsequent studies of metabolic fluxes in maize roots using labelling and enzyme activity measurements (Alonso *et al.*, 2005, 2007b) and direct measurements by *in vivo* NMR of ATP turnover fluxes and metabolic flow through nucleotide sugars and sugar phosphate pools (Roscher *et al.*, 1998) added support to the idea that the active turnover of sucrose and glucose pools in plant cells can dissipate a substantial proportion of cellular ATP. This conclusion has been challenged by Krueger *et al.* (2007b) who showed computationally that ^{13}C -MFA which relies on labelling in soluble sugars and ignores the compartmentation of sugar

pools and reactions into vacuolar and cytosolic components can potentially seriously overestimate the degree of futile cycling. The issue of sugar turnover and futile cycling highlights the way in which ^{13}C -MFA can point to biologically significant processes that are difficult to quantify by classical methods and which are not likely to be identified by computational approaches like FBA that assume objective functions. The disputed status of conclusions also emphasize the increased care and information that is needed to resolve fluxes in compartmentalized cells – especially in plants where both metabolites and fluxes are present in multiple compartments (Allen *et al.*, 2007, 2009a).

The syntheses of starch oil and protein in maize seeds have also been the subject of ^{13}C -MFA studies and these have yielded insights into the major fluxes that provide the reductant, ATP and precursors for storage production. By contrast with other seeds studied by ^{13}C -MFA, maize seeds present particular challenges. They require supporting maternal tissue (the cob), and they consist of two distinct seed tissues (a starchy endosperm and an oil- and protein-rich embryo). The first steady-state ^{13}C labelling studies of developing maize kernels (Glawischnig *et al.*, 2002; Spielbauer *et al.*, 2006) focused on measuring by NMR the ^{13}C labelling patterns in glucose units of starch (largely from the endosperm). The labelling data were interpreted using a simplified model of central metabolism that focused on routes by which label in hexose can be rearranged. It was deduced that the large majority of sugar supplied to the cultures had its label rearranged by flow through a combination of pathways before incorporation into starch. Subsequent ^{13}C -MFA studies (Alonso *et al.*, 2010b, 2011) highlighted the importance of taking into account the metabolic activities of the maternal cob tissue which was shown to transfer to the developing seeds in culture a rather different array of labelled substrate molecules to the simple labelled substrate(s) added to the culture medium. Thus much of the label rearrangement takes place in the cob. After taking this into account by direct measurement of rates and forms of substrate transfer and analysing a wider array of products (including oils and amino acids in proteins, as well as carbohydrates), Alonso *et al.* (2011) were able to fit the results to a full MFA model and derive insights into carbon conversion efficiency (yield), as well as revealing the routes of carbon flow into storage products. The model revealed the presence of a large flow of carbon into the plastid at the hexose level and subsequent efflux into the cytosol as well as identifying potential targets for increasing oil synthesis in maize endosperm – an attractive target for metabolic engineering. The separate culture of maize embryo also allowed the mapping of metabolic fluxes in this tissue and highlighted the differences in the cells of different tissue types within the same plant organ (Alonso *et al.*, 2010b).

Applications of MFA for the analysis of oilseeds

Among the various flux studies in higher plants, several were aimed at describing *in vivo* flux distribution in the central metabolism of developing seeds that store triacylglycerol as

a major storage compound. Such plant oils are of major economic importance (Dyer *et al.*, 2008). Developing seeds of rapeseed and *Arabidopsis* (Schwender and Ohlrogge, 2002; Schwender *et al.*, 2003, 2004, 2006; Junker *et al.*, 2007; Lonien and Schwender, 2009), soybean (Sriram *et al.*, 2004; Iyer *et al.*, 2008; Allen *et al.*, 2009b), sunflower (Alonso *et al.*, 2007a), and maize (Alonso *et al.*, 2010b) were studied based on culture of excised embryos in liquid media with different ^{13}C -labelled organic nutrients (Table 1). Flux patterns in central metabolism were quantified to understand partitioning and allocation of maternal carbon resources to oil, protein and other storage compounds during seed development (Table 1). As to the interpretation validity of the results for *in planta* seed development, one should be critically aware of the experimental conditions.

Precursors of fatty acid synthesis

Following basic nutritional aspects known for developing seeds *in planta*, cultured embryos are typically fed with multiple organic substrates (sugars and amino acids, Table 1). By using ^{13}C -label in the substrate mixtures, it was found that *de novo* fatty acid synthesis (FAS) is predominantly derived from hexose catabolism (Schwender and Ohlrogge, 2002; Allen *et al.*, 2009b). The amino acids present as substrates, while also serving as the nitrogen source for protein synthesis, were only used to a limited extent as carbon precursors for FAS (Schwender and Ohlrogge, 2002). This is not obvious *a priori* since Gln, Asp, and Ala, for example, can be readily converted to 2-oxoglutarate, oxaloacetate, and pyruvate, respectively. Those, in turn, are close to acetyl-CoA, the precursor of fatty acids. Therefore it appears that protein and lipid synthesis are separated to a certain degree by subcellular compartmentation of precursor pools. Considering that *de novo* FAS from acetyl-CoA is bound to the plastid compartment (Ohlrogge *et al.*, 1979), it becomes clear that, in flux studies to understand seed metabolism, it is important to resolve subcellular compartmentation. In fact all flux studies on oilseeds summarized in Table 1, except one (Allen *et al.*, 2009b), resolve subcellular compartmentation with respect to the cytosolic, chloroplastic, and mitochondrial compartments.

Rubisco bypass: the path of carbon in oilseeds

Developing embryos of rapeseed, *Arabidopsis* and soybean have photosynthetic capacity and light drives the conversion of sugars to oil (Ruuska *et al.*, 2004). A particular feature of this light-dependent seed filling is the bypass of upper glycolysis by an alternative metabolic route, converting hexose phosphate to the glycolytic intermediate 3-phosphoglyceric acid (3-PGA) via interconversions of the pentose phosphate pathway and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Schwender *et al.*, 2004; Goffman *et al.*, 2005). Due to the participation of Rubisco, CO_2 generated by catabolic and biosynthetic processes is refixed, resulting in a reduced net CO_2 loss, i.e. higher carbon conversion efficiency in the oil-synthesizing seed

Table 1. Generation of precursors and metabolic cofactors for storage synthesis in oilseeds

Data extracted from ^{13}C -MFA studies of cultivated developing embryos of various species and genotypes. 3-PGA, phosphoglyceric acid; NC, reaction not considered in flux model; OPPP, oxidative pentose phosphate pathway.

Oilseed species	Soy ^{a,b}	Soy ^c	Rapeseed ^{d–f}	<i>Arabidopsis thaliana</i> ^g		Sunflower ^h	Maize ⁱ
Cultivar/genotype	cv. Evans	cv. Amsoy	cv. Reston	Ws/pkp (wt vs. low-oil mutant)	Col/wri (wt vs. low-oil mutant)	cv. Ames 7576	LH 59
Conditions for embryo culture (temp; light; duration)	12 °C/ 20 °C/ 27 °C; 100 μmol m ⁻² s ⁻¹ ; 6 d	27 °C; 35 μmol m ⁻² s ⁻¹ ; 14 d	20 °C; 50 μmol m ⁻² s ⁻¹ ; 14 d	21 °C; 50 μmol m ⁻² s ⁻¹ ; 7 d	21 °C; 50 μmol m ⁻² s ⁻¹ ; 7 d	25 °C; dark; 5 d	25 °C; dark; 7 d
Organic nutrients	Sucrose/ Gln	Sucrose/ Glc/ Gln/ Asn	Sucrose/ Glc/ Ala/ Gln	Sucrose / Ala/Gln	Sucrose / Ala/Gln	Glc/Gln	Glc/Fructose/ Gln
Oil content in cultivated embryos (% of dry weight)	16 ^b	18	38 ^f	45/20	47/13	40	34
Rubisco bypass (3-PGA generated by Rubisco; mol%)	11–18 ^a NC	25/NC ^{k,j}	46–75 ^e 45 ^f	82/118	91/138	NC	NC
Generation of plastidic pyruvate, the precursor for <i>de novo</i> fatty acid synthesis ^j							
Pyruvate generated by plastidic pyruvate kinase (mol%)	–	–	74	73/73	88/33	93	70
Pyruvate generated by import from cytosol (mol%)	–	–	26	25/21	10/53	NC	NC
Pyruvate generated from plastidic malic enzyme (mol%)	–	–	NC	2/7	2/14	7	30
Production of energy cofactors, relative to demands of storage synthesis							
ATP production by TCA cycle activity via oxidative phosphorylation, relative to biosynthetic demands (mol%)	154 ^{b,k}	80	22	60; 100	71; 262	>100	200 ^k
NADPH production by OPPP activity, relative to demands of fatty acid synthesis (mol%)	200–350 ^{b,k}	NC/24 ^{k,j}	38 ^d	OPPP flux estimates not reliable	OPPP flux estimates not reliable	106 ^k	76 ^k
NADPH production by plastidic malic enzyme, relative to demands of fatty acid synthesis (mol%)		29/27 ^{k,j}	NC			7	30

^a Iyer *et al.* (2008).

^b Sriram *et al.* (2004).

^c Allen *et al.* (2009b).

^d Schwender *et al.* (2003).

^e Schwender *et al.* (2004).

^f Schwender *et al.* (2006).

^g Lonien and Schwender (2009).

^h Alonso *et al.* (2007a).

ⁱ Alonso *et al.* (2010b).

^j Soy models not considered since they do not fully distinguish pools of mitochondrial, plastidic and cytosolic pyruvate.

^k Numbers derived by J. Schwender from published data.

^l Allen *et al.* (2009b) considered two model variants. They mainly reported a model network with presence of Rubisco, but absence of oxidative reactions of the OPPP. The published supplement considers an alternative model with Rubisco absent and OPPP present.

(Schwender *et al.*, 2004; Goffman *et al.*, 2005). Theoretical considerations based on typical steady-state ^{13}C -labelling experiments show that reliable quantification of the flux through the bypass is not straightforward (Libourel *et al.*, 2007). This explains that in soy studies valid flux maps could be obtained with or without consideration of Rubisco

in the network (Table 1). In developing rapeseed embryos, by various complementary ^{13}C -tracer approaches, it was found that about 50% or more of 3-PGA is formed by the bypass (Schwender *et al.*, 2004, 2006) (Table 1). Beginning with a light intensity of 50 μmol m⁻² s⁻¹, the relative flux through Rubisco rises with light intensity (Schwender *et al.*,

2004), strongly suggesting a strict light dependency of the process (Schwender *et al.*, 2004; Hay and Schwender, 2011b). In developing *Arabidopsis* embryos, the Rubisco bypass appears to be even more active (Lonien and Schwender, 2009) (Table 1). For soybean embryos cultured at lower light levels than in the case of rapeseed or *Arabidopsis*, a smaller contribution of the bypass was found (Table 1) (Allen *et al.*, 2009b). In the various flux studies, quantification of the Rubisco bypass should be realistic since embryos cultures were kept under light levels that were intended to mimic the intensities received by embryos growing within a seed coat *in planta*. Altogether, the contribution of the Rubisco bypass to glycolytic catabolism of sugars in developing oilseeds appears to be substantial. With regards to the apparent light dependency of the Rubisco bypass (Schwender *et al.*, 2004), it is unclear why Rubisco seems to be present in non-green oilseeds that lack photosynthetic potential. In developing endosperm of castor, Rubisco enzyme activity has been found to be sufficient to support fatty acid synthesis flux (Simcox *et al.*, 1977). While in higher plants the expression of genes encoding for the Rubisco small subunit (*RbcS*) is strongly light regulated and often undetectable in non-green tissues (Dean *et al.*, 1989), substantial expression of *RbcS* was found for developing seeds of *Sesamum indicum* (sesame) (Suh *et al.*, 2003), in developing embryos of *Tropaeolum majus* (nasturtium) and in the oil-accumulating endosperm of *Ricinus communis* (castor) and *Euonymus alatus* (burning bush) (Troncoso-Ponce *et al.*, 2011). Could therefore the Rubisco bypass be operative in these non-green oilseeds? Simulations of a large-scale metabolic model of *Brassica napus* seed development (*bnas572*; Hay and Schwender, 2011a,b) can be used to further explore this question. For a published model configuration of heterotrophy (see Table 2, condition 'HO' in Hay and Schwender, 2011b) the overall conversion of substrate carbon to biomass can be calculated as 68.5% (carbon conversion efficiency, CCE; see also Table 2 in Hay and Schwender, 2011a). Flux through Rubisco is predicted to be inactive, i.e. any flux through the reaction would reduce overall inefficiency in terms of CCE. If, based on this simulated physiological condition, flux through Rubisco is constrained to a value of 0.1996 $\mu\text{mol h}^{-1}$ as obtained for photoheterotrophy (Supplement S13, mode 'PO'; Hay and Schwender, 2011b), a CCE of 67.8% is obtained, which is only 0.7% smaller than the 68.5% obtained for heterotrophy without Rubisco activity. This demonstrates that increasing Rubisco flux under heterotrophy in an oilseed might not be a substantial disadvantage in terms of carbon efficiency. Given apparent expression of the enzyme in non-green oilseeds like castor, the activity of the Rubisco bypass in non-green oilseeds certainly deserves further theoretical and experimental exploration.

Lower glycolysis provides pyruvate for FAS

Both upper glycolysis and Rubisco bypass merge at 3-PGA, which is further catabolized via reactions of lower glycolysis to yield phosphoenol pyruvate (PEP) and finally pyruvate,

the direct precursor of fatty acids in plastids. For the generation of pyruvate, three major pathways can be recognized: from plastidic PEP via plastidic pyruvate kinase (PK_p), from cytosolic PEP through a pathway involving PEP carboxylase, malate dehydrogenase, and plastidic malic enzyme (ME), or by uptake of pyruvate from the cytosol (Table 1). The route via PK_p has a dominant role in providing pyruvate: typically PK_p produces more than 75% of plastidic pyruvate, used for FAS (Table 1). Besides PK_p, flux studies recognized substantial contributions to pyruvate formation by ME (in soy and maize embryos, Table 1).

Major contributions to plastidic pyruvate apparently can also be made by transport of cytosolic pyruvate into the plastid (Table 1), but the existence of a reaction to transport pyruvate from cytosol to plastids was only considered in the *B. napus* and *Arabidopsis thaliana* flux models (Schwender *et al.*, 2006; Lonien and Schwender, 2009). In developing *B. napus* embryos, pyruvate transport across the inner plastid envelope has strong experimental support (Kang and Rawsthorne, 1996; Eastmond and Rawsthorne, 2000) and was therefore considered in the *Brassica* seed models and later in *Arabidopsis* (Lonien and Schwender, 2009). Due to lack of general molecular evidence for such a transporter in plants, it is unclear if this transport process is present in the other species reported in Table 1. Only recently the molecular identity of a pyruvate transporter was unravelled. In *Arabidopsis*, BASS2 (At2g26900) encodes for a protein that is localized to the plastid inner envelope and was characterized to have with Na⁺/pyruvate symporter activity (Furumoto *et al.*, 2011). *A. thaliana* BASS2 knock-out mutants appear to have reduced capacity for plastidic isoprenoid biosynthesis, which might be due to reduced supply of plastidic pyruvate (Furumoto *et al.*, 2011). Since *A. thaliana* microarray data (Schmid *et al.*, 2005) show expression of BASS2 in developing seeds, the transport modelled in *A. thaliana* developing seeds (Lonien and Schwender, 2009) is likely mediated by this protein. Yet, a seed phenotype for the BASS2 mutant has not yet been reported. In future flux studies the expression of a BASS2 homologue in embryos of other oilseed crop species (corn, soy) might be tested and the modelling of the transport in flux models be considered. The movement of pyruvate across the plastid envelope might be of further interest for metabolic engineering in particular since it might be an active transport. Initially orthologues of this transporter have been discovered in the context of C4 photosynthetic metabolism, where the Na⁺/pyruvate symport is proposed to be indirectly driven by a pH gradient generated via Na⁺/H⁺ antiport across the plastidic envelope (Furumoto *et al.*, 2011).

Cofactor supply for FAS

Both protein and lipid synthesis have substantial requirements of ATP (Schwender, 2008). Given flux rates of mitochondrial NADH producing reactions, the magnitude of potential ATP production via mitochondrial oxidative phosphorylation can be estimated and compared to

biosynthetic ATP demands, which can be inferred based on fluxes through the biosynthetic pathways (Table 1). Accordingly, in rapeseed embryos it was estimated that the oxidative reactions of the tricarboxylic acid (TCA) cycle provide about 20% of the biosynthetic ATP demand (Schwender *et al.*, 2006). No cyclic degradation of acetyl-CoA has been found and the TCA cycle appears to be mainly involved in anabolism, i.e. provision of cytosolic acetyl-CoA used for fatty acid elongation (Schwender *et al.*, 2006). For soybean, the TCA cycle activity is relatively higher and estimated to provide 80% of biosynthetic ATP demand (Allen *et al.*, 2009b), while for sunflower and maize embryos this number is about 100% and 200%, respectively (Table 1). It appears that catabolic TCA cycle activity is lower in photosynthetic embryos (rapeseed, soy) as compared to the non-photosynthetic (sunflower, maize), which parallels the common observation that mitochondrial respiration in leaves is inhibited in light (Atkin *et al.*, 2000).

In non-photosynthetic tissues, the oxidative pentose phosphate pathway (OPPP) is regarded as a major source of reductant (NADPH) for biosynthetic processes such as FAS (Neuhaus and Emes, 2000; Kruger and von Schaewen, 2003). After quantifying flux through OPPP and FAS, the NADPH production by the OPPP can be expressed relative to the NADPH demand in FAS (Table 1). For sunflower embryos, production and demand appear to match closely (Table 1). In case of *Brassica* and for one of the soy studies (Allen *et al.*, 2009b) the NADPH production by OPPP was found to be far below the biosynthetic requirements (Table 1). Since in both cases, photosynthetic capacity is present, a contribution of photosynthetic electron transport can be postulated to be a major additional source of NADPH. The study on maize embryos (Alonso *et al.*, 2010b) suggests that the OPPP and ME share the provision of NADPH in a 70:30 ratio (Table 1). The conversion of malate to pyruvate by NADP-ME with concomitant reduction of NADP provides both pyruvate and NADPH in a ratio of 1:1, which comes close to the requirement for the synthesis of long-chain fatty acids (e.g. 9:8 in the case of stearic acid). Earlier biochemical studies on leucoplasts isolated from castor endosperm had shown that malate can serve as a precursor that provides carbon and reductant at the same time to fatty acid synthesis. (Smith *et al.*, 1992; Eastmond *et al.*, 1997).

Comparing different flux states

Metabolic flux studies give a static picture of flux distribution under specific experimental conditions. Some flux studies on oilseeds assessed the effect of environmental or genetic perturbation by comparing different steady states, which in turn can lead to hypotheses about regulation of central metabolism in oilseeds.

Comparing *B. napus* embryos cultured with Gln and Ala as nitrogen sources to cultures growing with inorganic nitrogen demonstrated redirection of fluxes leading into and out of the TCA cycle (Junker *et al.*, 2007). In the presence of organic nitrogen, Gln is taken up and transformed to 2-oxoglutarate (OG) which enters the TCA

cycle; while under inorganic nitrogen OG has to be withdrawn from the TCA cycle to supply carbon precursor chains for the synthesis of Gln, Glu, Pro, and Arg. The adjustment between the two conditions appears to be made by significant reduction in mitochondrial NAD-ME and by significant increase in PEP carboxylase flux (Junker *et al.*, 2007).

Iyer *et al.* (2008) used ^{13}C -MFA to study the effect of temperature on protein and oil biosynthesis in developing soybean cotyledons. This was motivated by various reports that had indicated a significant response of developing soybean to temperature and the ultimate objective of understanding carbon regulation for improved protein and oil production (Iyer *et al.*, 2008). Their results suggest that the capacity for flux through certain components of central carbon metabolism can be influenced by temperature during early stages of embryo development *in planta*.

Mutations that severely affect seed oil accumulation can give insight into the regulation of central carbon metabolism. In *Arabidopsis*, a regulatory mutant wrinkled1 (*wril-1*; At3g54320) and a double mutant in two isoforms of plastidic pyruvate kinase ($\text{pkp}\beta_1\text{pkp}\alpha$; At5g52920 and At3g22960) were studied by ^{13}C -MFA of developing embryos (Lonien and Schwender, 2009). Both mutations are characterized by severely reduced seed oil content (Table 1) and accordingly for both mutants the flux of plastidic pyruvate into FAS was reduced. For both mutations, an increase of TCA cycle activity was found, very pronounced in *wril-1*. The effect on relative contributions to the synthesis of plastidic pyruvate differed between both mutants (Table 1). For the PK_p mutant there was no major change in relative fluxes. This means that the reduction in PK_p flux could not be compensated by the above-described import of cytosolic pyruvate or plastidic ME. However, in the *wril-1* mutant, the large reduction in PK_p flux was compensated in part by an increased import of cytosolic pyruvate and by plastidic ME (Table 1). It is unclear why this compensation is possible only for *wril-1* but not for $\text{pkp}\beta_1\text{pkp}\alpha$. To explain this difference one hypothesis can be brought forward considering that PEP, the substrate of PK, is known to be a strong allosteric inhibitor of ATP-dependent and pyrophosphate-dependent phosphofructokinase in plants (Plaxton and Podesta, 2006). In *wril-1*, a large reduction in flux through PK_p apparently is caused by decreased enzyme capacity at multiple steps along the glycolytic and lipid synthesis pathways (Lonien and Schwender, 2009). In $\text{pkp}\beta_1\text{pkp}\alpha$ it appears that a more severe and targeted reduction of PK_p has actually less effect on flux through the reaction (Lonien and Schwender, 2009), which might cause the concentration of PEP to rise, leading to a pronounced allosteric feedback inhibition of upper glycolysis, thus preventing compensatory flux via cytosolic glycolysis towards pyruvate.

Flux analysis beyond the metabolic steady state

Steady-state MFA is an extremely useful diagnostic tool set for determining the *in vivo* metabolic rates of cell tissues and simple organisms under metabolic steady-state conditions

where metabolic fluxes and the pools through which they flow are constant. Recent efforts have successfully expanded the applicability of isotopic MFA to systems in which metabolism is in steady state but not isotopic steady state and therefore the analysis of endpoint labelling patterns is impractical (Leighty and Antoniewicz, 2011; Young *et al.* 2011). In order to quantify the metabolic fluxes outside of these situations, it is necessary to look beyond the scope of MFA.

In plants there are many important metabolic processes that are inherently non-steady-state. The most common of these is the diurnal cycling of metabolism due to changes in light conditions. The interactions of the day and night metabolism of plant cells and the manner in which the cells transition between the two states is a naturally occurring and distinctly dynamic phenomenon of significant importance. This has been observed not only in carbon metabolism, but also in nitrogen and other nutrients (Tcherkez and Hodges, 2009; Gauthier *et al.*, 2010). Another type of non-steady-state metabolism of note is the response of plants under conditions of nutrient deprivation (Miller *et al.*, 2010). Nutrient deprivation is an important situation in agriculture (Schachtman and Shin, 2007). It is also employed in eliciting specific product production from plant and algal cell cultures of industrial relevance. A third class of non-steady-state metabolism meriting investigation is response to biotic or abiotic stresses. Stress responses are inherently non-steady-state but play a ubiquitous role in protecting plants from their environment (Bolwell *et al.*, 2002). In cases where product quality is tightly controlled, such as pharmaceutical protein production in plants, understanding these irregular but significant influences to metabolism is necessary.

The analysis of non-steady-state metabolism requires many of the same experimental and computational tools that are utilized in the analysis of steady-state metabolism in MFA. Isotopic labelling is necessary to estimate metabolic fluxes, although this must be done as a function of time and during transient changes it is also necessary to measure metabolite concentrations in order to capture the effects that changing pool size will have on the fluxes and labelling dynamics. Of additional utility is coupling the metabolic flux data with information from other 'omics' fields, such as transcriptomics and proteomics (Feng *et al.*, 2010; Matsuoka and Shimizu, 2010). A more complete understanding of the impacts that the different levels of regulation have becomes even more essential during transient periods because metabolites are expected to be at concentrations where small changes will have a significant impact, contrary to steady-state metabolism where most small changes in concentrations are controlled to limit their overall impact, thereby increasing the robustness of the steady-state system's stability (Gerosa and Sauer, 2011).

Ultimately, this information can be combined into a comprehensive, overarching kinetic model. Fully characterized kinetic models are capable of handling non-steady-state dynamics in metabolism and have been shown to properly predict the effects of metabolic perturbations and new steady states. Kinetic models have been used on multiple systems in

plants including photosynthesis (Tholen and Zhu, 2011), secondary metabolism (Boatright *et al.*, 2004; Rios-Estapa *et al.*, 2008; Colon *et al.*, 2010), and amino acid metabolism (Curien *et al.*, 2009) and have even sought to include compartmentation (Uys *et al.*, 2007). While models incorporating transient metabolic effects would be relatively small at first due to the difficulties in modelling plants' inherent complexity, the rapid advances which metabolic engineering has seen in the past two decades indicate that this level of modelling is well within reach.

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