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Metabolic flux analysis in plants: coping with complexity

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ABSTRACT

Theory and experience in metabolic engineering both show that metabolism operates at the network level. In plants, this complexity is compounded by a high degree of compartmentation and the synthesis of a very wide array of secondary metabolic products. A further challenge to understanding and predicting plant metabolic function is posed by our ignorance about the structure of metabolic networks even in well-studied systems. Metabolic flux analysis (MFA) provides tools to measure and model the functioning of metabolism, and is making significant contributions to coping with their complexity.

This review gives an overview of different MFA approaches, the measurements required to implement them and the information they yield. The application of MFA methods to plant systems is then illustrated by several examples from the recent literature. Next, the challenges that plant metabolism poses for MFA are discussed together with ways that these can be addressed. Lastly, new developments in MFA are described that can be expected to improve the range and reliability of plant MFA in the coming years.

Key-words: central metabolism; compartmentation; isotopic labelling; metabolic engineering; metabolic networks; optimal design; plant metabolism; plant systems biology; predictive modelling; regulation.

INTRODUCTION

Plant metabolic networks are substantially more complex than those of other organisms. This is because of several interlinked aspects of plant life: being sessile, ectothermic and autotrophic, and having vast chemical repertoires and a high degree of subcellular compartmentation. Therefore, it is not surprising that metabolic engineering (especially in primary metabolism) has had a low success rate with, for example, single gene alterations usually resulting in little of the desired change in composition, yield or growth. The relationship between phenotype and genotype is also inherently complex because the functioning of individual proteins or even pathways depends on the operational state of the larger metabolic network (Kruger & Ratcliffe 2008; Moreno-Sanchez *et al.* 2008). Genetic manipulation, followed by phenotypic – even 'omic' – analyses, is therefore also limited as an approach to understanding how plant metabolism works, and there is a clear need for tools to measure and model metabolic function (as distinct from metabolic components) at the network level.

Metabolic flux analysis (MFA) provides such tools. MFA quantifies the flow of material through metabolism, yielding flux maps and can aid engineering efforts by explaining phenotypes in detail. Experience in the metabolic engineering of improved productivity by bacteria, shows that MFA can make substantial contributions to biotechnology. Used in a cycle of genetic alteration followed by subsequent analysis, MFA has allowed bacterial strain improvement for industrial purposes (Kim, Kim & Lee 2008) by, for example, highlighting potentially wasteful metabolic processes (Petersen *et al.* 2000, 2001; Nielsen 2001; Koffas, Jung & Stephanopoulos 2003; Koffas & Stephanopoulos 2005). MFA has already yielded substantial new insights into the structure and function of plant metabolic networks and holds promise for guiding successful engineering for practical purposes in the coming years.

MFA approaches of interest here are those that focus on obtaining estimates and models of multiple fluxes through a metabolic network or more commonly, a sub-network, and include systems in steady state (a constant set of metabolic fluxes) and ones whose fluxes may be changing. MFA approaches can be divided into several categories that differ in the information required, the kind of model used and the type of information obtained and are outlined in Table 1. Whether the biological system can be evaluated in steady state, the (sub)network size and the level of detail with which reactions are described will determine which MFA approaches are best suited to each system. Flux analyses all begin with a reaction network description (set of equations) that stoichiometrically relate the substrates of each reaction to its products. Given that there are multiple routes through the network, the stoichiometric description represents the full range of feasible metabolic behaviours. Elementary mode analysis (EMA; Schuster, Dandekar & Fell 1999; Schuster, Fell & Dandekar 2000; Poolman, Fell & Raines 2003) and extreme pathway analysis (EPA; Schilling, Letscher & Palsson 2000) are structural methods that are used to explore this range and define the boundaries of feasible steady-state flux distributions.

At steady state, the range of flux patterns is finite but still very large. With estimates of input and output flux values

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Table 1. Methods of metabolic flux analysis

Method	Conditions and information required						Information yielded			Technique reference For overviews see: Stephanopoulos *et al.* (1998), Ratcliffe & Shachar-Hill (2006)	Recent plant applications For overviews see: Libourel & Shachar-Hill (2008); Rios-Estepa & Lange (2007), Poolman *et al.* (2004)
	Steady state conditions	Typical network size analysed	Stoichiometric matrix	Pool size measurements	Labelling measurements	Objective function	Net flux	Reversibility	Predictive		
Elementary mode analysis and extreme pathway analysis	Y	Full network	Y	N	N	N	Y	N	N	Schuster *et al.* (2000); Schilling *et al.* (2000)	Schwender *et al.* (2004a) Poolman *et al.* (2003) Rohwer & Botha (2001)
Flux balance analysis	Y	Full network	Y	N	N	Y	Y	N	Y	Varma & Palsson (1994)	Boyle & Morgan (2009) Grafahrend-Belau *et al.* (2009) Schwender (2008) Shastri & Morgan (2005)
Steady state isotopic labelling MFA	Y	50–100 reactions	Y	N	Y	N	Y	Y	N	Zupke & Stephanopoulos (1994) Schmidt, Carlsen, Nielsen, Villadsen (1997) Wiechert *et al.* (2001)	Allen *et al.* (2009) Williams *et al.* (2008) Alonso *et al.* (2007a) Junker *et al.* (2007)
Instationary state-MFA	Y/N	50–100 reactions	Y	Y	Y	N	Y	Y	Y	Noh *et al.* (2007) Antoniewicz *et al.* (2007) Zhao *et al.* (2008)	Shastri & Morgan (2007)
Kinetic/dynamic MFA	N	10–50 reactions	N*	Y	Usually	N	Y	Y	Y	Reviewed in Steuer (2007)	Matsuda *et al.* (2007) Heinzle *et al.* (2007) Uys *et al.* (2007)
Metabolic control analysis	N	5–20 reactions	N*	N	N	N	N	N	Y	Kacser *et al.* (1995) Heinrich & Rapoport (1974)	Moreno-Sanchez *et al.* (2008) Rohwer & Botha (2001)

*Although dynamic/kinetic metabolic flux analysis (MFA) and MCA do not explicitly use the stoichiometric matrix, the same information, in the form of network structure and reaction definitions is needed. (MCA is mathematically parallel to Biochemical Systems Theory, which uses a power-law description of kinetics for the reaction steps in a biological network; both representing methods of sensitivity analysis).
Definitions: metabolic steady state, when pool sizes and fluxes are constant; stoichiometric matrix, matrix containing the number of molecules of each metabolite made or consumed in each metabolic or transport reaction in the netwosrk; pool size, the concentration of an intracellular metabolite; objective function, a goal that the system is believed to maximize or minimize (usually growth rate) used in flux balance analysis (FBA) to predict the net fluxes through a system; net flux, overall flow of material from one metabolite to another (forward minus reverse fluxes); reversibility, the degree to which material flows in both directions through a reaction; predictive model: one that allows the calculation of fluxes under conditions different from those of the experiment(s) already performed; elementary mode analysis (EMA) and extreme pathway analysis (EPA), two ways to structurally analyse a network to obtain a subset of the infinite number of possible flux patterns through the network that is sufficient to describe the full range of possible patterns. Each elementary mode is a minimal set of enzymes that could operate together at metabolic steady state; elementary modes represent a superset of extreme pathways because non-decomposable modes in the interior (i.e. not on the boundary of the admissible flux region) are included in EMA; FBA, constraint-based approach that uses an objective function to restrict the range of possible flux patterns; steady-state isotopic labelling MFA, often called 'MFA' by its practitioners, an analysis that uses measurements of isotopic labelling made in a system at metabolic steady state after labelling patterns are no longer changing, together with direct measurements of uptake and output fluxes to generate a map of net and exchange fluxes; instationary state MFA, same as steady-state isotopic labelling MFA, except that labelling patterns are changing, requires differential equation description rather than linear equations for isotopomer balances, metabolic steady state is maintained; kinetic/dynamic MFA, analysis of metabolic fluxes in either steady state or not, using time-dependent measurements of pool sizes and (usually) isotopic labelling to define a predictive model and can be used for metabolic control analysis (MCA); MCA, quantifies the control/sensitivity of individual fluxes and concentrations throughout a pathway or network. MCA can be applied with or without a full kinetic analysis and is usually applied to individual pathways or small sub-networks.

and an objective function (i.e. an optimization that maximizes or minimizes some particular goal such as maximal biomass production), flux balance analysis (FBA; Varma & Palsson 1994) yields a set of net flux values from the feasible 'flux space'. The branching of networks creates more unknown fluxes than stoichiometric relationships, resulting in an under-determined system; the degree of under-determination is especially high in primary metabolism. The use of linear programming with an objective function is necessary to limit this range of solutions. Still, the result of FBA may be more than one equally optimal flux solution set, and energy balance analysis (EBA) and thermodynamics-based MFA (TMFA) are used to constrain FBA by imposing further thermodynamic considerations on the network. Together, they enforce free energy rules (e.g. the decrease in free energy through the network) and reduce the number of solutions obtained. In general, EMA, EPA and FBA approaches are often applied to the full metabolic network (~1000 reactions for a microbe). This is possible because only net fluxes are considered, no pool size or labelling measurements are needed for the metabolites, and because an acceptable outcome may entail multiple solutions. Experimentally based methods deal with sub-networks of different sizes (Table 1).

Incorporation of isotopic labelling data allows one to model the transition not only of metabolites, but also individual atoms through metabolism. Mapping the transition of atoms (almost always carbon) greatly enhances the information content for MFA. For steady-state isotopic labelling MFA, all fluxes are unvarying and labelling patterns in intermediates and products are allowed to reach stable values. Label distribution in the end products can then be used along with mass balances to determine fluxes throughout the network. Isotopic labelling-based steady-state MFA results in a mathematically over-determined system (for sub-networks of metabolism), with more information than flux parameters and allows both net and some exchange fluxes to be obtained without assuming an objective function. Fluxes are obtained by fitting their values in a stoichiometric model to the labelling data and uptake/efflux measurements through quadratic programming. For those tissues that can be maintained at or close to a metabolic steady state, this approach is attractive and indeed the term 'MFA' is currently often applied exclusively to steady-state analyses. Though steady-state MFA does not produce a predictive model, it is appealing because it yields flux maps without requiring measurements of metabolite pool sizes or the estimation of kinetic parameters, which are often difficult to obtain but which are required for dynamic (kinetic) MFA. Additionally, any set of reactions between branch points is combined into one step in steady-state MFA, which dramatically reduces the number of variables to be determined. In a kinetic/dynamic or unsteady-state approach, fluxes need not be constant (so metabolite pool sizes can change) and fluxes are established from time course measurements of pool sizes and labelling. The use of dynamic labelling experiments for single flux evaluations and for pathway elucidation is well established in plant biochemistry and has made enormous contributions, but is beyond the scope of this review. With dynamic MFA, a much larger number of independent parameters is used because individual enzymatic and transport steps are modelled, each involving multiple concentrations and rate constant values.

For steady-state MFA using labelling experiments, the system must be in a metabolic steady state long enough to reach isotopic steady state (a stable labelling pattern in metabolites). However, many plant tissues do not show steady-state metabolism and/or cannot be labelled to isotopic steady state under physiologically relevant conditions. In these cases, dynamic MFA is needed to quantify multiple fluxes though networks, and this approach has the further advantage of yielding models that can be used to predict the effects of genetic or other changes on metabolic fluxes and pool sizes (Morgan & Rhodes 2002; Poolman, Assmus & Fell 2004). Dynamic MFA is also important to identifying and analysing regulatory points within these pathways which is performed using metabolic control analysis (MCA; Rees & Hill 1994; Moreno-Sanchez *et al.* 2008). In MCA, the control of flux along pathways is quantitatively assigned to different enzymes (Heinrich & Rapoport 1974; Kacser & Burns 1981; Fell 1998). By revealing which enzymes maintain greatest control over flux, this method is a powerful predictive guide for metabolic engineering efforts. In Top Down Control Analysis (Hafner, Brown & Brand 1990), enzymatic reactions are grouped into blocks and the MCA analysis yields information on control of flux between, but not within those blocks.

Recent successes in MFA have been possible because of modern tools: nuclear magnetic resonance (NMR) and mass spectroscopies, the availability of a range of substrates positionally labelled with stable isotopes, and crucially, the development of modelling theory and computational methods. Detailed accounts of how different types of MFA analyses are performed using these tools are given in the literature: general overview (Stephanopoulos, Aristidou & Nielsen 1998), FBA (Schilling & Palsson 1998; Edwards, Covert & Palsson 2002), EMA (Schuster *et al.* 1999, 2000), EPA (Schilling *et al.* 2000), dynamic and steady-state MFA (Zupke & Stephanopoulos 1994; Schmidt *et al.* 1997; Wiechert *et al.* 2001; Ratcliffe & Shachar-Hill 2006; Rios-Estepa & Lange 2007; Steuer 2007) and MCA (Fell 1992; Kacser, Burns & Fell 1995). As shown in Fig. 1, the outcome of MFA may vary from a single set of flux values to a range of possible values in 'flux space'. The degree to which a range or absolute set of values is established is defined by the number and scope of constraints and experimental measurements available, as well as the network complexity and choice of MFA strategy. Different 'omics' data can establish a feasible range of values for fluxes, but for networks of any complexity, a substantial number of functional measurements are necessary to determine flux values accurately. From an MFA perspective, 'omics' data serve as input and constraints for model building and solving. From an 'omics' perspective, the fluxome is simply another level of system-wide description. From a Systems Biology perspective,

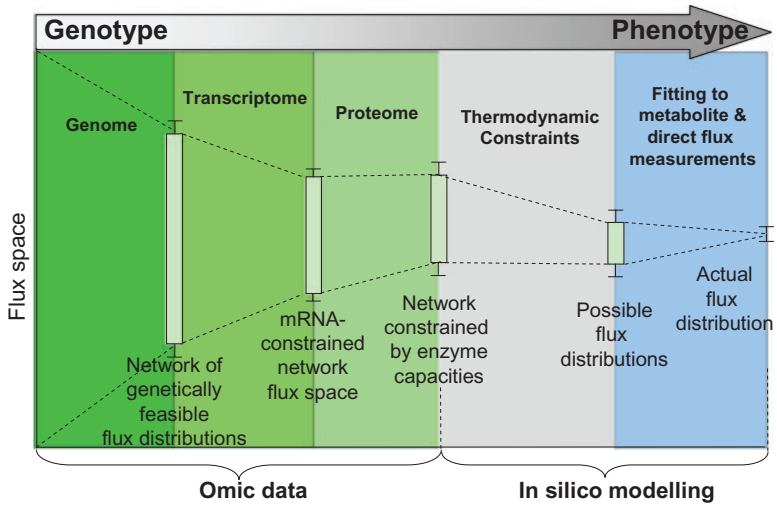

Figure 1. The relationship between the distribution of metabolic fluxes and the biological and physical constraints on the system and the measurements made on it. This is depicted in consecutive conceptual stages from left to right. Firstly, the range of all possible metabolic flux patterns (flux space) is successively restricted by the genome, transcriptome and proteome of the system. The genome defines the widest possible metabolic network for an organism, and the transcriptome and proteome further define the structure of the network and therefore all possible fluxes that could be conducted by the cells under consideration. In the next stage, physical and chemical constraints in the form of the stoichiometries and conservation of matter and energy must be taken into account, which constricts the flux space further. Finally, an estimate of the actual flux distribution (flux map) is derived using some combination of: (1) direct measurements of a subset of the fluxes (usually uptake, export and/or biomass accumulation rates); (2) measured metabolite levels and/or labelling patterns; and (3) assumptions about the functional target of the system (usually applied to microbial systems as the selection pressure to maximize growth rates in FBA analyses). At each stage, uncertainty about the possible flux distribution is introduced by uncertain gene function and measurement errors or omissions of different sorts. This uncertainty widens the range of possible flux distributions as represented by error bars. For some of the methods of MFA, such as FBA that are frequently mathematically under-determined (i.e. more unknown parameters than relationships and measurements), the use optimization by an objective function can produce more than one equally optimal flux distributions. These solutions are considered – preferably in the light of additional measurements – to determine which most accurately describes the biology of interest and which may be silent phenotypes. FBA, flux balance analyses; MFA, metabolic flux analysis.

MFA is qualitatively different from 'omics' in its attempt to describe function rather than structure and its aim of building predictive models. In this sense of quantitative modelling to make predictions, MFA is more like informatics, though in the latter the models are statistical and in MFA they are mechanistic. Others have recently provided surveys and guides to plant-based MFA (Ratcliffe & Shachar-Hill 2006; Rios-Estepa & Lange 2007; Schwender 2008; Sweetlove, Fell & Fernie 2008). Here, we discuss a small number of studies to illustrate the contributions of MFA to our understanding of the complexities of plant metabolism and then focus on the challenges facing MFA of plant systems, strategies to overcome them and new methods for future work.

MFA STUDIES ARE UNIQUELY INFORMATIVE ABOUT THE FUNCTIONING OF PLANT SYSTEMS

Plant tissues that have been studied using different MFA approaches, including cultured cell suspensions (Rontein *et al.* 2002; Baxter *et al.* 2007; Kruger *et al.* 2007a; Matsuda, Wakasa & Miyagawa 2007; Williams *et al.* 2008), microalgae (Yang, Hua & Shimizu 2002; Shastri & Morgan 2005, 2007; Boyle & Morgan 2009), developing seeds (Glawischnig *et al.* 2002; Schwender, Ohlrogge & Shachar-Hill 2003; Sriram *et al.* 2004; Ettenhuber *et al.* 2005b; Schwender, Shachar-Hill & Ohlrogge 2006; Spielbauer *et al.* 2006; Alonso *et al.* 2007a; Junker *et al.* 2007; Troufflard *et al.* 2007; Iyer *et al.* 2008; Allen, Ohlrogge & Shachar-Hill 2009; Grafahrend-Belau *et al.* 2009), stem (Rohwer & Botha 2001; Uys *et al.* 2007), root tips (Dieuaide-Noubhani *et al.* 1995; Alonso *et al.* 2005, 2007b,c), transformed root culture (Sriram, Fulton & Shanks 2007a), leaves (McNeil *et al.* 2000a,b; Poolman, Fell & Thomas 2000), flowers (Boatright *et al.* 2004; Orlova *et al.* 2006), tricomes (Rios-Estepa *et al.* 2008) and tubers (Matsuda *et al.* 2003, 2005; Heinzle *et al.* 2007). Several recent studies explore the potential for, and illustrate the challenges in, performing MFA in whole plants (Ettenhuber *et al.* 2005a; Huege *et al.* 2007; Romisch-Margl *et al.* 2007).

Seeds produce large amounts of storage reserves at steady rates during much of the filling period, and therefore meet the metabolic steady state requirement over a long enough labelling period to reach isotopic steady state. Together with their value as sources of protein, oil and carbohydrate, this makes developing seeds attractive for steady-state MFA. The work in developing rapeseed embryos has led to a number of insights into novel modes of operation of well-studied enzymes and pathways. MFA revealed that contrary to expectations, neither the oxidative pentose phosphate pathway (Schwender *et al.* 2003) nor

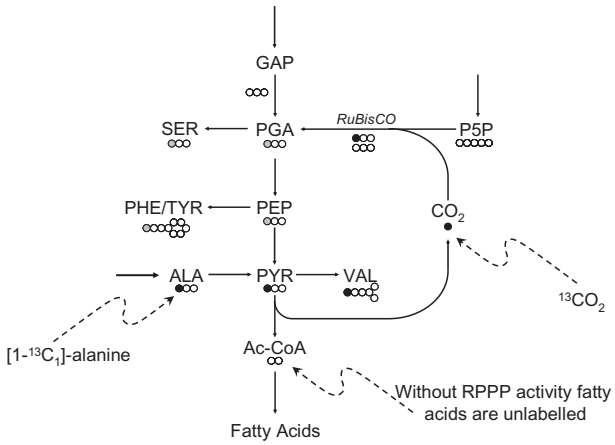

Figure 2. Re-assimilation of carbon and improvement of the carbon use efficiency in *Brassica napus* (after Schwender *et al.* 2004a). The provision of [1-^{13}C]-labelled alanine or $13CO_2$ resulted in labelling in readout metabolites of PGA and PEP as was supported through metabolic flux analysis. As the label did not end up in fatty acids, scrambling that occurs within reversible pentose phosphate pathways and Calvin Benson cycle activity was not present, supporting an uncommon operation of Rubisco in these oilseeds. Ac-CoA, Acetyl-Coenzyme A; ALA, Alanine; GAP, glyceraldehyde-3-phosphate; P5P, pentose phosphate; PEP, phosphoenol pyruvate; PGA, 3-phosphoglycerate; PHE/TYR phenylalanine/Tyrosine; PYR, pyruvate; RPPP, reductive pentose phosphate pathway (Calvin Benson Bassham pathway); SER, serine; VAL, Valine.

the tricarboxylic acid (TCA) cycle (Schwender *et al.* 2006; Junker *et al.* 2007) enzymes function in their conventional roles in heterotrophic tissues to fuel biosysnthesis. MFA was also able to explain the high carbon use efficiency during oil synthesis in developing rapeseeds by revealing that CO_2 is recycled through Rubisco without the operation of the reductive pentose phosphate pathway (Schwender *et al.* 2004a).

This latter discovery illustrates the ability of label-assisted steady-state MFA to reveal new functional aspects of complex metabolic networks. During fatty acid synthesis from carbohydrate, a third of the carbon is released as carbon dioxide [one CO_2 for each acetyl-coenzyme A (CoA) produced by pyruvate dehydrogenase]. In Schwender *et al.* (2004a), the rate of CO_2 evolution by brassica embryos developing in culture was found to be lower than expected from the lipid synthesis rates. Furthermore, it was not possible to quantitatively explain the results of ^{13}C labelling experiments through the action of known metabolic pathways. Alanine labelled with ^{13}C provided to embryos resulted in labelling in the first carbon position of phosphoglycerate (PGA) which was detected in aromatic amino acids (Fig. 2). This was explained by the successive actions of alanine aminotransferase and pyruvate dehydrogenase yielding $^{13}CO_2$ which is assimilated by Rubisco to produce labelled PGA. The lack of transfer of this label into fatty acids in this experiment, or when $^{13}CO_2$ was provided, showed that Rubisco was operating outside its usual context of the reductive pentose phosphate pathway. Steady-state MFA with other labelled carbon sources supported this result, was able to account for the labelling patterns observed and the high carbon use efficiency of these seeds, and was consistent with the finding that Rubisco exists in its active form in green oilseeds (Ruuska, Schwender & Ohlrogge 2004). Analysis of the capabilities of the network using EMA is another powerful tool for understanding patterns of flux through complex networks (Table 1), and in this case showed that the potential for this hitherto unrecognized role for Rubisco is inherent in the network structure (Schwender *et al* 2004a). Recent steady-state MFA work in soybean also supports a role for Rubisco in developing green seeds (though less than in brassica), despite the low levels of light reaching them (Allen *et al.* 2009).

Up to 45% of fatty acids in *Brassica* (cv. Reston) seed oil have very long chains with elongation taking place in the cytosol (Ohlrogge, Pollard & Stumpf 1978; Whitfield, Murphy & Hills 1993; Bao, Pollard & Ohlrogge 1998). Acetyl-CoA is not transferred across membranes (Liedvogel & Stumpf 1982), but it can be provided to the cytosol through the export of citrate from mitochondria to the cytosol, and subsequent cleavage by ATP-citrate lyase. To describe the fluxes involved, experiments utilizing ^{13}C-labelled substrates were performed and labelling in amino and organic acids as wells as lipids and carbohydrates were measured. Initial inspection of the results seemed consistent with the operation of known metabolic processes, but modelling of the data supported an atypical operation of the tricarboxylic acid cycle enzymes. In particular, isocitrate dehydrogenase, believed to be an irreversible step in plant metabolism, is apparently highly reversible and even carries net backwards flux, resulting in the carboxylation of oxoglutarate. The reversibility of this step was confirmed in experiments using fully labelled glutamine, which yielded citrate containing the intact five-carbon skeleton of the substrate. Modelling of labelling data in this steady-state MFA further revealed that the pattern of net flow through mitochondrial enzymes involves a substantial flow through malic enzyme, and that citrate formed by the acetylation of oxaloacetate (citrate synthase) is exported rather than being isomerized and then oxidized as in the conventional TCA cycle. The same reversibility of isocitrate dehydrogenase has since been observed in soybean (Allen *et al.* 2009), although in the seeds of this plant, the net flux through isocitrate dehydrogenase (ICITDH) is decarboxylating and the TCA cycle operates with a conventional forward flux.

Steady-state MFA can also improve our understanding of plant metabolism by characterizing the potentially complex changes in fluxes induced by environmental and developmental changes (Rontein *et al.* 2002; Alonso *et al.* 2007b; Junker *et al.* 2007; Williams *et al.* 2008). A recent investigation of cultured *Arabidopsis* cells (Williams *et al.* 2008) highlights the plasticity of metabolism to perturbations in oxygen level. Electron transport directly couples TCA cycle activity with the oxygen consumption of the cell and therefore changes in oxygen level drastically influence primary metabolism (Davies, Grego & Kenworth 1974). In the

Williams *et al.* study, the balance of respiratory and biosynthetic fluxes through primary metabolism was largely unchanged, though absolute fluxes through the network did increase with higher oxygenation rates. This work implies that cellular metabolism was oxygen-limited rather than carbon-limited.

To date, only a handful of MFA studies have examined the effect of genotype: Alonso *et al.* (2007b) explored the role of sucrose synthase in starch and cell wall production in root tips of mutant and wild-type maize; Spielbauer *et al.* (2006) compared flux patterns in maize kernels of 18 genotypes; and McNeil *et al.* (2001) used dynamic MFA to analyse the biosynthesis of glycine betaine in transgenic tobacco. Applications of MFA to transgenics can be expected to grow given the frequent finding of unexpected metabolic changes in genetically altered plants and the development of plant MFA over recent years (Kruger & Ratcliffe 2007, 2008). Additional factors making the growth of MFA in mutant and transgenic plants likely are the establishment of flux maps for model tissues, advances in MFA methods generally and for plants in particular, and the potential to use cell suspensions to compare genetically different lines (Rontein *et al.* 2002; Baxter *et al.* 2007; Kruger *et al.* 2007; Williams *et al.* 2008).

Work towards applying MFA to plants under physiologically normal conditions has taken several directions in recent years. For autotrophic cells or tissues, CO_2 is the natural substrate; however, labelling with CO_2 to isotopic steady state results in uniform and uninformative labelling, thus non-steady-state experiments and analyses are required. One approach to this challenge is to transiently label all internal pools with CO_2 and perform metabolic phenotyping of the subsequent label dilution in whole plants (Huege *et al.* 2007; Romisch-Margl *et al.* 2007). Though attractive from a physiological perspective, the mixed metabolism across plant tissues makes it unclear if this approach can lead to flux maps. In other labelling studies, the temperature (Iyer *et al.* 2008) or light (Ettenhuber *et al.* 2005a) was varied diurnally during labelling so as to make conditions more representative of normal plant growth than in studies in which constant conditions are maintained. The resulting end-point labelling patterns reflect metabolism that was changing dynamically during the experiment, and this temporal convolution makes the results of such studies more challenging to evaluate. Alternatively, the analysis of labelling over shorter time periods in dynamic labelling experiments can be performed more rigorously, and this has been applied to a range of more or less intact plant tissues to study secondary metabolism (discussed below). Recent developments at the interface of steady-state and dynamic MFA (Noh, Wahl & Wiechert 2006; Antoniewicz *et al.* 2007; Noh *et al.* 2007; Wahl, Noh & Wiechert 2008; Zhao *et al.* 2008) methods in microorganisms should increase the range of options for MFA of plant central metabolism, and are beginning to break down the conceptual divisions between dynamic and steady-state MFA approaches. In a recent study, Shastri & Morgan (2007) analysed transient labelling with CO_2 to develop dynamic flux analysis methods for unicellular microalgae at metabolic but not isotopic steady state.

Dynamic MFA studies are based on time course measurements of the concentrations and/or labelling levels of metabolic intermediates, and the kinetic properties of enzymes and transporters in the network (usually measured *in vitro*). Dynamic MFA has been successfully applied to study plant metabolism (reviewed in Morgan & Rhodes 2002; Poolman *et al.* 2004; Ratcliffe & Shachar-Hill 2006; Rios-Estepa & Lange 2007; Libourel & Shachar-Hill 2008). The availability of dynamic modelling software and analytical spectroscopies for measuring metabolite levels and stable isotopic labelling have, together, expanded the size of networks that can be studied by dynamic MFA, although steady-state MFA or FBA are still more often used for analysing larger central metabolic sub-networks. In plants, dynamic MFA methods have been more extensively applied to the study of photosynthesis (e.g. Zhu, de Sturler & Long 2007) and secondary metabolism (e.g. Rios-Estepa *et al.* 2008), and can result in intriguing predictions about potential metabolic engineering opportunities for improved plant performance (Rohwer & Botha 2001; Uys *et al.* 2007). For example, Uys and co-workers examined the accumulation of sucrose in maturing sugarcane stem. Using kinetic modelling and MCA, these authors were able to establish the reactions with the greatest control over futile cycles associated with sucrose accumulation in the vacuole.

This use of kinetic modelling with MCA provides a means for predicting the impact of changes to specific enzymes, but dynamic modelling in this way does not have to include knowledge of kinetic parameters. Indeed, power law descriptions of biochemical processes have resulted in a comprehensive mathematical framework known as Biochemical Systems Theory (Savageau 1998 and references therein). Heinzle *et al*. (2007) modelled the metabolism of phenylpropanoid metabolism, using data on potato discs labelled with phenylalanine (Matsuda *et al.* 2003, 2005) using a power-law kinetics representation. They were able to establish pathway fluxes and control coefficients that provide rationale for the self-protection of plants by comparative flux control analysis between the control and cells that received an elicitor (Fig. 3).

A recent study of monoterpenoid metabolism in peppermint illustrates how metabolic regulatory features can be discovered through the modelling of kinetic metabolite data with dynamic MFA (Rios-Estepa *et al.* 2008). Monoterpenoid metabolism is responsible for the biosynthesis of a range of plant secondary compounds and has been the target of substantial metabolic engineering efforts (Mahmoud & Croteau 2002). Rios-Estepa and co-workers developed a kinetic model of the metabolic sub-network using measurements of the levels of intermediates and products, estimating their concentrations in the secretory cells from microscopic analysis and using previous knowledge about the levels and kinetics of the enzymes involved. This kinetic model was used to analyse the effects of environmental stress (low light) on the accumulation of products and intermediates, which pointed to a hitherto

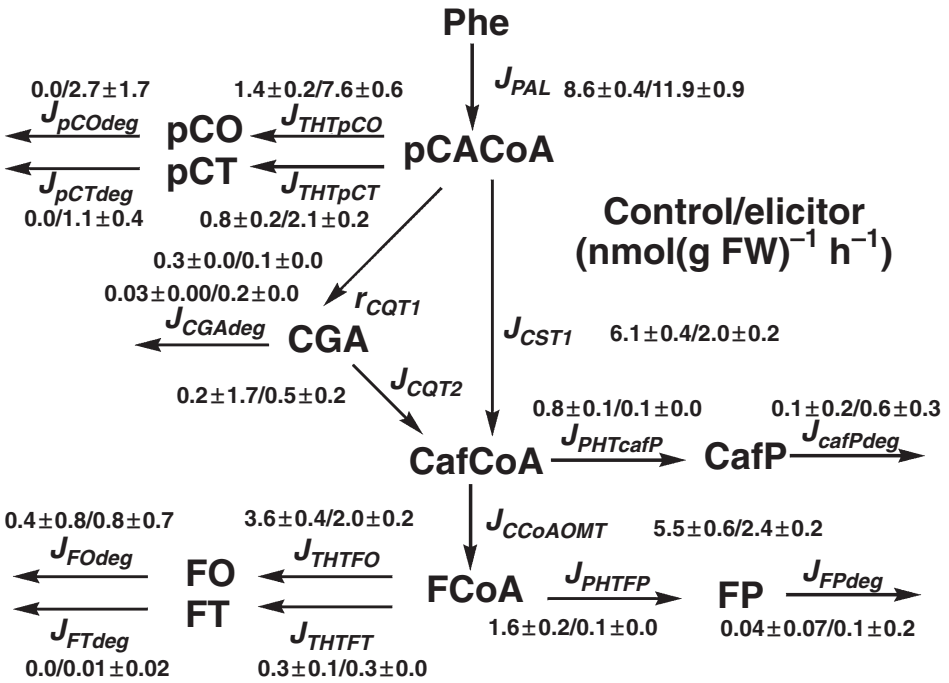

Figure 3. Flux map obtained by dynamic metabolic flux analysis for phenylpropanoid metabolism by Heinzle *et al.* (2007, with permission) for potato tuber tissue in the absence or presence of an elicitor of a chemical defence response. The dynamic model used to obtain the fluxes shown was based on power law rate equations and like other kinetic/dynamic models yields predictive insights into the regulation of metabolism.

unknown inhibitory effect of an intermediate (menthofuran) on a key branch point enzyme (pulegone reductase). This model-derived result was verified by *in vitro* assays on the enzyme. This and other studies serve to demonstrate the power of dynamic MFA to identify novel regulatory processes as part of plant metabolic engineering.

SUBCELLULAR COMPARTMENTATION

Subcellular compartmentation increases metabolic flexibility, specialization and regulation. It also presents challenges to metabolic analyses, with MFA being no exception. For MFA, compartmentation complicates the structure of the metabolic network, the localization and measurement of metabolite levels and the determination of metabolite labelling, which may differ for the same metabolite in different compartments (Fig. 4). In plants, the presence of large vacuoles and metabolically active plastids creates additional challenges compared with animal or some fungal systems. Failure to account properly for compartmentation can potentially lead to differing flux maps, models and conclusions, as was shown recently for the case of futile cycling

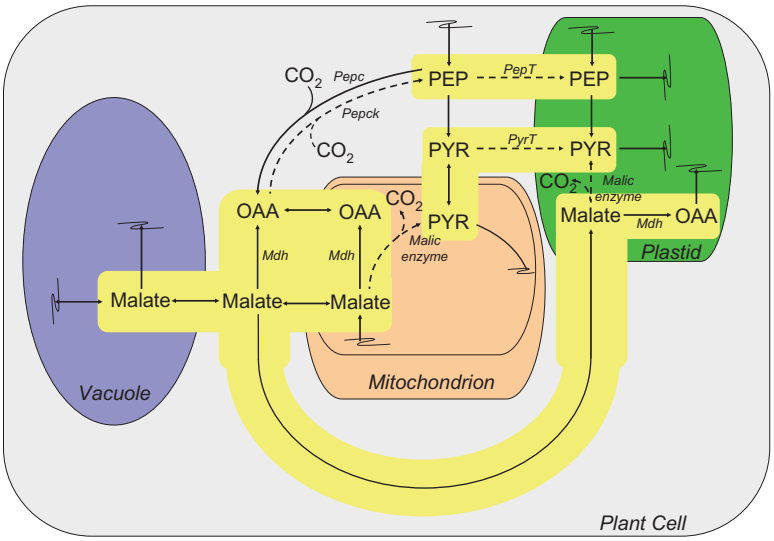

Figure 4. C3 and C4 carboxylic acid interconversions in plant central metabolism. The existence of multiple pools for each metabolite and poorly understood intercompartmental transport rates make flux analysis of this part of metabolism challenging. Frequently the C3 and C4 products are combined in flux analyses into a smaller number of pools as is shown by the three clusters in the yellow areas, to minimize the problems associated with flux identifiability in modelling. OAA, oxaloacetate; PEP, phosphoenol pyruvate; PYR, pyruvate.

associated with sucrose and glucose turnover by (Kruger, Le Lay & Ratcliffe 2007b). However, if compartmentation can be resolved, MFA studies have the potential to determine the relative contributions of different compartments to metabolic fluxes. Comparing the fluxes through parallel routes in the cytosol and plastid (like those connecting hexose- and triose-phosphates), is extremely difficult to do reliably by most approaches. MFA also has the potential to reveal the existence of multiple pools of the same metabolite as, for example, for choline in leaves (McNeil *et al.* 2001).

The structure of a metabolic network depends on the location of its enzymes and transporters, and the location of these proteins can be determined with varying degrees of confidence by microscopic immuno-histochemistry and fluorescence tagging, by organelle fractionation and proteomics, and by targeting predictions based on sequence. Incomplete, inaccurate and uncertain information on network structure is unfortunately the norm in plant metabolism, and care should be taken to check key assumptions. Such testing can be computational – to see whether models based on different network structures can account equally well for observed data – and ideally also experimental, by seeking evidence on the location of important proteins. Metabolite concentrations are used in dynamic MFA, and determining them usually depends on dividing the total levels determined after extraction by the volume they occupy – requiring knowledge of their compartmentation. Metabolite labelling measurements are used in both dynamic and steady-state MFA, and these can be different for the same metabolite in different compartments.

Organelle purification methods

The need for compartmental information on proteins and metabolites can be partially met using organelle purification methods (Winter, Robinson & Heldt 1993; Weise, Weber & Sharkey 2004; Farre, Fernie & Willmitzer 2008). In non-aqueous fractionation, tissues are frozen quickly and lyophilized under conditions where the levels and localization of metabolites are as little perturbed as possible (Stitt *et al.* 1989). Because the separation of organelles is incomplete, estimates of compartmentalized metabolite levels by this approach rely on deconvolution techniques using known protein markers for different compartments (Riens *et al.* 1991). It is not clear how successful such a deconvolution strategy would be for determining unknown labelling differences between compartments, though only small amounts of pure organelle fractions would be necessary for label determination using sensitive mass spectrometric methods. Aqueous fractionation methods (Keech, Dizengremel & Gardestrom 2005) involving separation by density-gradient centrifugation results in a better separation of organelle fractions, and this is important for protein localization efforts, but is unlikely to preserve the location of metabolites other than less mobile end products.

Compartment-specific readout metabolites

An alternative strategy for resolving metabolite labelling in different compartments involves the use of reporter or read-out metabolites that are specific to subcellular locations. For example, acetyl-CoA is an important metabolic intermediate that plays different roles in several compartments and is not transported across membranes (Weaire & Kekwick 1975; Roughan, Holland & Slack 1979). As a precursor for lipid synthesis, acetyl-CoA for de novo fatty acid synthesis is produced by plastidic pyruvate dehydrogenase (Bao *et al.* 2000), while acetyl-CoA for fatty acid elongation is generated in the cytosol (Ohlrogge *et al.* 1978; Whitfield *et al.* 1993; Bao *et al.* 1998). Labelling in these pools can be determined by analysing fatty acids that are made in the plastid and elongated in the cytosol (Schwender & Ohlrogge 2002; Allen, Shachar-Hill & Ohlrogge 2007). Thus, the labelling measured in 16 and 18 carbon fatty acids represents the labelling of plastidic acetyl-CoA, whereas labelling in the terminal carbons of 20 carbon or longer fatty acids represents the labelling of cytosolic acetyl-CoA.

Distinct readout metabolites can also be used to distinguish the labelling of key sugar phosphate pools located in the plastid and cytosol. Labelling in starch represents the isotopic state of its precursors in the plastid and sucrose, protein glycans and cell walls are imprinted with the labelling patterns of the cytosolic carbohydrates from which they are made. Although labelling in sucrose can be measured directly, enzymatic or chemical breakdown is used to facilitate the analysis of polymer-associated carbohydrates. Thus, for example, acid hydrolysis of starch and protein glycans yields levulinic acid, where enzymatic hydrolysis yields glucose whose labelling can be analysed by NMR (Sriram *et al.* 2007b). A distinct benefit of this approach is the ability to examine positional enrichments and long range coupling between carbons that provides valuable information for flux analysis (Sriram *et al.* 2007b). Labelling in monomers of cell walls, starch and protein glycans can also be analysed using gas chromatography-mass spectrometry (GCMS) with or without prior reduction of the sugars to their alditol derivatives (Allen *et al.* 2007). Several studies have reported the resolution of fluxes between cytosol and plastid based on readout metabolites (e.g. Sriram *et al.* 2004; Alonso *et al.* 2007a), although in other studies it was concluded that there was insufficient distinguishing information to resolve these with any confidence (e.g. Schwender *et al.* 2003; Allen *et al.* 2009).

Non-destructive methods for metabolite analysis

In vivo NMR spectroscopy and imaging are non-destructive methods that can provide information on the levels and labelling of detected metabolites (Ratcliffe & Shachar-Hill 2001; Kockenberger *et al.* 2004). Although NMR is limited by sensitivity to reporting on the more abundant metabolites, in favourable cases, it can also contribute to analysing the distribution of those metabolites among subcellular

compartments, usually between the vacuole and the rest of the cell. A compound that is located in multiple intracellular environments may give distinct signals depending on whether the signal is sensitive to any differences in pH, viscosity or ionic composition between those compartments (Vogel, Lundberg & Bagh 1999; Ratcliffe, Roscher & Shachar-Hill 2001). The pH dependence of NMR signals is commonly exploited for phosphorylated compounds and organic acids (Stidham, Moreland & Siedow 1983; Gout *et al.* 1993), and compartmental information has also been obtained on amino acids (Aubert *et al.* 1998, 1999) and ammonium (Lee & Ratcliffe 1991). *In vivo* NMR spectroscopy has also been used to measure steady-state fluxes directly by magnetization transfer; this has been very informative about the turnover of phosphorylated compounds in heterotrophic plant tissues (reviewed in Ratcliffe & Shachar-Hill 2001). *In vivo* NMR can also be used to obtain high-quality time course measurements of labelling e.g. (Troufflard *et al.* 2007) which directly reflect fluxes.

Fluorescent protein reporters with fluorescence microscopy provide an additional non-destructive *in vivo* method (Lalonde, Ehrhardt & Frommer 2005; Okumoto, Takanaga & Frommer 2008) to measure metabolite levels in different compartments. Sensitive reporters have been developed for a number of metabolites (mainly sugars and amino acids), and they can be selectively targeted to several different intracellular compartments. These reporters should contribute to dynamic MFA by providing measurements of subcellular metabolite concentrations and their changes in response to perturbations (Okumoto *et al.* 2008).

METABOLIC BRANCH POINTS

The presence of branch points in a network creates multiple options for fluxes between different intermediates and this presents one of the largest challenges for MFA. In central metabolism, such branch points are the rule rather than the exception (Figs 2–4), making this one of the most challenging areas to study. Because of their structural significance, enzymes at such branch points are frequently important in regulation and have been the target of metabolic engineering as well as flux analyses that often represent sets of linear steps as combined single processes.

Pentose phosphate pathway

A longstanding challenge for MFA is the branch point at glucose-6-phosphate between the pentose phosphate pathways and glycolysis (Kruger & von Schaewen 2003). The pentose phosphate pathways are important to redox status, and to providing substrates for the synthesis of ribonucleotides, histidine and aromatic amino acids and shikimic acid products. For plants, the latter can represent large and variable fluxes because of the production of lignins and flavonoids. The availability of glucose labelled in different positions has been important for resolving fluxes at this branch point, first with the use of [1-^{13}C]-glucose and [6-^{13}C]-glucose (Willis, Williams & Schleich 1986; Kingsley-Hickman, Ross & Krick 1990), and later [2-^{13}C]- and [1,2-$^{13}C_2$]-glucose (Lee *et al.* 1998), which provide greater sensitivity for resolving the split of fluxes (Schwender, Ohlrogge & Shachar-Hill 2004b; Libourel, Gehan & Shachar-Hill 2007). However, fluxes through other branch points and reversible fluxes can obscure the labelling imprint of the glycolysis oxidative pentose phosphate pathway (OPPP) split. Additionally, ketolase and aldolase enzymes can act on multiple substrates, complicating label interpretation (Flanigan *et al.* 1993; Williams & MacLeod 2006). Thus, even in thorough investigations of simpler systems, uncertainty (confidence ranges) about the split ratio can be large (Dauner, Bailey & Sauer 2001; van Winden *et al.* 2005). For example, studies of the same microbe by different well-known MFA groups have reported ranges from 33 to 75% for the glycolysis/OPPP split ratio (Christensen & Nielsen 2000; Christensen, Thykaer & Nielsen 2000; van Gulik *et al.* 2000; van Winden *et al.* 2003; Kleijn *et al.* 2006), although genetic and growth conditions are likely to have contributed to this.

This analysis is further complicated in plants by the duplication of PPP and glycolytic enzymes in both cytosol and plastid (Nishimura & Beevers 1979; Schnarrenberger, Flechner & Martin 1995; Kruger & von Schaewen 2003; Caillau & Quick 2005), with fluxes between compartments via hexose, pentose or triose transporters (Eicks *et al.* 2002; Weber 2004). The presence of both oxidative and reductive PPP (the Calvin Benson Bassham cycle) enzymes in plastids – whose activities can result in labelling patterns that are hard to distinguish – is another issue that complicates MFA of green tissues. It turns out that in plant metabolism, the simultaneous operation of oxidative and reductive pentose phosphate pathways is strongly inhibited at multiple levels of regulation (Buchanan 1980; Buchanan & Luan 2005). However, light-dark changes and cellular heterogeneity in a tissue require the issue to be borne in mind, especially for photoheterotrophic tissues. Distinguishing between oxidative and reductive penstose phosphate pathway fluxes is aided in the context of MFA by measurements of net CO_2 evolution (Allen *et al.* 2009).

Three and four carbon carboxylic acid interconversions

The fluxes connecting PEP, pyruvate, oxaloacetate (OAA) and malate also present substantial challenges to metabolic engineering and flux analysis in plants (Fig. 4). Here again, compartmentation and duplication of enzymatic steps, multiple branch points and alternative routes that result in the same labelling patterns in the analytes, all contribute to the difficulty in resolving fluxes in this part of metabolism. Relatively few examples of compartmentalized flux analysis for this part of metabolism exist and those have been based upon labelling in amino acids that are known to be compartmentally distinct in other systems such as yeast (Gombert *et al.* 2001), but not necessarily in plants. Nevertheless, these reactions are crucial to anaplerosis, amino

acid metabolism and the direction of carbon into biosynthesis and catabolism, they also influence the energy and redox balances of the cell. Manipulation of anaplerotic fluxes has resulted in increased production of amino acids in microbes such as *Corynebacterium glutamicum* (Peters-Wendisch *et al.* 1998; Petersen *et al.* 2001) and in *Vicia*, the overexpression of phosphoenolpyruvate carboxylase (PEPC) resulted in increased partitioning of carbon to protein (Rolletschek *et al.* 2004; Radchuk *et al.* 2007). FBA (Table 1) has recently been used to show that where amino acid uptake is significant, an anaplerotic flux for protein biosynthesis is not required (Schwender 2008). Plant MFA studies have not been able to resolve all the fluxes through these branch points, but progress has been made using a combination of: analysing amino acid labelling as readout metabolites, measuring labelling in organic acids directly, making simplifying assumptions about the equilibration of labelling between different pools of four-carbon dicarboxylates and using multiple labelled substrates. Thus, plant MFA studies have been able to estimate, for example, fluxes into and out of the TCA cycle and fluxes that convert C3 to C4 carboxylic acids by CO_2 fixation and to analyse the contribution of malate decarboxylation reactions to mitochondrial pyruvate supply (Schwender *et al.* 2006) and plastidic fatty acid synthesis (Alonso *et al.* 2007a).

MFA-based conclusions about the type, quantitative contribution and location of fluxes through PPP and among the 3C and 4C organic acids should be received with attention to the rigor with which they were obtained. The reliability of such findings depends on the complexity of and depth of prior knowledge about the structure of the network under study, the number and quality of labelling and input/output measurements, the sensitivity of the substrates used (discussed in the following section), the care with which confidence intervals were evaluated, and the degree of independent testing to which the model and its results were subjected. Indeed, although methods and standards for plant MFA are still being refined, these criteria should be borne in mind when evaluating all MFA studies. We next consider ongoing technical developments in MFA that can improve the reliability and predictive ability of plant analyses.

Pathway-tailored isotope label design

The ability to distinguish between parallel pathways located in different organelles largely depends on the availability of compartment-specific readout metabolites. The *precision* with which fluxes can be estimated depends heavily on the sensitivity of label measurements to fluxes. Because of the many scrambling reactions in most metabolic networks, positional labelling of downstream metabolites tends to approach the average label content. This implies that the dynamic range for labelling intensities is reduced, which lowers the measurement sensitivity. Therefore, the proximity of measured metabolites to the fluxes of interest is important in determining precision. A distant relationship between label measurements and fluxes compounds uncertainties and in practice exchange fluxes relatively close to measurements are the only ones that can be well resolved. It follows that the quality of flux estimates is best if labelled substrates that enter metabolism at multiple points are used, and label measurements are made from all parts of the network.

Whether all fluxes in a pathway can be resolved *in principle* for a given set of measurements, is determined using structural identifiability analysis (van Winden *et al.* 2001; Isermann & Wiechert 2003; Chang, Suthers & Maranas 2008). Usually, multiple label measurements from multiple label experiments are necessary for each degree of freedom as single labelling experiments are unlikely to uniquely establish fluxes (Suthers *et al.* 2007). Once it has been established that the set of available measurements is sufficient to identify the entire flux map or a pathway of interest, the sensitivity of the label measurements for the flux estimates can be optimized by the choice of substrate label composition. Because the precision of a flux map is a quality of the whole system, an objective function must be formulated to represent overall precision. An optimal experimental design of substrate label is therefore in essence an optimization (fitting) problem that minimizes a chosen optimization criterion.

To accomplish this goal, the expected label measurements from using different mixtures of available labelled substrates and the sensitivity of the measurements to the fluxes are calculated. This sensitivity information is contained in the flux covariance matrix, and an optimal label design is chosen based on a quality of the covariance matrix expressed as a criterion. Optimal experimental design theory is well advanced and has described many such criteria, all suitable for specific aims (Pazman 1986; Pukelsheim 1993). Libourel *et al.* (2007) have shown that optimizing the choice of how much of each of the available and physiologically relevant labelled substrates to use in a steady-state MFA experiment can greatly increase the precision of flux measurements both for the network as a whole and for particular fluxes of interest.

The sensitivity of measurements for fluxes is not linear, which makes the optimal substrate mixture dependent on the actual flux values. As a consequence, the establishment of precise flux maps is an iterative process. Rough initial flux estimates can be used to design a labelled substrate mixture that is subsequently used for more precise flux measurements. Because the choice of labelled substrates yields a different distribution of sensitivities across the network, multiple labelling experiments can help improve the precision of flux estimates. This method can be especially helpful if parallel pathways need to be resolved without compartment-specific readout metabolites. Two or three label experiments have therefore been used in some studies to improve the quality of flux maps in plants (McNeil *et al.* 2000b; Schwender *et al.* 2004a, 2006; Alonso *et al.* 2007a; Allen *et al.* 2009). However, criterion-based optimal design for multiple experiments (Libourel *et al.* 2007) has yet to be applied.

HARNESSING INSIGHT FROM COMPLEX FLUX MODELS

A flux map is an emergent property of the underlying physical components: the enzymes, transporters and metabolites. A steady-state labelling-based metabolic flux map, however, is established without any knowledge of these underlying components beyond the network stoichiometry and yields a description of the fluxes without mechanistic detail. This means that such maps do not predict how metabolism will adapt to a change in the underlying components, such as a change in an enzyme activity. This inherited limitation of a descriptive model makes it non-trivial to use flux maps for hypothesis testing or *in silico* evaluation of metabolic mutants and transgenics. Hypothesis testing is important for the evaluation of our fundamental understanding of metabolism, and *in silico* evaluation of metabolic mutants will be key to rational engineering of transgenic plants (Libourel & Shachar-Hill 2008). Although detailed dynamic/kinetic models are inherently predictive, there have been important recent developments towards making steady-state MFA predictive.

Minimization of metabolic adjustment (MOMA) and regulatory on/off minimization (ROOM)

Two methods have been developed that aim to predict flux adaptation in response to a given change in a particular flux, such as in a knockout mutation. The first approach is based on MOMA. This is accomplished through minimization of the sum of the squared differences between the original and adjusted flux map. MOMA predictions are often in good agreement with experimental results, and its easy applicability makes MOMA an attractive tool for metabolic engineering (Segre, Vitkup & Church 2002). A description of MOMA is provided in Fig. 5. The second method, ROOM, is based on the minimization of the number of flux changes (Shlomi, Berkman & Ruppin 2005). The concept behind ROOM is based on the observation that gene expression dramatically alters immediately after a metabolic perturbation, but gradually returns to a state, close to the one before the perturbation. ROOM predictions are also in good agreement with experimental data for bacteria, and ROOM predictions outperform MOMA predictions in experiments where an adaptation period was included. In contrast to MOMA, ROOM usually finds multiple equivalent solutions, which makes the practical application of ROOM for metabolic engineering less straightforward, especially for the more complex networks of plant metabolism. ROOM, which is based on a biological observation, does provide insight into how metabolic networks are regulated.

FBA

FBA (see Table 1 and Introduction) is a largely theoretical alternative approach to deducing metabolic flux values within a network. FBA flux values are determined on the basis of reaction stoichiometries, measurements of

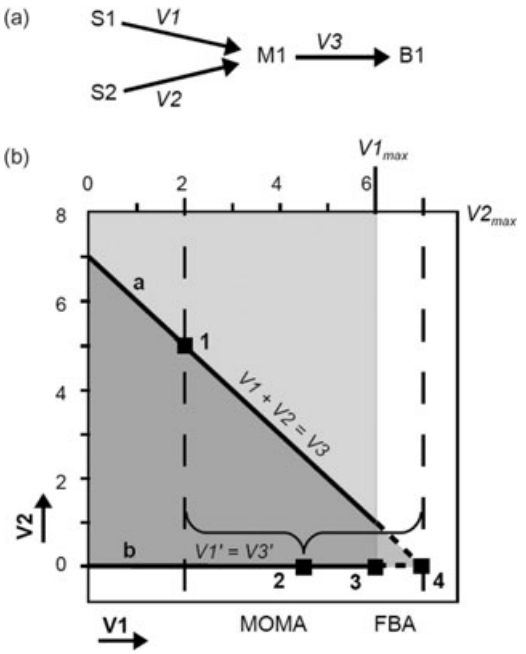

Figure 5. Example network with one internal metabolite, two substrates and one product (biomass). (a) Graphical representation of the flux space of network a (b). The light grey area shows the range of values that *V1* and *V2* may assume, the dark grey area limits the value for *V3*. Line **a** represents the equivalent FBA solution space given the objective function to maximize *V3*, and a *V3max* of 7. Line **b** represents the stoichiometrically feasible solution space following a knockout mutation in *V2*. Point **1** represents an example wild-type flux map and points **2** and **3** the mutant prediction using minimization of metabolic adjustment (MOMA) and flux balance analysis (FBA), respectively. Because *V1′* has now become limiting, selective pressure may eventually yield a flux map close to point **4**. Note that the MOMA prediction for *V1′* falls right between the original values *V1* and *V3* (vertical dashed lines).

substrate and biomass fluxes and inferred selective pressures (optimization of an objective function). By including the assumption that selective pressure has optimized metabolic efficiency, FBA is focused on the functionality of a metabolic network. Conversely, this approach makes it possible in principle, not only to infer internal metabolic fluxes, but also – when the flux map is known from experimental MFA – to investigate the selective pressures that shaped metabolism. FBA- and labelling-based flux maps can be compared for this purpose, and for *Brassica napus*, interesting differences between FBA and labelling-based flux maps were recently discussed (Schwender 2008).

FBA flux maps for wild type and mutant are established in the same way, with the implicit assumption that the network still operates to maximize the objective function after a perturbation. This suggests that either adaptive evolution occurs or redundancies exist in networks. Indeed, experimental flux values of mutant bacteria matched the FBA prediction better after an adaptive growth period and flux values of strains that had very recently been perturbed tended to more closely reflect the sub-optimal MOMA

prediction (Shlomi *et al.* 2005). Because FBA requires no label measurements to predict flux values, it can handle very large networks. In fact, genome-wide networks are becoming almost routine for FBA studies of prokaryotes (Reed & Palsson 2003; Feist *et al.* 2007). With the growing availability of completed plant genomes and the steady improvement of orthologue-based annotation, many more FBA flux maps including of plant systems can be expected in the future. Indeed, FBA was recently used to analyse metabolism in barley seeds (Grafahrend-Belau *et al.* 2009) and *Chlamydomonas* (Boyle & Morgan 2009). However, the incomplete annotation of plant genomes and imperfect compartmental targeting predictions still present considerable challenges to reconstructing metabolic networks from plant genomes (reviewed in Sweetlove *et al.* 2008).

Developments in predictive modelling

There are two significant limitations of the FBA method. First, it requires knowledge of the objective function, which for microbes is usually maximal growth rate, but is often unclear for plant tissues. This issue can be tackled by comparing different objective functions, although this has yet to be demonstrated in a plant system (Burgard & Maranas 2003). Secondly, the predicted flux maps obtained from FBA are typically not unique, but instead form a solution space (Fig. 1). Equivalent flux solutions have been compared with silent mutations, where change in gene expression does not affect the functionality of the organism (Reed & Palsson 2004). Although this property takes little away from the ability to aid network functionality, it does hamper the applicability of FBA for biotechnological purposes, where the outcome of a genetic intervention must be unequivocal to be useful.

FBA is also referred to as constraint-based flux analysis and it is the lack of sufficient constraints on a metabolic network that is the cause of the existence of a solution space instead of a singular flux solution. Identifying additional constraints to shrink the solution space remains a focus of FBA development (Bonarius, Schmid & Tramper 1997; Covert, Famili & Palsson 2003; Price, Reed & Palsson 2004). Current FBA flux models are augmented with metabolic and physiological information from the literature – bibliome (Duarte *et al.* 2007) – as well as with gene and protein expression and localization data derived from network reconstructions (Schilling, Edwards & Palsson 1999; Feist *et al.* 2009). Current FBA models also include regulatory information to restrict the actual flux map for a given tissue and/or condition (Covert & Palsson 2002).

A different line of attack was taken by groups that sought to include the intrinsic biophysical constraints that operate on metabolism. Work of this kind uses the functional group contributions of metabolites (Mavrovouniotis 1990, 1991; Jankowski *et al.* 2008), in combination with estimates for cellular pH and ionic strength to calculate their Gibbs free energy (Maskow & von Stockar 2005). With this information, the directionality of reactions can be estimated [Energy Balance Analysis (EBA); Beard, Liang & Qian 2002; Qian, Beard & Liang 2003; Beard *et al.* 2004] and thermodynamically feasible metabolite concentration ranges can be established. Likewise, the method thermodynamic-based FBA (TMFA) uses group contributions to trim the number of likely metabolic routes from substrate to product (Henry, Broadbelt & Hatzimanikatis 2007), although other algorithms directly include metabolite concentration ranges as part of the optimization criterion (Hoppe, Hoffmann & Holzhutter 2007). The relationship between free energy changes and flux reversibility was the topic of two recent theoretical studies. These studies addressed how enzyme mechanisms affect exchange fluxes as determined by labelling-based MFA (Beard *et al.* 2004; Wiechert 2007). The same relationship can be used to extend thermodynamic constraints to MOMA predictions (Libourel *et al.*, unpublished results).

CONCLUSIONS

Plants are indispensable sources of food, feed and fibre production and offer potentially invaluable resources for making renewable feedstocks and fuels. Manipulating plant metabolism to better serve these and future needs requires an improved understanding of the links between genotype and phenotype. Fluxes through metabolism directly report on cellular physiology at the network level and their analysis has received increasing attention in recent years. A range of different MFA methods has been applied to plant systems, yielding unique insights into the operation of plant metabolic networks. Importantly, MFA allows emergent properties of the network (e.g. overall efficiency) to be analysed, and offers the prospect of better linking functional phenotypes with genotype than has been possible to date.

The MFA toolset continues to grow, with developments in the microbial metabolic engineering community pointing the way towards predictive flux modelling and whole-genome network analyses. Importing both existing and emerging MFA methods for plant studies faces considerable hurdles because of the greater complexity of plant metabolic networks and our ignorance of them at the gene, protein and subcellular compartment levels. Structural and functional genomic investigations will continue to unveil the metabolic capability of plants, but the ability to estimate fluxes confidently and accurately will require measurements that are compartment-specific. As experimental techniques are enhanced to better discern compartmentalized behaviours, the resulting metabolic model can be expected to be more accurate and serve to better aid rational metabolic engineering efforts in plant and other complex systems.

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