Chapter 9

METABOLIC FLUX MAPS OF CENTRAL CARBON METABOLISM IN PLANT SYSTEMS

Isotope Labeling Analysis

V. V. Iyer[1], G. Sriram[2] and J. V. Shanks[1]
[1]*Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA;* [2]*Department of Human Genetics and Department of Chemical and Biomolecular Engineering, UCLA, Los Angeles, CA 90095, USA*

Abstract: Metabolic flux analysis (MFA) quantifies carbon flow in a biological system, which is an important characteristic reflective of physiology. Nodal rigidity of the metabolic network at branchpoints can be assessed from flux ratios to compare genetic and environmental variants and identify targets for potential genetic manipulations. MFA coupled with systems-wide tools such as transcriptomics and metabolomics have significant potential for building predictive models of plant metabolism. This chapter aims to explain the methodology behind MFA using carbon labeling experiments (CLE), nuclear magnetic resonance spectroscopy and a comprehensive mathematical framework (NMR2Flux) for a better understanding of central carbon metabolism in plants.

1 INTRODUCTION

Genetic engineering marked the advent of modifying specific enzymatic reactions using recombinant DNA technology. Early genetic engineering manipulations showed that a single gene transformation can result in unexpected changes in the metabolic pathways and phenotypic behavior and gave credence to a systems-level understanding of physiology. Consequently, the field of metabolic engineering emerged, which dealt with a systematic approach towards pathway modification to understand the underlying physiology (Stephanopoulos et al., 1998). The significance of metabolic engineering lay in the fact that the metabolic network was considered in its entirety as opposed to a single reaction.

B.J. Nikolau and E. Syrkin Wurtele (eds.), Concepts in Plant Metabolomics, 125–144.
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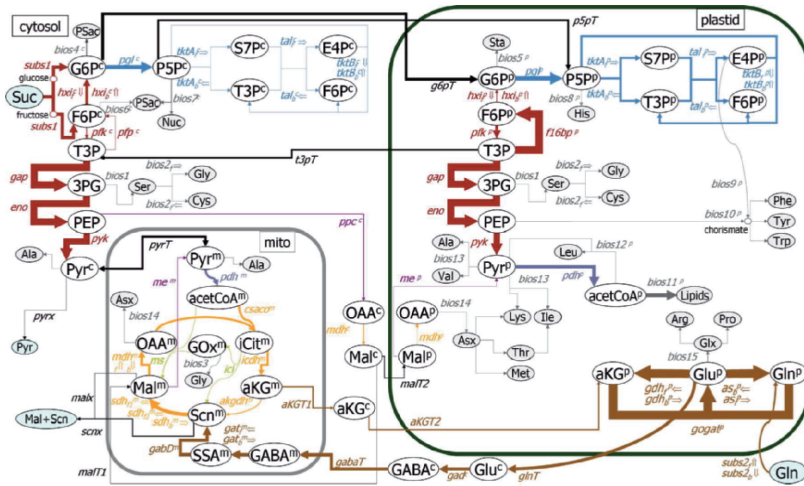


Figure 9-1. Metabolic network for central carbon metabolism in embryos of soybean (Glycine max). Parallel pathways for glycolysis and the pentose phosphate pathway exist in the cytoplasm and the plastid and communication between them occur through three transporters: glucose 6-phosphate (g6pT), pentose 5-phosphate (p5pT), and triose 3-phosphate (t3pT). The thickness of the arrows is directly proportional to the flux values. Reprinted from Sriram et al. (2004) with permission of the American Society of Plant Biologists.

The importance of metabolic fluxes as a fundamental determinant of cell physiology was promoted by metabolic engineering (Stephanopoulos, 1999). Metabolic flux is defined as the net rate of conversion of a precursor metabolite to a product in a metabolic pathway. The quantification of intracellular metabolite fluxes in a network of metabolic pathways is termed as metabolic flux analysis (MFA). In particular, MFA has been applied to network models of central carbon metabolism due to it importance in cellular physiology (Stephanopoulos, 1999). Central carbon MFA calculates steady-state intracellular fluxes using a stoichiometric model supplemented with extracellular measurements such as substrate intake and effluxes of metabolites. In larger network models for which additional measurements are required, constraints in the form of labeling data from Nuclear Magnetic Resonance (NMR) spectroscopy or Mass Spectroscopy (MS) can be applied (Section 2 of this chapter). The result of MFA is a metabolic flux map (Figure 9-1) which indicates the steady-state fluxes through various reactions of the metabolic pathway. Such metabolic flux maps can be effectively used for comparing flux differences in genetic or environmental variants. Subsequently, once the effect of a genetic or environmental manipulation is analyzed, further hypotheses are developed and tested (genetic modification followed by analysis) in an interactive cycle to further characterize the cellular physiology (Nielsen, 1998).

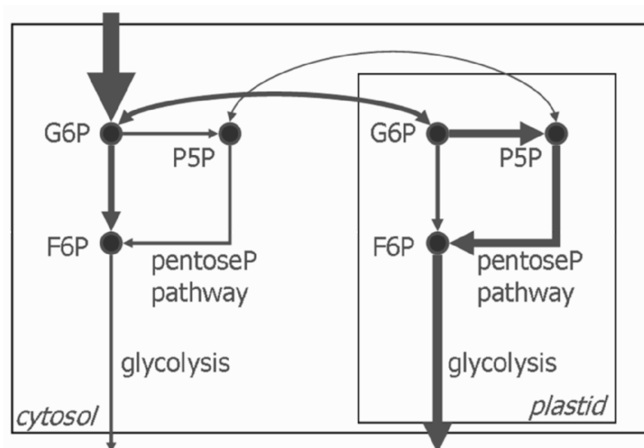


Figure 9-2. Parallel glycolytic and pentose phosphate pathways in cytosol and plastid. Transporters facilitate plastidic and cytosolic interactions.

Although the application of MFA in central carbon metabolism in microbes has been appreciable, unfortunately the same cannot be said in plants. Metabolic networks are more complex to analyze in plants than in microbes. One of the major factors contributing to the complexity of plant networks is compartmentation. In plants, the same reaction pathway may occur in more than one compartment as shown in Figure 9-2. Transporters facilitate the exchange of metabolites between compartments thus making intracellular transport processes important. Hence, the quantification of fluxes in parallel compartments becomes vital (Shanks, 2000). Additionally, higher plants are separated on various levels such as the tissue (roots, stems, and leaves) and cellular levels within a tissue. Furthermore, the topology of plant networks is often incomplete.

As a result of aforementioned complexity of plant metabolic networks, the few "flux" labeling studies in plants have focused on either the identification of metabolic network topology (Glawschnig et al., 2002; Krook et al., 1998; Schwender et al., 2004) or flux quantification using analytical or a highly simplified ^{13}C NMR constrained analysis (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Schwender et al., 2003). Plant systems biology has reemphasized the importance of fluxes (Girke et al., 2003; Stitt and Fernie, 2003; Sweetlove et al., 2003) in achieving the "in silico" plant (Minorsky, 2003). Thus, it has become more essential that application of MFA to different plant systems be promoted. Toward this goal, a comprehensive flux analysis tool for central carbon metabolism, NMR2Flux, was developed using recent mathematical advances from our research group (Sriram et al., 2004). This chapter aims at explaining the theoretical background and the methodology that NMR2Flux employs in the

evaluation of intracellular fluxes using the example of the developing soybean embryo.

2 METABOLIC FLUX ANALYSIS

Metabolic flux analysis (MFA) involves the quantification of intracellular steady-state fluxes in the cell, using metabolite balances and extracellular measurements.

2.1 Stoichiometric flux analysis

Metabolic flux analysis relies on the principle of conservation of mass: mass cannot be created or destroyed. Stoichiometric MFA is the most basic approach of metabolic flux analysis and requires details of the reaction stoichiometry. Consequently, mass balances around each intracellular metabolite are written to generate a system of linear equations (Stephanopoulos et al., 1998; Varma and Palsson, 1994). Thus in vector notation,

$$d\mathbf{X}/dt = \mathbf{r} - \mu\mathbf{X} \tag{1}$$

where, $\mathbf{X}$ represents the concentration of the metabolite under consideration; $\mathbf{r}$, the rate of formation of the metabolite and μ is the biomass growth rate. Assuming a pseudo-steady-state, where the rate of turnover of X (left-side of equation (1)) is smaller than the sum of the rate of metabolite formation and dilution due to cell growth (right-hand side of equation (1)), we have,

$$\mathbf{r} - \mu\mathbf{X} = 0 \tag{2}$$

The dilution due to biomass growth is generally small and the second term can be neglected and we have,

$$\mathbf{r} = \mathbf{G}^{T}.\ \mathbf{v} = 0 \tag{3}$$

where, $\mathbf{v}$ is the vector containing the fluxes and $\mathbf{G}$ is the stoichiometric matrix. If the network model has J reactions and K internal metabolites, the degrees of freedom F, is represented by,

$$F = J - K \tag{4}$$

Hence, to solve for the intracellular fluxes, some measurements such as substrate consumption, metabolite effluxes etc. have to be supplied. Thus, the measured and calculated fluxes can be partitioned into $\mathbf{v}_m$ and $\mathbf{v}_c$,

respectively. Correspondingly, the stoichiometric matrix can be partitioned into $\mathbf{G}_m$ and $\mathbf{G}_c$. Thus, knowing $\mathbf{v}_m$ and $\mathbf{G}$, we can calculate $\mathbf{v}_c$, the set of unmeasured intracellular fluxes. If the number of supplied measurements is same as F, it is an exactly determined system; if greater than F, an overdetermined system; and if less than F, an underdetermined system. The exactly determined and overdetermined systems will have a unique solution for the distribution of fluxes through the metabolic network. In addition, for an overdetermined system, the extra measurements can be used to check the validity of the metabolic network.

On the other hand, to solve an underdetermined system, cofactor balances (NADPH/NADH) may need to be supplemented as additional constraints (Varma and Palsson, 1994). However, the NADPH, NADH and ATP balances are not closed in reality due to futile cycles and incomplete pathway knowledge. Stoichiometric MFA also fails in certain cases of parallel pathways and metabolic cycles (Wiechert, 2001). It is hence essential to provide further information and also elucidate flux distribution at branchpoints. For larger networks with an increase in the number of reactions, flux analysis becomes more difficult as the number of measurements required correspondingly increases. Consequently, ^{13}C labeling experiments can be used to complement stoichiometric balancing and extracellular measurements, thereby providing a rigorous alternative to traditional flux analysis.

2.2 ^{13}C metabolic flux analysis

Carbon labeling experiments (CLE) involve feeding a combination of labeled (^{13}C or ^{14}C) substrates along with ^{12}C substrates such as glucose or sucrose to the biological system of interest. The label gets distributed throughout the network when the substrate is assimilated into metabolites. The labeling pattern of various metabolites depends on the network topology and the intracellular fluxes. The labeling patterns can be detected by Nuclear Magnetic Resonance (NMR) spectroscopy (Marx et al., 1996; Szyperski, 1995) or Mass Spectroscopy (MS) (Christensen and Nielsen, 1999) or a combination of the two (Klapa et al., 1999). The labeling data can be translated into flux information, using the concept of isotopomers as explained in Section 3.3 (Klapa et al., 1999; Schmidt et al., 1997).

Plant systems exemplify complex metabolic networks due to compartmentation issues, futile cycling, and anaplerotic reactions. Consequently, additional measurements are required in plant systems and the number of isotopomer balances increases, further increasing the computational burden. Due to the mathematical burden required for quantification of flux, most papers that have reported labeling studies in plants have focused on the identification of metabolic network topology

rather than flux quantification (Glawschnig et al., 2002; Krook et al., 1998). In an elegant example of the use of labeling for network topology, Schwender et al. demonstrated the use of labeling studies to identify a new pathway in *Brassica napus* embryos (Schwender et al., 2004). They characterized the role of Rubisco in the absence of Calvin cycle in improving the efficiency of carbon utilization during oil synthesis. Earlier studies of flux quantification in plants have used analytical or a highly-simplified ^{13}C NMR constrained analysis (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Schwender et al., 2003). These simplified analyses may lead to erroneous fluxes – a comprehensive analysis from an abundance of data is needed to verify the assumptions (Sauer, 2004). Recently, we have been able to execute comprehensive flux analysis of central carbon metabolism in plant tissues (Sriram et al., 2004). Section 3 of this chapter describes our analysis methodology in detail.

3 FLUX EVALUATION METHODOLOGY

Fluxes in a biological system can be quantified from isotopomer abundances, extracellular measurements, and biomass accumulation data coupled with a mathematical framework, using the evaluation methodology as explained below.

3.1 Experimental design

The selection of the type of labeled substrate, i.e., selective or uniformly labeled is a fundamental component of experimental design (Schmidt et al., 1999). In addition, it is essential that the relative extents of the labeled and unlabeled substrate be decided *a priori* to get adequate information from the NMR data (Stephanopoulos et al., 1998; Szyperski, 1995). In the case of selectively labeled substrates, a large percentage of labeling (as high as 90%) has to be used to obtain meaningful data (Park et al., 1999). On the other hand, when a mixture of uniformly labeled and unlabeled substrates is used, carbon bond-bond connectivities are traced as opposed to fractional enrichments. Hence, percentages of uniformly labeled substrate required are much lower (approximately 10%) for adequate NMR data (Szyperski, 1995).

Once the type of labeled substrate and their extents are decided, the cells are cultured with the mixture of labeled and unlabeled substrate. The experiment is carried out at metabolic (the rate of change of intracellular metabolite concentrations is much less than that of fluxes in and out of the metabolite) and isotopic (labeling patterns of the metabolites do not change with time) steady-states. Finally, the biomass from the plant tissue is extracted and broken down into its corresponding components. Depending on the

network topology and intracellular fluxes, different labeling patterns of the metabolites will be reflected from the biomass components (e.g., protein or starch sample), which can be detected using NMR or MS (Christensen and Nielsen, 1999; Klapa et al., 1999; Szyperski, 1995). The conversion of NMR data to fluxes using both type of substrates involve the concept of isotopomers as explained in section 3.3. Details of the experimental setup for labeling studies in developing soybean embryos and NMR sample preparation have been discussed in our recent work (Sriram et al., 2004).

3.2 NMR spectroscopy

NMR spectroscopy has proved to be an efficient analytical technique to gain significant insights into plant metabolism (Ratcliffe and Shachar-Hill, 2001). In an NMR measurement, the spin of the ^{13}C nucleus is detected and gives rise to a signal. The signal to noise ratio (S/N) in an NMR experiment depends directly on the concentration of the nuclei (C) and the number of scans (N_s).

$$\mathrm{S/N} \propto C*(N_s)^{0.5} \tag{5}$$

The accumulation time (T_a) for the signal depends on N_s and the pulse interval T_p (Shanks, 2000),

$$T_a = T_p * N_s \tag{6}$$

T_p is given by the following equation,

$$T_p = t_p + T_{acq} + T_{rd} \tag{7}$$

where, t_p is the length of radiofrequency pulse, T_{acq} is the acquisition time and T_{rd} is the relaxation delay (Shanks, 2000). Hence, for example, to double S/N, the number of scans needs to be increased four times. Also, T_a depends directly on N_s and will increase proportionately. Thus, it is essential to balance the parameters T_a, N_s and S/N to keep the NMR analysis cost at a reasonable limit without compromising on the S/N ratio. Using the developing soybean embryo system as an example, some of the key parameters for the NMR analysis have been discussed below and two dimensional (2D) experiments for detection of labeling patterns have been suggested.

In the soybean *in vitro* experiment, only 10% uniformly labeled sucrose was fed to the soybean cotyledons. Assuming this 10% labeling randomly distributes through the network, the probability that two adjacent atoms are

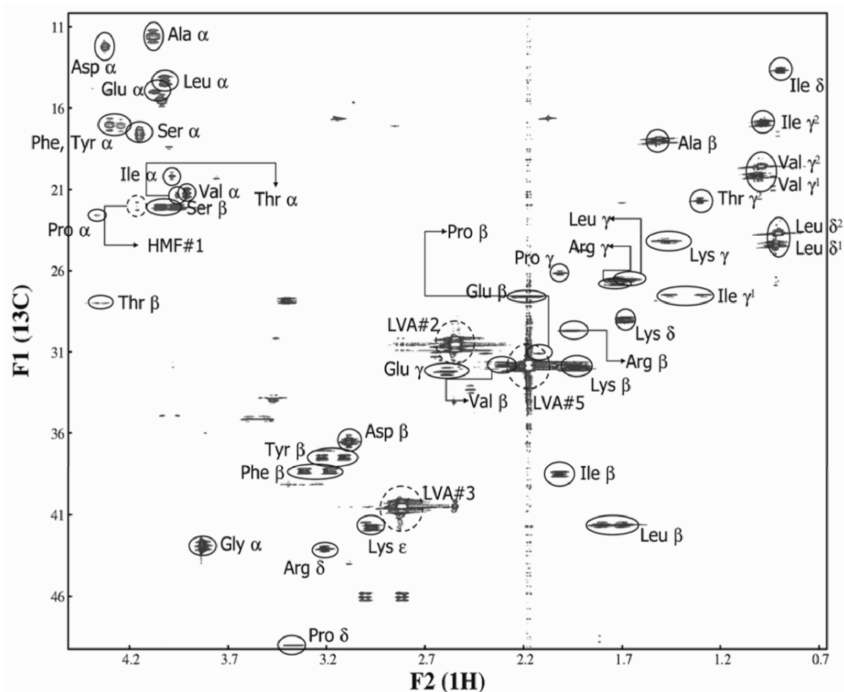


Figure 9-3. 2D [^{13}C, ^{1}H] HSQC spectrum of protein hydrolysate from soybean cotyledons cultured on sucrose (10% w/w U-^{13}C) and glutamine. Cross peaks represent carbon atoms of hydrolysate constituents (proteinogenic amino acids and hydrolysis products of sugars from glycosylated proteins – 5-hydroxymethyl furfural (HMF) and levulinic acid (LVA)). Reprinted from Sriram et al. (2004) with permission of the American Society of Plant Biologists.

labeled, is about 1% and the probability that two adjacent atoms originating from the same metabolite are labeled is 10% (Szyperski, 1995). From the specifications of the 500 MHz spectrometer, the minimum concentration for a 2D NMR analysis was determined to be 1 mM. The amino acid with the lowest concentration in the soybean protein hydrolysate was methionine (2 mol%). Hence, for a 20–22 hour [^{1}H, ^{13}C] Heteronuclear Single Quantum Correlation (HSQC) experiment, taking the aforementioned parameters into consideration, the minimum amount of soybean protein required for an adequate S/N ratio was 20 mg.

Two experiments, the HSQC and [^{1}H, ^{1}H] Total Correlation Spectroscopy (TOCSY) were performed on a Bruker Avance DRX 500 MHz spectrometer at 298 K on the soybean protein hydrolysate. For more details on the parameters of the NMR experiment, the reader is referred to our previous work (Sriram et al., 2004). The HSQC analysis determines the labeling pattern between the adjacent carbon atoms (Szyperski, 1998). Also, since we have unlabeled glutamine as a carbon source in addition to the

labeled sucrose, there is a dilution of ^{13}C in the system. The 2D TOCSY analysis detects the protons attached to ^{12}C and ^{13}C, thus providing the enrichment of each carbon atom of amino acids.

NMR spectra were acquired and processed using the Xwinnmr (Bruker) software. Peak assignments were verified using 2D [^{1}H, ^{1}H] TOCSY and 3D [^{13}C, ^{1}H, ^{1}H] TOCSY (Braunschweiler and Ernst, 1983) experiments on 100% labeled protein sample, with a pH of 1.0. Hence, the pH of the soybean sample was also adjusted to 1.0 to avoid a change in the chemical shift data caused by the environmental variation. The 2D HSQC spectrum of the hydrolyzed soybean protein with peak assignments of the carbon atoms of amino acids is shown in Figure 9-3. The HSQC and TOCSY spectra were analyzed using the free software NMRview (Johnson and Blevins, 1994). The deconvolution of the multiplet peaks was carried out using software based on the spectral processing proposed by van Winden et al. (van Winden et al., 2001). The isotopomer theory employed to convert the NMR data to intracellular fluxes using the software NMR2Flux (Sriram et al., 2004) is explained below.

3.3 Isotopomer theory

The concept of isotopomers arises from a combination of the terms isotope and isomers, which represent various labeling patterns of a given metabolite. For example, for a three carbon metabolite, there are $2^3 = 8$ isotopomers possible (Figure 9-4). Hence for a metabolite with n carbons, there are 2^n labeling patterns possible. As mentioned before, 2D HSQC detects the labeling patterns of adjacent carbon atoms. The peak fine structure obtained from the HSQC experiment shows multiplet patterns proportional to the isotopomer abundances (Figure 9-5). The labeling data is converted to flux data by comparing the experimental data with simulated

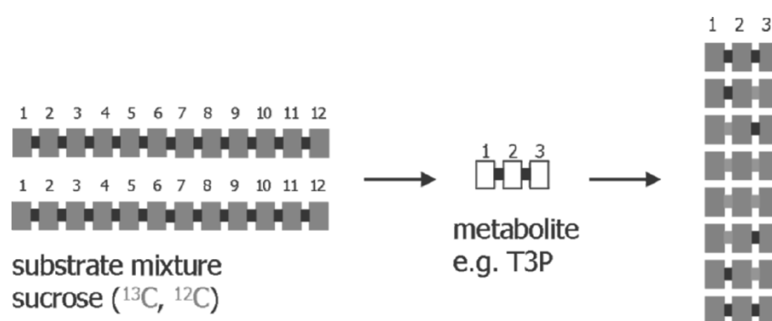


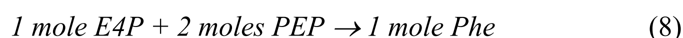
Figure 9-4. Isotopomers of a three carbon metabolite from a mixture of uniformly labeled and unlabeled sucrose.

isotopomer abundances generated using isotopomer mapping matrices (IMM). Assuming a set of intracellular fluxes, IMM uses the concept of isotopomer distribution vectors (IDV) and reaction stoichiometry to generate the simulated isotopomer abundances (Schmidt et al., 1997). The "best" set of intracellular fluxes satisfies the reaction stoichiometry and also shows the least mean square error between experimental and simulated isotopomer abundances. IMM are analogous to the Atom Mapping Matrices (AMM) used to calculate the TCA flux ratios in a hybridoma cell line (Zupke and Stephanopoulos, 1994).

3.4 Additional measurements

In addition to NMR labeling data, extracellular measurements such as substrate intake, product effluxes, and fluxes contributing to biomass accumulation are essential inputs to the model. The substrate intake and product effluxes can be measured by carrying out a high performance liquid chromatography (HPLC) of the culture media. It is also required that the biomass composition be known so that the carbon balance of the system is adequately accounted for. For example, in the soybean embryo culture, in addition to protein, lipids, and starch, a major constituent of the biomass were seed coat carbohydrates. The sugars that contributed to the carbohydrates were estimated from literature values (Mullin and Xu, 2000). The dry weight of the embryo and the fractions of protein, lipids, and starch were measured using standard protocols (Sriram et al., 2004). The fatty acid composition of the lipid fraction was estimated from literature (Dey and Harborne, 1997). When the molecular formula of the biomass is known, it can be used to close the carbon balance more efficiently.

The external fluxes contributing to protein were determined from the amino acid HPLC analysis, coupled with the precursor-amino acid stoichiometry (Szyperski, 1995). To elucidate, let us consider the synthesis of the amino acid phenylalanine (Phe) from erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP),



$$1 \text{ mole } E4P + 2 \text{ moles } PEP \rightarrow 1 \text{ mole } Phe \quad (8)$$

Thus, from protein data and HPLC analysis of the amino acids, the total number moles of Phe in the sample are known. Consequently, from equation (8) total moles of the precursor metabolite PEP required for synthesis of Phe can be calculated. Additionally, we know that tyrosine (Tyr) and tryptophan (Trp) are the other amino acids synthesized from PEP. Hence, the total external flux from PEP can be calculated from the sum of the moles of PEP required for synthesis of the corresponding three amino acids (Tyr, Trp, and Phe). Similar analysis can be carried out for remaining precursor metabolites

(for example, Pyr, OAA, P5P, etc.) associated with the synthesis of amino acids.

3.5 Metabolic reaction network

It is essential that a metabolic network mimicking the underlying physiology be proposed to convert the labeling data to intracellular fluxes. Figure 9-1 shows a metabolic network that describes sucrose metabolism in developing soybean embryos. The fluxes in a reaction network are stoichiometrically related to each other and can be expressed in terms of flux parameters. The selection of flux parameters is important to solve the metabolic network (Sriram et al., 2004). Some potential candidates for flux parameters are the independent reactions of the network (Stephanopoulos et al., 1998), reversibilities of key reactions and also scrambling extents of parallel reactions (Szyperski, 1995). Product effluxes, substrate consumption, and biosynthetic reactions are additional essential extracellular measurements required for accounting for complete carbon balance, thereby providing a better estimate of the intracellular fluxes.

Further, labeling data which give key information about branchpoints are additional important inputs. In the event that the labeling data does not satisfy the proposed reaction network, certain reactions may need to be added or removed from the proposed network to satisfy the labeling data. Also, sometimes the error in the experimental NMR data can translate to a very high probability distribution of the flux, leading to "identifiability" problems (Wiechert et al., 2001). For example, flux analysis of the

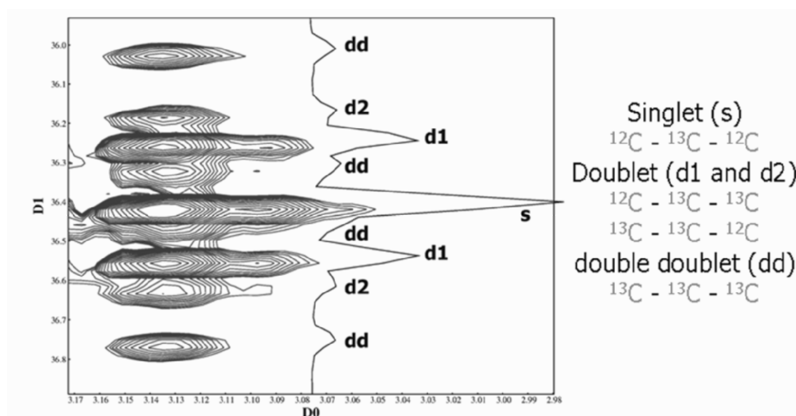


Figure 9-5. Peak fine structure of Aspβ. The multiplet intensities are proportional to the isotopomer abundances. Reprinted from Sriram et al. (2004) with permission of the American Society of Plant Biologists.

reversibilities of the transketolase and transaldolase reactions in the pentose phosphate pathway depend primarily on the labeling information obtained from the PEP family of the amino acids and histidine. If the NMR data is not sufficient to estimate these fluxes or if the error in the measurements is large, then the fluxes become “structurally unidentifiable”. The problem of structural identifiability can be solved by increasing the number of external measurements pertaining to that particular part of the metabolic network or providing low error NMR data. However, in some cases, the relationship between the NMR measurements and the fluxes are highly nonlinear. In such cases, the fluxes become “statistically unidentifiable” and a very low noise level can translate into large probability distributions of the corresponding fluxes. Thus, in the case of a statistically unidentifiable flux, the model cannot estimate the flux irrespective of redundant measurements pertaining to that flux. Such issues need to be studied in detail in the course of developing the experimental design of the biological system.

3.6 Mathematical modelling of the reaction network

The metabolite balances from the metabolic network coupled with the carbon skeleton rearrangements are fundamental in enumerating the isotopomers of the metabolites in the network. Both analytical approaches (Klapa et al., 1999; Park et al., 1999; Rontein et al., 2002) and numerical solutions (Schmidt et al., 1999; Wiechert and De Graaf, 1997a; Wiechert et al., 1999; Zupke and Stephanopoulos, 1994) have been used to solve isotopomer abundances for calculating intracellular fluxes. A generic software using the concept of isotopomer balancing for flux analysis is also available (Wiechert et al., 2001).

More recently, a generic tool NMR2Flux (Figure 9-6) has been developed in our lab by employing recent mathematical advances, that can be extended to complex plant systems (Sriram et al., 2004). The tool chooses an initial set of flux parameters (independent net fluxes, reversibilities, and scrambling extents) that are stoichiometrically feasible (Sriram et al., 2004; Stephanopoulos et al., 1998). From the feasible set of flux parameters, the remaining fluxes can be calculated. These fluxes are converted to isotopomer distributions using a recently developed efficient Boolean function mapping method (Figure 9-7), coupled with explicit solution methods (Wiechert and Wurzel, 2001). Boolean function mapping is a novel method of simulating isotopomer distributions. Carbon skeletal rearrangement steps are modeled as Boolean or arithmetic operations on the decimal representation of an isotopomer. The Boolean function mapping method is based upon the fact that all reactions in a metabolic network can be represented as occurring between two reactants (R_1, R_2) to give two

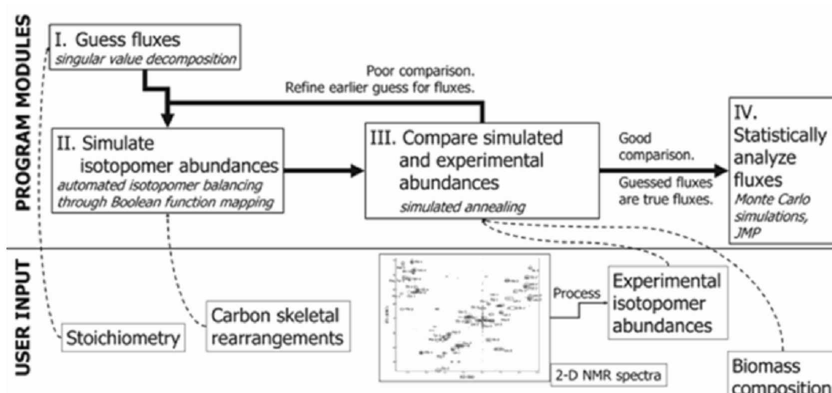


Figure 9-6. Flux evaluation methodology.

products (P_1, P_2), i.e., they can be represented as "bi–bi" reactions (Wiechert and Wurzel, 2001). Reactions steps in this schema can be described as a function of four different "moves": fragmentation, reversal, transposition, and condensation. The simulated and experimental (from NMR data) isotopomer abundances are compared and the error between them is minimized using a global optimization routine (employing simulated annealing).

The reduction in computation time achieved using the Boolean function mapping method allows additional statistical analysis of fluxes. The errors in the NMR input intensities are used to perform multiple Monte Carlo estimation of fluxes (Press et al., 1992); thereby generating probability distribution of the intracellular fluxes (Sriram et al., 2004). For further information on the tool and comprehensive explanation of the mathematical details, refer our recent work (Sriram et al., 2004).

Recently, a new concept, bondomer was introduced which can be used in case of single carbon substrate experiments only. Bondomers are similar to isotopomers except that the bonds instead of the carbon atoms are being followed (Sriram and Shanks, 2002; van Winden et al., 2002). Bondomers are molecules of the same metabolite, which have different bond integrities for different carbon–carbon bonds (Sriram and Shanks, 2004). Bondomer analysis is advantageous in plant tissue cultures that require only sucrose or glucose as a carbon source.

4 INSIGHTS FROM MFA INTO PLANT METABOLISM

Metabolic fluxes form the most important link in translating transcript and metabolite information to the existing physiology (Sauer, 2004). Flux

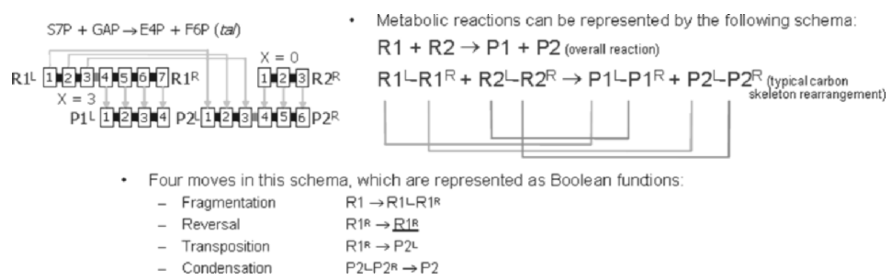


Figure 9-7. Boolean function mapping.

ratios can be used to analyze different nodes in the reaction network where there is a partitioning of the flux into multiple branches. The node under consideration can be either rigid or flexible. A node is said to be "flexible" if the ratio of the carbon flow into multiple reactions changes with a change in the incoming flux. In the case of flexible nodes, the distribution of the precursor metabolite can be modified inherently by the system without the need of any major genetic modification. For a "rigid" node, the ratio of the carbon flow into multiple branches remains the same irrespective of changes to the total flux coming into the node. Subsequently, genetic modifications will prove more effective in altering the metabolic flow in the desired direction in the case of rigid nodes (Stephanopoulos and Vallino, 1991). Network topology is less understood in plants as compared to microbes and the application of MFA can help elucidate plant reaction pathways. Examples of application of flux analysis in revealing network topology has been discussed below.

4.1 Segregation of pathways

As mentioned before, plant metabolism is compartmented, and features multiple copies of the same reaction of a pathway in different subcellular compartments. A classic example is the glycolysis/pentose phosphate pathway subnetwork, which exists in both the cytosol and the plastid in plant cells. In our flux analysis, it was therefore, critical to determine if these pathways were in equilibrium (i.e., they exchanged metabolites so rapidly that for all practical purposes, they could be considered one consolidated pathway) or were segregated (the fluxes through the cytosolic and plastidic pathways are significantly different, and the pathways did not rapidly exchange metabolites).

The segregation or equilibration of cytosolic and plastidic pathways can be ascertained by examining isotopomer abundances or ^{13}C enrichments of

metabolites synthesized in those compartments. Previously, Krook et al. have also reported significantly different ^{13}C enrichments of cytosolic and plastidic hexose pools in *Daucus carota* cells (Krook et al., 1998), which showed that cytosolic and plastidic pathways were segregated. However, hexose phosphate pools were found to be in equilibrium in tomato cells (Rontein et al., 2002) and *B. napus* embryos (Schwender et al., 2003), which showed that the cytosolic and plastidic pathways were in equilibrium.

In our work, on comparing the isotopomer abundances of the carbon atoms of glucosyl units from protein hydrolysate (which are derived from the cytosolic glucose-6-phosphate pool ($G6P^c$) and starch hydrolysate (which are derived from the plastidic glucose-6-phosphate pool, ($G6P^p$) from soybean embryos, we found that they were significantly different, and not in equilibrium (Sriram et al., 2004). In contrast, we did find that the isotopomer abundances of Ala α and Phe α in soybean embryos were similar. Phe α is obtained from the second carbon atom of PEP and is exclusively synthesized in the plastid; whereas Ala α is synthesized in all three compartments, cytosol, plastid, and mitochondrion, respectively from the second carbon atom of pyruvate. From biochemistry, we know that the three carbon atoms of PEP translate to the three carbon atoms of pyruvate without any rearrangement. This result indicated that the T3P pools in the plastid and cytosol were exchanging rapidly between the two compartments (Sriram et al., 2004).

To account for the above observations, we developed a compartmented model of the metabolic network, with separate glycolysis and pentose phosphate pathways in the cytosol and plastid. This model, when used in conjunction with NMR2Flux, was able to explain the observed isotopomer abundances well (see Sriram et al., 2004). Additionally, a fructose-1,6-bisphosphatase reaction had to be included in the plastid to fully account for the experimental isotopomer abundances (see below). Thus, labeling-based flux analysis is competent in segregating pathways in multiple compartments thereby accounting for complex compartmentation inherent in plant systems.

4.2 Identification of new pathways

In the case of the pyruvate family of amino acids the δ^1 carbon of Leu, the β carbon of alanine and γ^1 carbon of Val reflect the same carbon atom of pyruvate respectively (Szyperski, 1995). Hence, the multiplet intensities should be similar for these carbon atoms in the above-mentioned amino acids. However, our recent soybean work (Sriram et al., 2004) indicated that the δ^1 carbon of Leu shows a 30% difference from Ala and Val. This disparity in the isotopomer abundances of the Pyr family of amino acids has been observed in our labeling experiments on another plant system, *Catharanthus roseus* hairy roots as well (Sriram, G., and Shanks J. V.,

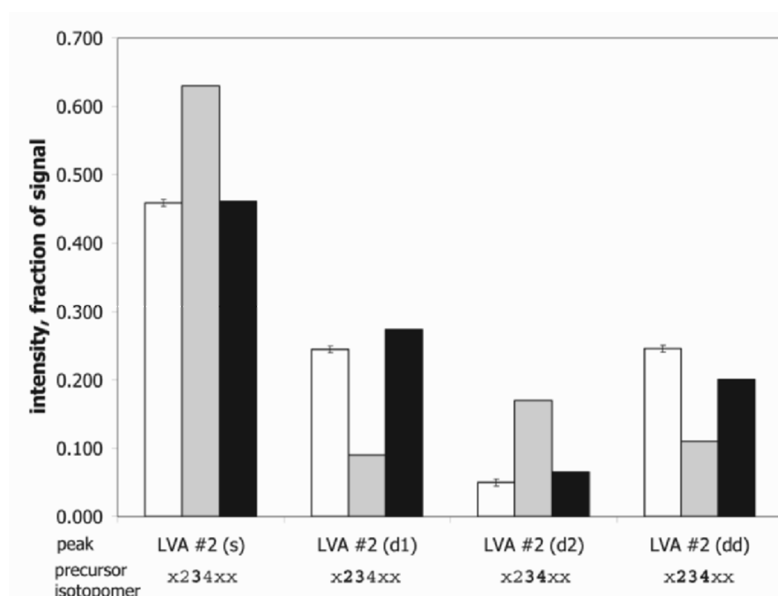


Figure 9-8. Identification of fructose-1, 6-bisphosphatase in the plastid. White bars are the experimental isotopomer abundances of levulinic acid atom 2 (LVA #2) from starch hydrolysate. This atom reflects the isotopomer abundances around carbon #3 of plastidic glucose-6-phosphate. Grey bars are simulated isotopomer abundances from a compartmented model with glycolysis and pentose phosphate pathways in the cytosol and plastid that included no plastidic fructose-1, 6-bisphosphatase. Black bars are from a similar compartmented model that included plastidic fructose-1, 6-bisphosphatase.

unpublished data). Its cause still remains a mystery and we believe that it may involve a currently unknown reaction or pathway related to Leu metabolism.

In addition, we identified the fructose-1,6-bisphosphatase (F16BP) reaction, which converts T3P to F6P, in the plastid. Although acompartmented model with separate glycolysis and pentose phosphate pathways in the cytosol and plastid accounted for most of the isotopomer abundances of the glucosyl units from protein and starch hydrolysates, we found that the isotopomer abundances around levulinic acid atom 2 (LVA #2) were not accounted for (compare white and grey bars in Figure 9-8). LVA #2 is derived from atom 3 of plastidic glucose-6-phosphate pool ($G6P^p$), and the above observation hinted that some pathway or reaction that significantly affects atom 3 of $G6P^p$ was absent in our initial compartmented model. This led us to hypothesize that a significant flux from T3P to F6P may be present in our system. Since such a reaction would cause two three-carbon T3P molecules to form a six-carbon F6P (and eventually a G6P) molecule in the plastid, it may result in isotopomer patterns different from those resulting

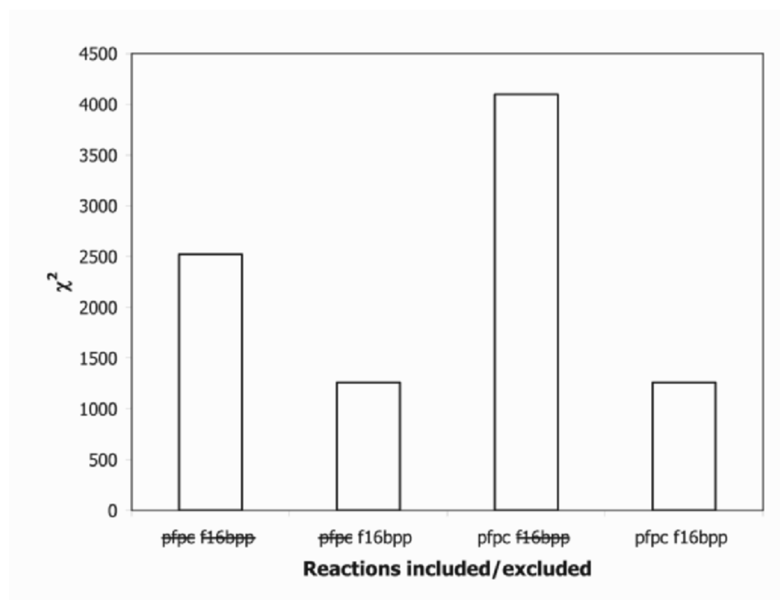


Figure 9-9. Identification of fructose-1,6-bisphosphatase in the plastid: Effect of the inclusion of plastidic fructose-1,6-bisphosphatase (f16bpp, catalysing F6P → T3P in the plastid), and corresponding cytosolic enzyme, pyrophosphatase (pfpc, catalysing F6P → T3P in the cytosol), on the χ^2 error.

due to the presence of the pentose phosphate pathway alone. Such a reaction is usually not present in nonphotosynthetic plant tissues. However, on including this reaction into our compartmented model, we found that observed isotopomer abundances for LVA #2 were well-accounted for (compare white and black bars in Figure 9-8).

Figure 9-9 depicts the improvement in the χ^2 error between experimental and simulated isotopomer abundances, due to the inclusion of plastidic F16BP and the corresponding cytosolic reaction, pyrophosphatase (pfpc). Only the plastidic conversion of T3P is evident in our system, and the cytosolic flux may be small or negligible as it does not significantly improve the χ^2 error. The example of fructose-1,6-bisphosphatase illustrates a systematic approach to pathway identifiability. More recently, Schwender et al. characterized the role of Rubisco in the absence of Calvin cycle (Schwender et al., 2004). They found that Rubisco improves the carbon efficiency during the formation of oil synthesis in *Brassica napus* embryos using an alternative pathway.

5 SUMMARY

The flux evaluation methodology described in this chapter is a promising powerful tool for understanding plant physiology. We expect that the generic computer program NMR2Flux (Sriram et al., 2004) available for calculating fluxes from the labeling data encourages the applicability of flux analysis in plants. Furthermore, once the methodology is established for a particular plant system, the tool can be used to compare the plants environmental and genetic variants. Currently, flux analysis of both environmental and genetic variants of plants is in progress in our laboratory. The ability of the labeling method to establish key regulatory nodes of metabolism thereby enabling identification of potential targets for genetic manipulations makes MFA important from a metabolic engineering perspective (Stephanopoulos and Vallino, 1991). Quantification of fluxes thus is an important tool, which when complemented with metabolite, transcript, and genomic data can contribute toward an overall correct picture of plant physiology (Sanford et al., 2002; Sauer, 2004; Schwender et al., 2004).

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