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Metabolic Flux Analysis in Plants: From Intelligent Design to Rational Engineering

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Abstract

Metabolic flux analysis (MFA) is a rapidly developing field concerned with the quantification and understanding of metabolism at the systems level. The application of MFA has produced detailed maps of flow through metabolic networks of a range of plant systems. These maps represent detailed metabolic phenotypes, contribute significantly to our understanding of metabolism in plants, and have led to the discovery of new metabolic routes. The presentation of thorough statistical evaluation with current flux maps has set a new standard for the quality of quantitative flux studies. In microbial systems, powerful methods have been developed for the reconstruction of metabolic networks from genomic and transcriptomic data, pathway analysis, and predictive modeling. This review brings together the recent developments in quantitative MFA and predictive modeling. The application of predictive tools to high quality flux maps in particular promises to be important in the rational metabolic engineering of plants.

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INTRODUCTION

Stoichiometry: the stoichiometry of a reaction is the molar balance between metabolites consumed and produced

Metabolism is a complex network of interdependent chemical reactions catalyzed by highly regulated enzymes. Metabolic flux analysis (MFA) aims to characterize fluxes through the network and to give insight into their regulation (107). The resulting flux maps form a detailed metabolic phenotype that is more closely related to biological function than phenotypes defined by metabolic profiles or other biological parts lists. Flux mapping goes beyond providing a rich phenotypic description, and has yielded a great deal of novel insight into the metabolic operation of plant cells (67, 81, 87). MFA has contributed to our fundamental understanding of metabolism by helping to determine which of the known alternative routes carry fluxes through the metabolic network (13, 64), and by uncovering novel routes and cycles (3, 98). Flux maps obtained under different growth conditions or from mutants also aid in the generation of hypotheses about metabolic regulation (2, 28, 43, 91).

Defining the structure and stoichiometry of the network is the first step in MFA; for microbial systems, this first step can be performed in an objective, almost routine way from the annotated genome together with transcript, protein, and metabolite datasets. The structure of the network can be used to define the range of possible flux maps that a network can support, and to determine which of these best meet particular cellular objectives (evolutionary selection pressures), such as maximizing growth (77, 78, 83, 115). The comparison of fluxes estimated from experimentally based MFA and those calculated to best meet evolutionary incentives can be a valuable tool to evaluate the validity of the postulated incentives. Experimentally based approaches to quantify fluxes through cells and tissues are based on either steady-state or kinetic isotopic labeling experiments and their interpretation by computer-aided modeling. Two recent reviews (81, 87) provide detailed tutorial-style explanations of kinetic and steady-state MFA approaches, whereas we focus on the principles involved, and on illustrating the sort of information these approaches have yielded when applied to plants. Because of the diversity of approaches used for MFA of plant systems, we include a discussion of the statistical confidence of the flux values reported in plant MFA studies.

In the microbial world, MFA is the basis of rational metabolic engineering (73). Plant metabolic engineering, which has thus far been largely based on intelligent tinkering, is severely hampered by a lack of understanding of metabolic network structure, function, and regulation (19). Indeed, with the exception of some notable successes (for example Reference 124), the great majority of plant metabolic engineering efforts are unsuccessful. Thus, the quest for better methods for predicting the effects of genetic or environmental alterations is important. Predictive metabolic modeling techniques that derive from MFA can be highly effective in microbial systems (23, 46, 101, 104, 115), which suggests that predictive modeling is poised to make significant contributions to rational metabolic engineering in plants. The current interest in plant-based biofuels, whose production is expected to require the development of plants with altered biomass composition (122), adds impetus for improved predictive modeling. An increasingly urgent need to foresee the effects of climatic changes on plants will also stimulate efforts in predictive metabolic flux modeling.

Metabolic flux analysis also has a central part to play in plant systems biology (51), a field in which the development and application of high-throughput analytical technologies still exceedes our ability to use the resulting data to understand plant function. The use of metabolic network analysis in plant systems biology is currently hampered by the lack of a common platform for the dissemination of models. Such a platform is widely used in microbial and mammalian flux modeling studies and we believe that its adoption by plant researchers would accelerate the integration of metabolic modelling into plant systems biology.

This review focuses on subjects that have so far received little attention in the plant literature and whose importance has been outlined above: (*a*) network reconstruction, (*b*) quantitative aspects of flux mapping, and (*c*) predictive modeling. These are exciting times in plant metabolic flux analysis, as illustrated by the recent appearance of an entire issue of *Phytochemistry* devoted to theoretical analyses, experimental findings, and methodological developments. Our hope is to stimulate the interest of plant biologists in what we believe is an important area of research, and to commend to current and future plant MFA practitioners some of the techniques developed in the microbial field.

NETWORK DEFINITION

The first requirement for the analysis of metabolic fluxes is to define a network; values obtained through the use of a model that is based on an erroneous network are likely to be wrong, even if the model explains the measured data well. Network reconstruction has traditionally been done using the physiological, biochemical, and molecular genetic literature. The present availability of annotated genomes and rich databases of transcription and protein composition information makes it possible to assemble such networks in a more efficient and unbiased manner, and allows networks to be created for systems that have not been studied intensively by traditional means (8, 17, 18, 54, 55).

The most complex network reconstructed to date is *Homo sapiens* Recon 1 (21). This genome-scale model is based on the primary literature (>1500 papers, referred to as the bibliome) and its creation was guided by the Kyoto encyclopedia of genes and genomes (KEGG) database (44). Recon 1 is a network reconstructed from the bottom up, where relationships between gene expression and protein function are deterministic (82). Recon 1 includes eight subcellular compartments and consists of 1496 open reading frames (ORFs), 2004 proteins, 2766 metabolites, and 3311 metabolic reactions. This bottom-up approach of starting with the molecular components allows the application of gap analysis to identify metabolites or enzymes that are unconnected or incompletely connected to the network. In the human metabolic network,

this approach was used iteratively to identify missing components. The 356 metabolites that still remain disconnected in the published version serve to highlight areas where more detailed work is required.

In the world of microbial network analysis, genome-scale network construction began with *Escherichia coli* and is now well advanced (18, 78, 82). Genome-scale network construction is applied in an almost routine way to an increasing number of organisms (8, 54). Gap analysis was used to assign putative function to 55 ORFs (86) for *E. coli* K-12 MG1655, and was followed up with the development of algorithms to predict missing reactions to reconcile the model with experimental data. This work led to the assignment of function to 8 more ORFs, 5 of which were verified experimentally (85). Genome-based network reconstruction is thus not only a starting point for metabolic flux analysis but also a powerful tool for functional genomics.

In *Arabidopsis thaliana*, gene annotation and deterministic gene-to-protein-to-metabolic reaction descriptions are not yet available to the same extent as in bacterial or mammalian models (108). Annotation of plant genes is often putative and based on sequence similarity. The need for a system-wide approach to network construction in plants has been recognized (34) and the most comprehensive plant (*Arabidopsis*) network to date (33) integrates the regulatory and metabolic networks and contains 7635 nodes (6176 genes and 1459 metabolites) in total and 230,900 interactions between these nodes (**Figure 1**).

Correlation-based information from omics approaches can also be used to generate candidates for network components (44). Metabolic genes involved in the same pathway are often expressed at the same time and in the same tissue (95). Using this observation, clustering analysis techniques have been applied in the plant fields (7, 123) to find unknown genes involved in secondary metabolism. Coexpression analysis holds promise for metabolic gene discovery, which is especially important in plant systems where metabolic networks are not yet fully resolved.

METABOLIC FLUX ANALYSIS AND THE STOICHIOMETRY OF THE NETWORK

MFA is the investigation of the flow of metabolites through a metabolic network. Biological model systems that have a nearly constant metabolism, in which fluxes and the levels of metabolites (except stored or excreted ones) are constant, are said to be in metabolic (pseudo) steady state. Examples of such systems include cell cultures during exponential growth and many mature differentiated tissues. Certain growing plant tissues, such as developing embryos during seed filling (99), also have nearly constant patterns of metabolic flux for extended periods. Computationally, steady-state models are easiest to manage, and steady-state systems have been used productively in plant studies over the last dozen years to yield extensive and detailed flux maps of central metabolism. For recent summaries and discussion of the findings of such plant studies see References 81 and 87.

The stoichiometry of the network is defined in a stoichiometric matrix $S_{(m*n)}$, which is constructed from mass balance equations around the internal metabolites. Usually a metabolic network contains more reactions (n) than metabolites (m) and is thus said to be underdetermined. Because no metabolites build up during steady state, multiplication of the stoichiometric matrix (S) with the flux vector (v) returns a null vector:

$$S.v = 0$$

This all-important relationship constrains the value that the internal fluxes (those within the metabolic network) may assume. The number of underdeterminacy (n-m) defines the number of free variables (fluxes) in the metabolic network model that can be varied independently without violating the steady-state assumption. However, the free variables do constrain each other, reducing the allowed

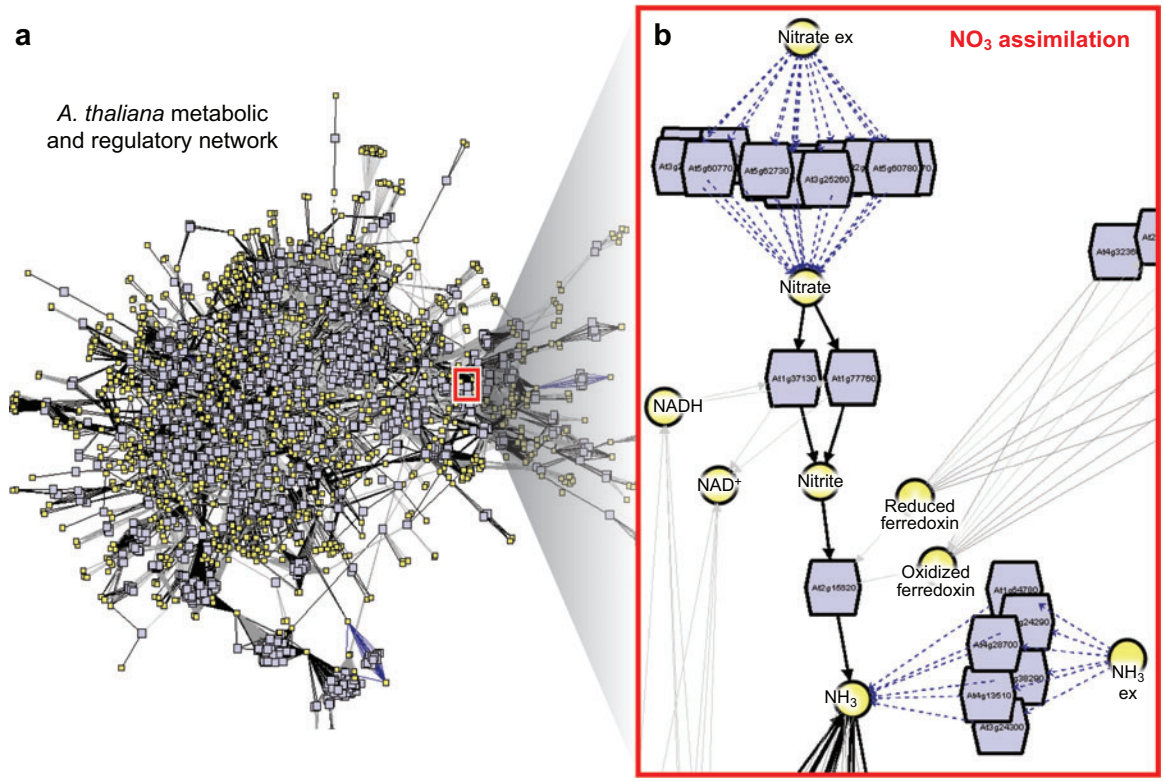

Figure 1

Reconstruction of a qualitative network model of a generic plant cell (taken from Reference 33 with permission). (*a*) A birds-eye view of the integrated network model. Metabolic data were compiled from the KEGG and Aracyc databases. In addition to interaction information pooled from three different databases, interactions were predicted or experimentally determined. A detailed description of this network is posted on the VirtualPlant website (**http://www.virtualplant.org**). (*b*) A close-up view of the genes and metabolites involved in the NO_3^- reduction and assimilation pathway. Yellow circles represent metabolites. Grey hexagons represent genes coding for transporters or enzymes. From top to bottom: extracellular NO_3^- is connected by blue dotted arrows to several genes that code for known or putative transporters. The transporters are in turn connected to intracellular NO_3^-. Intracellular NO_3^- is converted to NO_2^- by the action of nitrate reductase, for which there are two genes in *Arabidopsis*. NO_2^- is then reduced to NH_3 by the single gene enzyme nitrite reductase. Black arrows and thin grey arrows denote association through enzymatic reactions.

relative values that fluxes may assume to a hyper cone in the m-n dimensional flux space (**Figure 2**). Stoichiometric analysis can be used to reveal network properties such as complexity using singular value decomposition of S (21) and flux-coupling analysis (12, 14). Flux-coupling analysis is a method that identifies metabolic reactions that exclusively belong to a single metabolic pathway, which can be instrumental in the identification of targets in a given pathway available for manipulation.

Examining the stoichiometries of a pathway directly can also be informative. For example, stoichiometric balancing of protons and the cofactor NADH was used to resolve a longstanding error in the field of plant responses to hypoxia/anoxia. The balancing of protons and cofactors demonstrated that during anoxia, nitrate reduction in roots

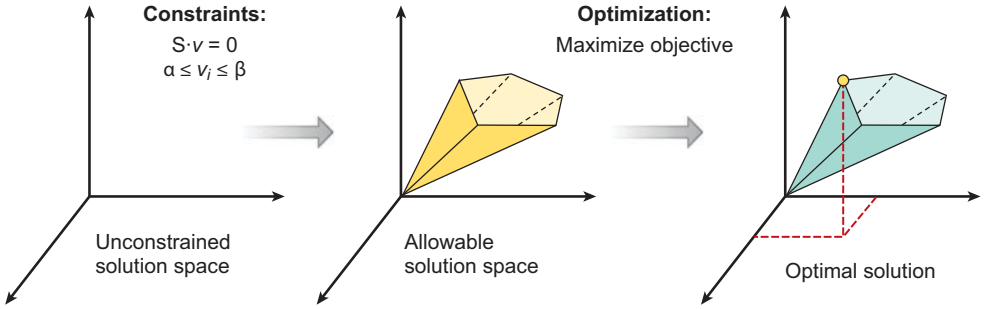

Figure 2

Computation of flux values using flux balance analysis (FBA) (taken from Reference 86 with permission). In the three-dimensional unconstrained flux space of a hypothetical network, fluxes can assume any value. After considering the stoichiometry and enzymatic limitations of the network's reactions, the feasible solution space is reduced to a cone. Using an objective function, such as maximizing biomass accumulation, FBA can identify a coordinate (set of flux values) on an edge of the feasible flux cone.

contributes to acidification instead of reducing it, despite the consumption of a proton in the conversion of nitrate to nitrite (110). Follow-up research (57) showed that the reduced cytoplasmic acidification observed in the presence of nitrate (88) could be mimicked by nitrite alone, which suggests a possible signaling role for nitrite or a downstream metabolite of nitrite, and provided support for the initial assertion based on stoichiometric balancing (57).

Elementary Mode Analysis and Extreme Pathway Analysis

Insight into the functional capabilities of a metabolic network can also be obtained from its stoichiometry by deriving operating modes of a metabolic network. Two of these methods, elementary mode analysis (EMA) and extreme pathway analysis (EPA) (**Figure 2**), are based on analysis of the flux space (96). The EMA description of a flux map is exhaustive and unique (72); each mode is a minimal pathway that can operate independently. The weighted sum of these minimal pathways describes the entire flux cone (97) (**Figure 2**). The number of modes of complex networks is often very large (30), but fortunately many elementary modes can be combined into families that share the overall mass balance. EMA has been applied to plant flux maps and has provided insight into futile cycling in sugarcane (89) and the role of photosynthesis and a unique role of Rubisco in the carbon conversion efficiency in developing *Brassica napus* embryos (98).

EPA is closely related to EMA and plays an important role in flux balance analysis (FBA), which is discussed below. EPA and EMA methods were recently reviewed and compared in depth (72). The primary distinctions between EMA and EPA are as follows: in EPA (*a*) the flux cone of feasible flux values is capped by restricting the pathways not to exceed the V_{max} deduced from external flux measurements, (*b*) futile substrate cycles are omitted, and (*c*) no linear combinations of modes are included. This results in a closed hyper volume rather than a cone, of which only the edges are feasible solutions. As a consequence, the number of extreme pathways is much smaller than the number of elementary modes for a typical metabolic network (72).

Flux Balance Analysis

FBA is a constraint-based method that solves the underdetermined mass balancing problem by finding the set of flux values that is optimal for a particular goal, referred to as an objective function (114). The use of an objective function allows for solutions of internal

fluxes, which are represented as points on the edge of an n-dimensional flux cone (83, 86) (**Figure 2**). Biomass accumulation is a popular choice as an objective function for microbial models because for fast-growing laboratory or industrial strains it is reasonable to argue that biomass accumulation is indeed the prevalent selective pressure.

Many bacterial networks, as well as those of yeast (22) and mammalian cell cultures (21), have been analyzed with FBA, amongst which the photosynthetic prokaryote *Synechocystis* sp. PCC 6803 (102) is of special interest to plant biologists as a model system. Under photoautotrophic conditions all carbon is converted to biomass, which led to the selection of light-use efficiency as the objective function. In applying FBA to heterotrophic growth conditions a two-step approach was chosen. Firstly, the efficiency of biomass accumulation from substrate was used as the objective function, followed by efficiency of light use for biomass accumulation. The mixotrophic mode of operation is of particular interest for developing plant embryos, where the balance between photosynthesis and carbon substrate uptake influences the carbon conversion efficiency during seed filling (32). FBA has yet to be applied to a plant model but promises to be a useful tool for developing embryos, in which optimal biomass accumulation and light use-efficiency are also reasonable assumptions. Academic software packages such as Fluxanalyzer (48) and MetaFluxNet (52) and proprietary software (SimPheny) have been developed to facilitate FBA. For a more comprehensive list of software tools see References 54 and 87.

The development of FBA as a technique continues because solutions to the FBA objective function are typically not unique, and mixed-integer linear programming techniques have been used to identify equivalent flux maps (53, 84, 86). Within the FBA framework, solutions having identical flux values are said to be silent phenotypes (79, 84). Efforts have been ongoing to increase the number of constraints used in genome-scale FBA models to reduce the number of equivalent solutions, with attention directed to thermodynamic constraints on extreme pathways (energy balance analysis; see Reference 11) (**Figure 2**) and measured transcript levels in the cells under study (11, 16, 35). Such information is also available for plant systems and we expect them to be productively applied there as well.

Steady-State Isotope Labeling Experiments

In experimentally based MFA, isotopically labeled substrates are supplied to a biological system and the resulting labeling patterns are analyzed to obtain internal flux information. Internal metabolic fluxes—including their reversibilities—can be estimated on the basis of modeling analysis of the labeling data. In an isotope labeling experiment (ILE), isotopically labeled substrates are supplied to cells in metabolic steady state until the pattern of labeling has stopped changing noticeably (isotopic steady state). The isotopes used are usually nonradioactive ones (^{2}H, ^{15}N, and most commonly, ^{13}C) and isotopic enrichment in metabolic intermediates and products is detected by nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry. Isotopic labeling information is used in conjunction with measurements of fluxes into (uptake) and out of (excretion, growth, and storage) the network. The details of such labeling experiments and the analysis of the labeling results in plant systems are explained for the nonspecialist in a recent review (81).

For simple (sub)networks, algebraic expressions can often be derived that directly relate the flux values to measurable positional isotopic labeling. In such fully determined cases the flux values in the system can be obtained by inserting the labeling measurements into the derived expressions (93). This approach has been used to obtain information on the fluxes of glycolysis/gluconeogenesis and the oxidative pentose phosphate pathway in heterotrophic plant cells, and specifically

to shed light on the role of fructose 2,6-bisphosphate in regulating fluxes in this part of metabolism (28). Solutions for larger metabolic networks are hard to obtain manually; Dieuaide-Noubhani and coworkers (20, 91) used algebraic solving software to derive relationships between positional labeling levels and the values of metabolic fluxes. These studies yielded flux maps for growing maize root tips and tomato cell suspensions and provided evidence for a dramatic rate of ATP dissipation via the turnover of sucrose.

This analytical approach is attractive because it is mathematically tangible, but it has at least two significant disadvantages. The first is that solutions have to be obtained afresh for every alteration in the network architecture that one wishes to explore. The second is that this approach does not easily allow the use of all the available experimental information. This is because the number of measurements from a labeling experiment is usually much larger than the number of fluxes to be measured, which makes the flux estimation an overdetermined problem, whereas the analytical solution approach uses only the amount of labeling information required to solve the relationships directly.

To take advantage of all available data and to provide flexibility in exploring alternative network models, theory and software have been developed to convert the problem of obtaining fluxes from measurements into one of optimizing the fit between predicted and observed labeling patterns. The principle of this approach is to construct a model based on the network structure that includes the stoichiometries of the metabolic reactions and tracks the transformation of positional isotopic labeling in reactions. The model is used to compute the expected labeling patterns from the network architecture, the values of the fluxes, and the labeling of the substrate(s) provided. The computed labeling patterns are compared with the measured ones and the flux values are iteratively changed to minimize the difference between the computed and observed labeling (117). The details of obtaining flux estimates based on the cumomer concept are well explained in a series of papers by Weichert and coworkers (66, 117, 118, 121). Alternative methods, such as the bondomer (111) and elementary metabolite units (5) concepts that aim to reduce the computational cost, have since been developed.

A significant number of studies in plant systems using ILE and variations upon it have appeared in the plant literature in the last several years (for reviews see References 81 and 87). Developing plant embryo cultures in particular has been the subject of many of these studies because they are amenable to in vitro growth under (pseudo) steady-state conditions. The striking findings to date include the following: the discovery of a new metabolic route in developing *B. napus* embryos (98), the observation that mitochondrial metabolism in these embryos operates differently than the canonical modes found in other biological systems (100), and the finding of a new substrate cycle involving the phosphorylation and dephosphorylation of glucose in maize roots (3).

QUANTITATIVE FLUX ANALYSIS USING ISOTOPE LABELING EXPERIMENTS

The number of the internal fluxes to be determined is the total number of internal fluxes minus the number of internal metabolite balances. In addition, ILEs also resolve the reversibility of fluxes, implying that reverse fluxes must be estimated as well. If more measurements are available than the number of fluxes to be estimated, the flux estimation problem is said to be overdetermined and can, in principle, be solved. However, if the label measurements report on only part of the network, it is possible that the flux estimation may still not be fully resolved, in which case the problem is said to be structurally undeterminable (41, 112, 113). In practice, many more measurements are required than the number of fluxes to be determined if one is to be able to estimate all the network fluxes.

Once flux estimates have been made, each flux value must be associated with a confidence interval to make quantitative interpretation possible. Because the estimation of internal flux values is an indirect method, the parameters of interest are not measured directly, but are inferred from measurements of input and output rates (external fluxes) and labeling data. As a result, confidence estimation for internal flux measurements is less straightforward. Fortunately, the theory of obtaining confidence intervals for inverse problems is well established and is based on the measurement sensitivity matrix (See Quantitative Flux Measurements). The sensitivity matrix contains the predicted responses of all measurements to a small change in the value of any of the fluxes. Together with the confidence range of each of the measurements, this information determines the flux covariance matrix, which contains linear approximations of all flux variances (See Quantitative Flux Measurements). Because the relationship between fluxes and label measurements is highly nonlinear, linear flux confidence estimates can easily be in error by a factor of two or more (4). To improve the quality of the confidence estimates, Wiechert and coworkers (121) transformed forward and reverse fluxes to net and exchange fluxes, and used a compactification mapping of the exchange fluxes (See Quantitative Flux Measurements). For a detailed description of flux confidence estimation see References 4 and 121.

Alternatively, flux variance estimates can be obtained using Monte Carlo sampling. For this approach, each measured label and external flux value must be replaced with a chance distribution of values generated from the mean and variance of each (label) measurement. By performing thousands of optimization runs using appropriately sampled measurement values, a distribution of flux values, and therefore variance estimates, will be found for each flux. Given enough trials, this method is very precise (4). In practice this method is complicated by the inability of optimization algorithms to reliably find the global best fit for each run. In fact, one can never be certain that a global minimum (minimal difference between actual and simulated measurements) has been found, and many optimization trials using the mean measurement values are usually performed to increase the likelihood of finding the global best fit.

The probability that the determined flux map differs from nature is usually determined using the χ^2-test. This test evaluates the goodness of fit of the model by considering the difference between the number of label measurements and the number of estimated fluxes, the precision of the label measurements, and the difference between the simulated and observed label measurements.

Interpretation of Flux Estimates in the Plant Literature

Owing to the specialist nature of internal flux estimation, flux values and their associated confidence intervals presented in the plant literature are not always straightforward to interpret. A flux estimate value with a standard deviation on the order of the largest flux values in the flux map is obviously poorly determined. However, evaluation of the validity of the reported confidence interval is more difficult. In the plant literature, reported flux standard deviations are determined in many different ways and often do not represent true confidence estimates. Several plant studies do not report variance estimates and therefore values in these studies cannot be interpreted quantitatively. In other studies, standard deviations associated with fluxes are a description of the size of the feasible solution space of underdetermined labeling systems (90). Even in one of the more advanced studies published to date (105), the reported standard deviations associated with the fluxes in developing soy beans do not represent confidence intervals. In this study a few hundred optimizations were run from different initial flux values, and the standard deviation of the distribution of optimized flux estimates was reported. Because no chance distributions of

a Relationship between label measurements and flux values:

Label measurements (y) are a function of the forward ($V^{\rightarrow}$) and reverse ($V^{\leftarrow}$) fluxes (117). Assuming that ε_y is normally distributed, the matrix Σ_y describes the variance σ^2 associated with y.

$$y = \Gamma\left(\frac{V^{\rightarrow}}{V^{\leftarrow}}\right) + \varepsilon_y$$

It should be noted that good estimates of measurement σs must be based on $n > 30$. Therefore, it is good practice to enter σs associated with the measurement technique used to prevent bias in the fitting procedure. Flux maps should still be based on multiple labeling experiments to ensure reproducibility and better mean labeling values.

$$V_{net} = V^{\rightarrow} - V^{\leftarrow}, \quad V_{ex} = \min(V^{\rightarrow}, V^{\leftarrow})$$

$$\Sigma_y = \begin{bmatrix} \sigma^2 & \cdot \\ \cdot & \sigma^2 \end{bmatrix}$$

b The inverse problem:

An optimizer is used to minimize the residuum, which is the sum of the weighted residuals (differences between simulated and observed values) where Θ are the flux estimates, $F_w(\Theta)$ and $F_y(\Theta)$ are simulated flux and label measurements, and w and y are flux and label measurements. At the minimum residuum value, flux values are said to be best estimates ($\hat{\Theta}$).

$$\min_{\Theta} \left\| F_w(\Theta) - w \right\|^2_{\Sigma_w} + \left\| F_y(\Theta) - y \right\|^2_{\Sigma_y}$$

c χ^2-test

To test whether the flux model cannot explain the measurements and must be rejected, the residuum must fail (be larger than) the χ^2-test. The χ^2-test value is calculated on the basis of the degrees of freedom (number of independent measurements minus parameters) and a defined confidence level α.

d The weighted sensitivity matrix of the label measurements:
This matrix expresses the change in measurements in response to a change in flux. Variances of the label measurements are used to weight the sensitivities.

$$Sens_{\Theta}^{w,y} = \Sigma_y^{-\frac{1}{2}} \cdot \left(\frac{\partial F_y}{\partial \Theta}\right)$$

e The flux covariance matrix:
The diagonal entries of the covariance matrix form a linear approximation of the flux estimates. Estimation of the confidence intervals is improved by nonlinear compactification mapping of the exchange fluxes (121). An even more precise estimate of flux variances can be calculated using an algorithm presented in Reference 4.

$$Cov_{\Theta} = \left[\left(\frac{\partial F_y}{\partial \Theta}\right)^T \cdot \Sigma_y^{-1} \cdot \left(\frac{\partial F_y}{\partial \Theta}\right)\right]^{-1}$$

$$V_{ex}^{[0,1]} = \frac{V_{ex}}{1 + V_{ex}}$$

f Individual flux confidence intervals:
Here $\hat{\theta}_i$ is the ith flux estimate and $\hat{\theta}_{ii}$ is the i^{th} diagonal entry of the flux covariance matrix (flux variance of $\hat{\theta}_i$).

$$[\hat{\theta}_i - \Delta, \hat{\theta}_i + \Delta]$$
$$\text{where } \Delta = \sqrt{\chi^2_1(1-\alpha) \cdot Cov(\hat{\Theta}_{ii})}$$

g The system variance:
The system-wide variance can be described by the D- or A-criterion, which are scaled to the average geometric or arithmetic mean, respectively. Criterion values have been used for optimal design studies (9, 56, 66).

$$D_{crit} = \sqrt[2n]{\det(Cov)}, \quad A_{crit} = \sqrt{\frac{tr(Cov)}{n}}$$

QUANTITATIVE FLUX MEASUREMENTS

Variance estimates of internal flux measurements are indispensable for a quantitative interpretation of internal flux estimates. An overview of the steps required for a quantitative assessment of an isotope labeling experiment (ILE) is presented as follows: (*a*) definition of the relationship between fluxes and measurements, (*b*) finding best estimates for internal fluxes using an optimization routine, (*c*) statistical evaluation of whether the model must be rejected, (*d*) calculation of the flux covariance matrix on the basis of the weighted sensitivity matrix (using nonlinear mapping of exchange fluxes), (*e*) calculation of confidence intervals from flux covariances, and (*f*) description of system-wide statistics that can be used for optimal design studies.

This section is intended to give a feeling for the theory involved. Comprehensive coverage of the theory involved can be found in parameter estimation textbooks (74).

measurements were used (as in Monte Carlo analysis), the ranges reported for the flux values reflect an evaluation of the performance of the optimization routine. In addition, taking the mean values of normal distribution fits to the flux distributions that were found this way has led to the presentation of a flux map that is not mass balanced. Although standard deviations obtained by this approach are likely qualitatively indicative of the determinability of the fluxes, they cannot be interpreted as flux confidence intervals.

Plant studies that used the 13C flux software suite (119) reported a nonlinearly mapped linear approximation of flux confidence estimates (121) as discussed above (See Quantitative Flux Measurements). Finally, flux value ranges should always be interpreted with caution, even if the method of calculation is appropriate. Systematic errors in label measurements and simplifications of the true metabolic network also contribute to error and are not easily estimated. A pragmatic approach to errors is therefore necessary, but this should not conceal the large variation in quality and meaning of flux standard deviations presented in the plant literature.

Design of Optimal Substrates and Labeling Measurements

Once a flux map has been determined, the quality of the flux estimates can be greatly improved by designing an optimal experimental strategy (66). The quality of the flux estimates of a metabolic flux map depends on the following: (*a*) network structure, (*b*) flux values, (*c*) design of the labeling substrate, and (*d*) label measurements performed. The first two parameters are dictated by nature, but the choice of the substrate label and the measurements made are to some extent controlled by the experimenter. Recently, two studies considered optimal designs for models of interest to the plant community. One study used the metabolic flux map of developing *Brassica napus* embryos (56). This study explores the value of optimal substrate label designs obtained with statistical criteria based on properties of the flux covariance matrix of simulated label designs. A partial optimal design aimed at best resolving the oxidative branch and the flux carried by Rubisco presented in this study confirmed the previous theoretically derived importance of 2-^{13}C-glucose (93). In a separate optimal design study, based on the assessment of simulated experimental data, Shastri & Morgan (103) determined optimal conditions and measurements for a transient labeling study of photoautotrophic bacteria. These studies show that effort spent in optimizing the choice of labeled substrate(s) is well repaid by improved resolution of the resulting flux maps. This is especially the case for the medium- or high-throughput studies that we expect for plant systems in the next several years, similar to those recently carried out for microbial systems (94).

KINETIC MODELS

As discussed above, the use of steady state metabolic flux analysis requires a system to be in metabolic and isotopic steady state. However, with some notable exceptions, plants and their tissues are usually not in metabolic steady state long enough to reach an isotopic steady state during labeling. Fortunately, two types of approach allow metabolic flux analysis to be carried out effectively in these situations: kinetic (or dynamic) and instationary metabolic flux analysis methods. Indeed, such "explicit kinetic models still allow for the most detailed and quantitative evaluation of the dynamics and function of metabolic systems" (109). Of these dynamic methods the approach that is in principle most generally applicable, kinetic modeling, is also the best established, because it has been applied to microbes since the 1960s and to plant systems shortly thereafter (45); see References 67 and 81 for reviews.

In studies of this type, a sequence of samples is taken while the labeling patterns, metabolite levels, and/or metabolic fluxes are changing. Time course data on metabolite

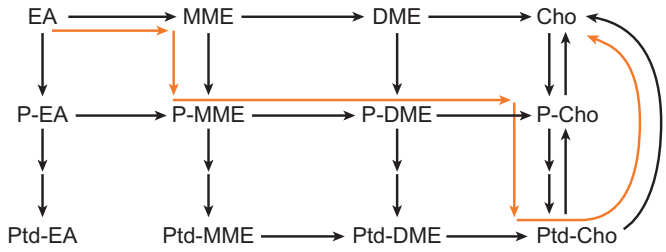

Figure 3

The network of reactions that could participate in choline (Cho, trimethylethanolamine) synthesis in flowering plants (adapted from Reference 64 with permission.). Methylation reactions proceed from left to right whereas reactions involving the making or breaking of phosphoester bonds (phosphorylation, phosphotransesterase, and phosphodiester hydrolysis) proceed from top to bottom (or in the case of hydrolysis, from bottom to top). The predominant route of synthesis as found by McNeil and coworkers (64) is shown in orange. Abbreviations: EA, Ethanolamine; MME, monomethylethanolamine; DME, dimethylethanolamine; P-base, phospho-bases; Ptd-base, phosphatidyl-bases.

levels and their labeling are then analyzed using rate equations written to represent the metabolic and transport steps of the network under investigation.

An example of this approach is a study to determine the route by which choline is synthesized in leaves (64). The metabolic network under consideration is shown in **Figure 3**, which shows the existence of multiple routes by which the precursor, ethanolamine, could be converted to choline. In one set of experiments researchers supplied ^{33}P-radiolabeled phosphoethanolamine or phosphomonomethylethanolamine to follow the phosphorylated species; in another set of experiments they provided ^{14}C-labeled formate to track methylation. In these experiments they obtained time courses of labeling and of the levels of the intermediates and products of the network. The model that was used to analyze the data is illustrated in **Figure 4**. By fitting the model parameters to the experimental data these authors showed that the first methylation step occurs solely at the phospho-base level, the second and third methylations occur largely at the phospho-base level (the remainder occur

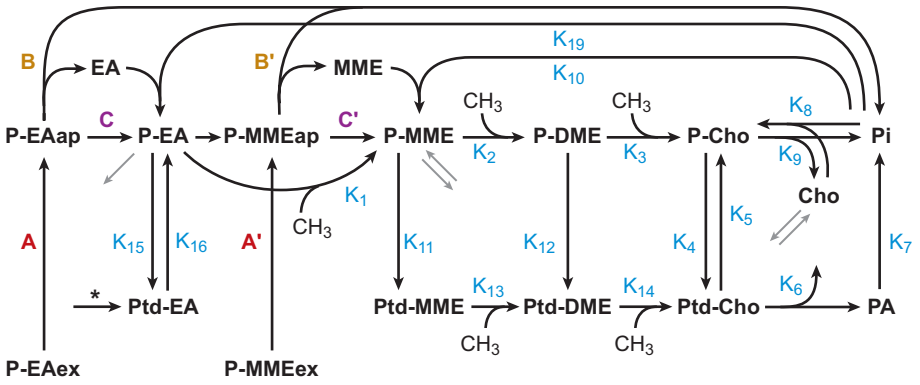

Figure 4

Illustration of the model of the kinetics of flux through the network shown in **Figure 3** that was used to analyze the results of labeling experiments (adapted from Reference 64 with permission). The notation K1–K16, A, A′, B, B′, C, and C′ next to the flux arrows refer to fluxes whose sizes were used as parameters in modeling the data. All fluxes were assumed to remain constant (metabolic steady state) except for rates A, A′, B, B′, C, and C′, which describe the uptake and apoplastic hydrolysis of supplied phosphomonomethylethanolamine (P-MME) and phosphoethanolamine (P-EA). These rates were set to be proportional to the substrate pool sizes. Thus, exogenously supplied ^{33}P-MME (33P-MMEex) or ^{33}P-EA (33P-EAex) was taken up into the apoplast at a rate proportional to the exogenous pool size ($A = [33P\text{-}EAex] \times K_A$; $A' = [33P\text{-}MMEex] \times K_{A'}$ where K_A and $K_{A'}$ are first order rate constants that were used as parameters and whose values were assigned to optimize the fit to the time course data). Diagonal arrows (*grey*) designate transport to and from separate storage pools. Abbreviations: EA, ethanolamine; DME, dimethylethanolamine; Cho, choline; PA, phosphatidic acid; P-base, phospho-bases; Ptd-base, phosphatidyl-bases.

at the phosphatidyl-base level), and that free choline originates predominantly from phosphatidylcholine rather than from phosphocholine. This study contributed significantly to our understanding of choline metabolism in plants, and was instrumental in metabolic engineering of increased stress tolerance by introducing betaine synthesis from choline.

Other studies by Rhodes and his collaborators over several decades used more or less similar kinetic modeling approaches and contributed substantially to pathway delineation and understanding the regulation of primary nitrogen metabolism (63), different aspects of one-carbon metabolism (65, 70), and more recently, the synthesis of floral scent compounds through the complex network of benzenoid metabolism (13, 71). Other significant recent studies utilizing similar kinetic flux analysis methods include work by Matsuda and coworkers (60, 61) on pathway delineation and regulation of fluxes of phenylpropanoid synthesis after wounding or exposure to an elicitor. These and other kinetic flux analysis studies, of which a substantial proportion are aimed at understanding the regulation of photosynthesis, have been reviewed elsewhere (81, 87). Additional studies analyzing tryptophan biosynthesis in cultured rice cells (62) and describing the kinetics of label distribution through central metabolism in heterotrophically growing *Arabidopsis* cells (10) have appeared since then.

Here we consider the study of choline synthesis in tobacco (64) in greater detail because it serves to illustrate several general features of this class of kinetic model. First, kinetic modeling is a powerful tool for determining the route that material takes through branched and interconnected parts of metabolism. This can be challenging to study by other means, because the complexity of time course data makes it impossible to discern the active route through the network by eye. Second, kinetic rather than steady state modeling is the toolset of choice when the substrate contains only one suitable atomic position for labeling. This is the case for important plant nutrients such as sulfate, nitrate, ammonia, carbon dioxide, or as in the study under consideration here: phosphate or methyl groups. For such substrates the labeling pattern reached in metabolic intermediates and products at isotopic steady state contains no information on fluxes because they all become equally labeled.

A third important issue in kinetic modeling encountered in the work of McNeil and coworkers (64) is the choice of the form of rate terms used in the model. Rate equations describing flow through a network are coupled linear differential equations whose parameters may be of various levels of complexity. In a related study by McNeil and coworkers (65) of choline conversion to betaine, rate equations were written with Michaelis-Menten terms, where each rate depends on substrate concentration and two parameters (K_m and V_{max}) that were fitted during modeling. In that study the smaller size of the network under consideration permitted, and the predictive aim of the study required, the use of more realistic rate terms with an attendant increase in the number of parameters.

A fourth issue illustrated by the study of McNeil and coworkers (64) is encountered in every flux analysis study of eukaryotic systems, whether dynamic or steady-state in character: compartmentation. To account for the fact that the cells were exposed to the externally supplied label gradually because diffusion into the leaf tissue proceeds at a finite rate, the analysis required that an apoplastic compartment be included. Cell suspensions present no such challenges (e.g., References 10 and 62), nor do studies in isotopic steady state in which the tissue under study can be treated as approximately uniform (such as those on root segments or embryos in culture) (2, 20, 105). However, compartmentation should not be neglected when there may be significant metabolic conversion of the substrate either in the apoplast or in tissue through which label passes before reaching the tissue under study (26). Nor can intracellular compartmentation be ignored when multiple pools of the same metabolite contribute significantly to the

redistribution of label, measured pool sizes, or specific enrichments. McNeil and coworkers (65) showed that three pools (vacuolar, cytosolic, and chloroplastic) are involved in determining flux to betaine and should be included in the model. The large artifacts that could arise in steady-state experiments from a failure to account for the potential effects of different metabolic pools of sucrose was recently explored (49). Obtaining information on labeling in different pools of the same metabolic intermediates, such as hexose and triose phosphates and acetyl-CoA, in different subcellular compartments can therefore be of central importance. Advances in making such measurements by analyzing compartmentally specific products as reporters were recently reported by Allen and coworkers (1). An exception to the requirement for a model to include multiple pools of the same metabolite is the case in which pools in different compartments are in rapid exchange relative to the rates of the relevant metabolic conversions (100).

Another important issue for both kinetic and steady-state analyses that arose in the work of McNeil and coworkers (64) concerns the simplification of a model compared with the actual metabolic network. In this study, the model represents the conversion of phospho-bases to phosphatidyl-bases as a single kinetic step, omitting the cytidyldiphospho-bases that are intermediates in this conversion (the omission is represented by double arrows in **Figure 3**). Reactions are collapsed in this way in kinetic, steady-state, and other flux models in which there are no measurements to allow meaningful modeling of the process. In kinetic flux analysis this occurs frequently when measurements on levels and/or labeling in extracted intermediates are unavailable owing to their low concentrations, lability, and/or uncertain or mixed compartmental origins. In steady-state labeling studies and other stoichiometric analyses, the network model is simplified relative to the actual metabolic network so that fluxes between metabolic branch points are represented as single quantities without regard to the number of enzymatic steps that actually take place between these points.

Instationary State Models

The successes of steady-state experimental MFA have motivated a number of leading microbial flux analysis groups to develop theoretical frameworks and experimental methods to study systems that are either in metabolic but not isotopic steady state (26, 68, 69, 120), or in neither metabolic nor isotopic steady state (6). Although they treat the same nonsteady-state conditions as the classical kinetic modeling approaches described in the preceding section do, the new instationary approaches, whose formalisms derive from steady-state flux analysis, use positional labeling information to greatly increase the information content of experimental data. To apply the approach of Noh and coworkers (68, 69), the concentrations of most of the intracellular metabolic intermediates, in addition to their labeling patterns, must be measured. In prokaryotic systems this can be achieved using modern mass spectrometric tools and we anticipate that flux analysis of photosynthetic prokaryotes will soon be forthcoming. Indeed, a recent study (103) describes a detailed experimental design for studying cyanobacterial photosynthetic metabolism using this approach. According to this design, cells are labeled with $^{13}CO_2$ and the labeling and pool sizes for metabolic intermediates are analyzed by gas chromatography–mass spectrometry (GCMS) for a time course leading to isotopic steady state. Analysis of the design shows that useful information on the kinetics and ultimately on the regulation of photoautotrophic metabolism could be obtained by this instationary approach. Provided that sufficient subcellular compartmental information can be obtained on the levels and labeling of plant metabolic intermediates, this instationary approach would allow kinetic flux mapping in higher plant systems. The success of this approach would allow for flux analysis

of metabolic states that are not sustained for long enough to be analyzed with established isotopic steady-state techniques.

In contrast, the instationary approach recently presented by Antoniewicz and coworkers (6) requires neither pool size measurements, information on labeling in intermediates, nor metabolic steady state. This approach uses experimental steady-state MFA with the analysis modified to include dilution factors that account for changing labeling in precursor and product pools. The underlying assumption is that the metabolic intermediates reach isotopic pseudo steady state rapidly compared with changes in the metabolic flux values through the network. This assumption makes the approach suitable for slow rather than abrupt changes in conditions or development and it may prove useful for studying some plant tissues. However, because the turnover rates of plant metabolites are often on the order of hours rather than the seconds or minutes that characterize bacterial metabolite turnover, the pseudo–steady-state assumption may not be valid for many plant systems.

INTEGRATION AND CROSS-VALIDATION OF FLUX MODELS

The existence of different experimental and theoretical approaches to predicting or estimating fluxes through metabolic networks invites comparison between their results. In the microbial fields, ^{13}C-based flux measurements from steady-state flux analysis have been compared with FBA predictions (104) and isotope label–derived flux measurements have also aided to constrain FBA models (116). A schematic of the how MFA technologies and concepts relate to each other is presented in **Figure 5**.

Flux models of central metabolism can be validated with simple physiological measurements, as was done with gas exchange rates in a recent study of central metabolism in developing sunflower embryos (2). In that case, the O_2 consumption rate was compared with the total NADH production rate predicted by the flux map, and measured CO_2 emission was compared with the total CO_2 production estimated from the flux map. Alternatively, key enzyme activities may be checked to verify whether a certain pathway branch has the capacity to carry the estimated flux (43).

Other methods exist that could be used to directly measure fluxes to validate the results of metabolic flux analysis. For example, exchange spectroscopy (EXSY) is an NMR technique that can provide direct measurements of forward and reverse fluxes in vivo. In principle, EXSY measures the chemical exchange rates between metabolites directly, without the requirement of a metabolic network. However, the insensitivity of this powerful technology has so far limited its application to biological systems (reviewed in Reference 80). The only in vivo ^{31}P-EXSY study reported to date in plants resolved reactions of glycolysis, hexose transformations, and ATP metabolism in maize root tips (92).

Metabolic flux analysis is a core component of systems biology (47) and its integration with other systems analyses requires that its models be accessible beyond flux analysis research groups. Cross-platform compatibility facilitates the integration of information obtained by different methods, and allows the systems biologist to begin integrating metabolic models into broader cellular, physiological, and ultimately, ecological contexts. The need for the adoption of a platform such as systems biology markup language (SBML) is even more pressing for flux analysis because of the incompatibility among, and great variety of, software used. The systems biology community has developed the extensible markup language (XML) standard SBML to communicate all systems biology data, and SBML definitions for kinetic models (37) were among the first to appear. Flux models in the microbial community have been published in SBML for some time now (125) and current metabolic flux analysis software generally accepts and exports SBML files

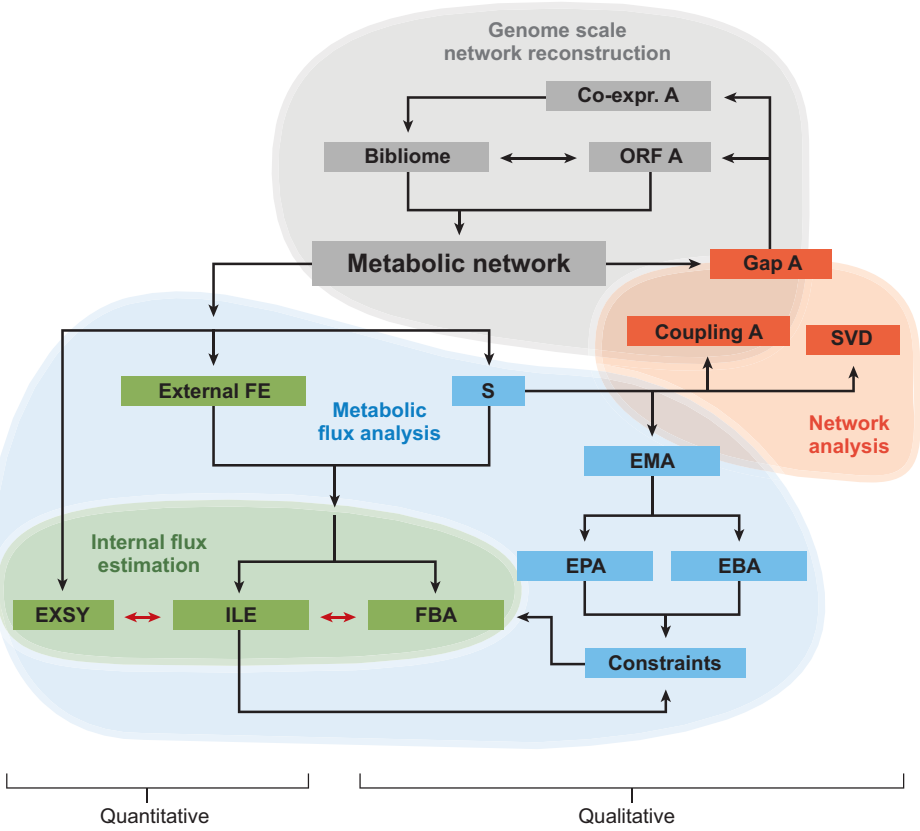

Figure 5

Schematic overview of metabolic network analysis. The different aspects of metabolic analysis are grouped into four functionally interconnected clusters: i) reconstruction of the network (*gray*), ii) analysis of the network structure (*red*), iii) metabolic flux analysis (*blue*), and iv) internal flux estimation (*green*). Genome-scale network reconstruction is an iterative process used to reconstruct the metabolic network from the literature (bibliome). Network analysis is used to evaluate the integrity of the metabolic network by gap analysis (Gap A). Network gaps prompt a targeted search for missing genes through open reading frame analysis (ORF A) and coexpression analysis (Co-expr. A). Additional network properties, such as coupling of pathways (Coupling A) and the more abstract determination of network complexity by singular value decomposition (SVD), can be determined via the use of the network stoichiometry (S). Only external flux estimation (External FE, direct measurements of input and output fluxes), internal flux estimation through in vivo nuclear magnetic resonance exchange spectroscopy (EXSY), and isotope label experiments (ILE) are quantitative (i.e., include confidence estimates). Flux balance analysis (FBA) forms a qualitative constraint-based alternative approach to determine internal flux values. Constraints can be based entirely on qualitative metabolic flux analysis of the network, such as extreme pathway analysis (EPA) and energy balance analysis (EBA). EPA and EBA are both related to elementary mode analysis (EMA), which is based on S, together with information about the reversibility of fluxes. EXSY, ILE, and FBA are largely independent methods used to estimate internal fluxes and can therefore serve to mutually validate findings (*red arrows*).

(54, 76). Unfortunately, with rare exceptions (58), plant flux models have not been made available in SBML, which hampers cross-comparison and validation of flux models.

PREDICTIVE MODELING

The next level of development for any set of methods, and a prime objective of plant systems biology, is to make useful predictions

about the effects of genetic or environmental interventions. The ability to make quantitative predictions of flux maps in response to a perturbation is also an excellent test of our understanding of the regulation of metabolism. Furthermore, the ability to predict the effects of a pathway manipulation provides a powerful tool for rational design of flux maps. Predictive modeling of the functioning of metabolic networks is already possible in plants using kinetic models, and several other approaches have been applied to microbial systems and successfully make qualitative and quantitative predictions of the effects of metabolic perturbations on flux maps.

Predictions Using Mechanistic Models

The most sophisticated kinetic models form complete mechanistic descriptions of metabolic networks and therefore contain all the information needed to predict the effects of environmental or genetic change. Such a model is thus the ultimate description of a metabolic network. However, to produce a useful model all the kinetic parameters of each enzyme must be included in the model. In practice this makes kinetic models of even moderate size underdetermined. Predictive kinetic models have been constructed and successfully tested for the limited and well-studied metabolism of mammalian red blood cells but the complexity of metabolic networks in most cells makes comprehensive predictive kinetic models unmanageable. One mechanistically realistic kinetic model in a plant system is that of glycine betaine production from choline (65). As part of a multistudy metabolic engineering effort by Hanson and coworkers to increase stress tolerance by introducing the synthesis of osmoprotectants, mechanistic kinetic modeling revealed that several factors in the structure and kinetic parameters of the network would need to be engineered before large amounts of betaine could be produced in a naturally nonproducing species. This model provides an excellent illustration of the potential uses of mechanistic models in plants and is available online in a user-friendly, interactive form that allows one to explore the predicted effects of putative interventions (**http://www.hort.purdue.edu/cfpesp/models/mo00014.htm**). A recent mechanistic model of C3 photosynthetic carbon metabolism uses kinetic parameters from the extensive literature on photosynthetic enzymes to successfully model CO_2-fixation rates under different conditions (126). Importantly, this model indicates that rising atmospheric CO_2 levels make the relative enzyme levels in C3 plant leaves suboptimal for carbon fixation.

Flux Balance Analysis (FBA)

Flux balance analysis is innately predictive because the computed fluxes are calculated on the basis of the network structure and one or more assumptions about the functional goal(s) of its operation. Indeed, predicted optimal metabolic operation modes for different culturing conditions have been confirmed experimentally (23). FBA also predicts the fluxes through a perturbed metabolic network, assuming that the network is still operating to maximize fluxes toward the initial objective (46). Knockout mutations can be simulated using the original network by including an additional constraint in which the gene knockout corresponds to a zero flux through the coded enzyme (24). This approach has been used to predict the lethality and other effects of knockouts. For microbes, FBA-based predictions of metabolic flux adaptations in response to perturbation are close to experimentally determined ones (36, 42, 75, 101, 115).

The cause of the predictive success of FBA in microbes is suggested to be twofold: (*a*) Redundancy in the network design and regulatory mechanisms adjust metabolism so that the original objective function is still closely approached, and (*b*) microbial cultures undergo adaptive evolution after a perturbation and evolve to optimize the original objective function (biomass accumulation) (24, 25, 29, 39). For plants, the first mechanism likely also applies. Adaptive evolution is not

applicable to most plant systems on an experimental time scale, except for models such as unicellular algae or cell cultures. However, the applicability of the first mechanism should provide sufficient impetus to the plant community to apply FBA to a plant model and evaluate the predictive potency of FBA for plant models. We note, however, that the choice of system will be important, because the objective functions of many plant tissues are not obvious and are likely not as straightforward as maximal growth rate.

Minimization of Metabolic Adjustment (MOMA)

A promising method for the prediction of metabolic flux adaptation in response to a perturbation is based on the principle of minimization of metabolic adjustment (MOMA) (101). In this approach, only the original flux values are used to determine the nearest feasible flux map after imposing the perturbation. The nearest feasible flux map is defined as the closest Euclidian location on the feasible flux cone to the location of the original flux map, which is found using quadratic programming and thought to be unique (101). Because often a genetically perturbed organism is not functioning optimally, MOMA is an important alternative predictive tool in the microbial world (46, 59, 106) (**Figure 6**).

MOMA has the great advantage that only flux values are required, which makes this technique directly applicable as soon as a flux map of a model has been established. When put to the test, MOMA often outperforms other methods such as FBA and ROOM (see below) in matching experimental results obtained with knockout mutants (101, 104). We believe that MOMA has significant promise for use in those plant systems where flux maps have now been established (87).

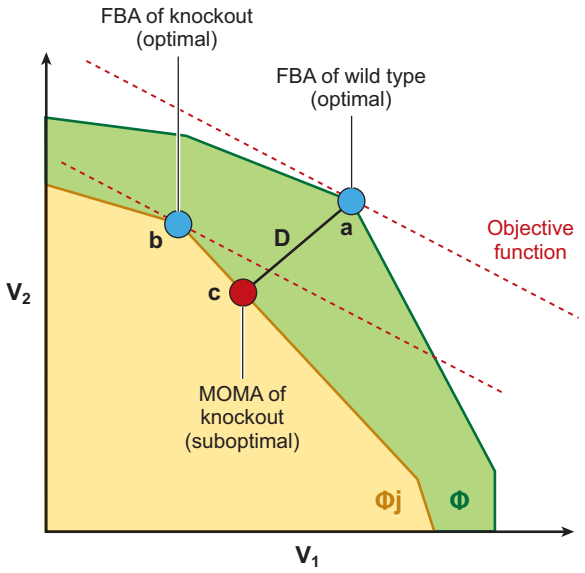

Figure 6

Predictive modeling of a knockout mutant using the minimization of metabolic adjustment (MOMA), taken from Reference 101 with permission. The green and yellow areas define the feasible flux space for the values of two fluxes V1 and V2 in a metabolic network. The outer red line is defined by the flux balance analysis (FBA) objective function, which forms a tangent to the feasible space and solves for V1 and V2 at point a. For a knockout mutant, for which the feasible space is reduced to the yellow area, the original FBA objective function now solves V1 and V2 at point b. In contrast with the outcome predicted by FBA, MOMA (which minimizes the metabolic adjustment) predicts that V1 and V2 for the mutant will be defined by point c. Thus, c is the nearest feasible point to a, and D is perpendicular to the nearest edge of the allowed flux space. Φj, feasible space of knockout (*yellow*); Φ, feasible space of wild type (*green*).

Regulatory On/Off Minimization (ROOM)

Another recently developed flux-based method to predict the metabolic state of an organism after a perturbation is regulatory on/off minimization (ROOM) (104). Related to but distinct from MOMA, ROOM is a constraint-based method that aims to minimize the number of changes required to adjust the flux map following a perturbation using mixed integer linear programming. The development of this method was inspired by the observation that organisms undergo a depression in growth shortly after a (genetic) perturbation that coincides with a large change in gene expression. After an adaptation period, gene expression often returns to a state close to the state prior to perturbation (31, 40). By analogy to the minimal change in gene regulation, ROOM minimizes the number of significant flux changes compared with the unperturbed state

(104). ROOM-based predictions of knockout mutant phenotypes closely resemble FBA prediction and match flux patterns after the adaptation phase better than MOMA, but during the adaptation phase MOMA predictions most closely match experimental results (104). A drawback of ROOM-based prediction, as is the case for FBA predictions, is that the solutions are often not unique.

The applicability of ROOM to plant models will depend on the nature of the changes that occur during the adaptation phase. If all changes are regulation based, similar results can be expected for plant systems as those that were found for yeast (104). However, if point mutations (adaptive evolution) underlie the predictive success, perturbations in plant systems cannot be expected to be predicted by ROOM successfully.

Flux-Based Plant Metabolic Rational Design is Within Reach

The approach suitable for rational design of a metabolic pathway depends on the context. Mechanistic kinetic modeling is appropriate for subnetworks in which the understanding of the pathway is detailed. Plant one-carbon and amino acid metabolism, as well as phenylpropanoid and other better-studied areas of secondary metabolism, are good examples. We note that multiple studies in each area are likely needed before the relevant network structure and enzymatic kinetic parameters are sufficiently well understood for more than occasional success.

ROOM and MOMA require only a flux map of the system of interest to make predictions of the effects of perturbations. Both methods can therefore be applied directly to plant models in which flux maps have been generated. Successful prediction of the metabolic adaptation following a perturbation will depend on the quality and the level of detail of the map (current flux maps of central carbon metabolism in plants contain 50–150 reactions). Their potential use in predictive modeling should provide a strong incentive for the production of detailed, validated flux maps of high statistical quality.

Other theoretical approaches to understanding the regulation of metabolism that are not covered in this article also have a part to play in guiding successful rational design. In particular, the lessons of metabolic control analysis should be considered (for explanation and review see References 27 and 87). Especially important is that control over flux is exerted throughout a pathway, so single gene overexpression rarely results in great increases in flux. Consequently, gene overexpression is less likely to be a successful approach to pathway manipulation than gene knockdowns. Mechanistic kinetic models yield flux control coefficients for all the elements in a network, but these coefficients can be obtained independently of such modelling efforts by piecemeal experiments that measure fluxes in perturbed systems.

CONCLUDING REMARKS

Metabolic flux analysis in plants provides a rich description of plant cellular function. Studies to date have yielded insights into the nature and regulation of integrated metabolic processes and have led to the discovery of novel routes and cycles that operate in plant cells and tissues. Metabolic network reconstruction, analysis, and predictive modeling methods developed in microbial systems have seen rapid progress in recent years and there are grounds for considerable optimism about their fruitful application to plants.

With the creation of metabolic flux maps in plants now becoming routine, extra attention should be paid to the quality of flux estimates. The wider use of appropriate and transparent confidence metrics in plant studies will greatly facilitate the interpretation and portability of flux maps. Transparency of flux data will aid in integration into plant systems biology, which provides another incentive for quality and consistency as well as for

the adoption of a common format for the exchange of flux models.

The plant community can utilize the tools for network generation and predictive flux analysis that have been developed by microbial engineers. Predictive modeling is a potent tool for hypothesis testing with regards to the regulation of metabolism, and allows for the systematic in silico evaluation of perturbations, which promises to improve success in the genetic alteration of plant metabolism.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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