Review

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Systems analysis of metabolic phenotypes: what have we learnt?

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Flux is one of the most informative measures of metabolic behavior. Its estimation requires integration of experimental and modeling approaches and, thus, is at the heart of metabolic systems biology. In this review, we argue that flux analysis and modeling of a range of plant systems points to the importance of the supply of metabolic inputs and demand for metabolic end-products as key drivers of metabolic behavior. This has implications for metabolic engineering, and the use of *in silico* models will be important to help design more effective engineering strategies. We also consider the importance of cell type-specific metabolism and the challenges of characterizing metabolism at this resolution. A combination of new measurement technologies and modeling approaches is bringing us closer to integrating metabolic behavior with whole-plant physiology and growth.

Systems biology of metabolism

A key goal of metabolic research is to understand the relation between genotype and metabolic outputs in the context of plant growth and chemical composition [1–4]. This requires detailed comparative analysis and modeling of the behavior of the metabolic network. Changes in the abundance of transcripts, enzyme activity, and metabolite levels have been extensively analyzed in this context [5], largely because their measurement has become routine. However, ultimately, it is metabolic fluxes rather than the levels of individual metabolites or enzymes that determine tissue composition and growth. The relationship between metabolite levels and flux, for example, is difficult to predict, even for simple networks, and is often highly nonintuitive [6]. In fact, in some cases, there may be no relationship, because fluxes can change substantially without significantly perturbing steady-state metabolite levels [7]. The reasons for the apparent disconnect between the accessible measures of the metabolic system (i.e., transcripts, enzyme activities, and metabolite levels) and flux have been discussed elsewhere [8–10]. However, given that it is not possible to predict one from the other with any certainty, measuring or modeling metabolic system fluxes has become a crucial activity in attempts to better understand metabolic behavior. Owing to the technical challenges of quantifying fluxes *in vivo*, it has been a somewhat neglected component of metabolic analysis [11]; however, this has changed in recent years with the maturing of metabolic flux analysis (MFA) methodologies and the emergence of flux-balance analysis (FBA) as a complementary modeling approach. Both these methods generate quantitative models of flux distributions at a network scale and have provided a wealth of information about the metabolic phenotype in a range of plants and tissues. Moreover, both methods are true to the core values of systems biology in that they require an integration of experimental data into computational models that predict flux (Box 1).

In this review, we focus principally on metabolic flux analysis because it is the only systems biology approach that provides direct access to the metabolic phenotype. Moreover, there have been several excellent reviews that have comprehensively covered the systems analysis of metabolism at the levels of transcripts, enzyme activities, and metabolites [5,12]. We also summarize what has been learnt from recent flux analyses and modeling, outline some of the challenges that must be met to obtain a true picture of the metabolic phenotype, and discuss some of the practical limitations of metabolic phenotyping.

Metabolic network flux maps

Metabolic flux maps from stable isotope labeling have been generated for a range of heterotrophic plant tissues, mainly cultured seed embryo systems or cell suspension cultures [13] and the first forays into network flux analysis in photosynthetic tissues of plants [14] and microorganisms [15] have been made using dynamic labeling. In addition, flux-balance models of plant metabolic networks are being generated at an accelerating rate and these can provide comprehensive (up to genome-scale) flux maps with no practical restriction on tissue type or scale [16,17]. Thus, it is possible to integrate flux models of different cell types [18]. Indeed, whole-plant models are conceivable and, ultimately, it should be possible to integrate multitissue metabolic models with whole-plant growth models [19]. There is now a substantial body of careful work in which

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Box 1. Principles of metabolic flux analysis and flux modeling

The two main approaches for the detailed analysis of metabolic network fluxes are stable isotope MFA and FBA. In both methods, certain experimental measures are used to constrain the flux solution space, but different approaches are used to derive quantitative estimates of fluxes.

Steady-state stable isotope MFA

The steady-state stable isotope MFA approach involves heterotrophic or mixotrophic cells or tissues that are supplied with a labeled precursor substrate, typically positionally ^{13}C-labeled glucose. Cells or tissues are cultured for several days on this substrate until all intracellular carbon-containing compounds are labeled to an isotopic steady state. The labeling pattern of a range of molecules (i.e., the fractional enrichment of specific carbon atoms within the molecule or fragments of the molecule) provides information about flux ratios at branch points in the network, because flux through different branches will lead to different labeling patterns. Typically, the molecules that are analyzed include amino acids (both from protein hydrolysate and free amino acids), sugars (from cell wall and starch hydrolysate and free sugars), and carboxylic acids. The inputs and outputs of the metabolic network are also quantified to place bounds on the possible intracellular fluxes. The inputs will comprise the rate of consumption of glucose and other macronutrients (such as nitrogen) and, in the case of mixotrophic tissues, light intensity. The outputs are the main biomass polymers (starch, cell wall, lipid, and protein) and abundant metabolites, such as carboxylic acids and sugars. To estimate fluxes from these data, a metabolic network model is constructed comprising the stoichiometry and carbon transitions of all known reactions in the network. This model is used to generate predicted labeling patterns of molecules given a random set of fluxes and these patterns are compared with the measured labeling patterns. The fluxes are varied, the labeling patterns compared again, and the process iterated until the difference between predicted and measured labeling patterns has been minimized.

FBA

FBA has some similarities with MFA, namely the requirement for a stoichiometric network model and experimental measurements of inputs and outputs to constrain the flux solution space. However, rather than derive fluxes from labeling data, fluxes are estimated by making the assumption that network fluxes have been optimized during the evolutionary history of the plant by the forces of natural or artificial selection. The optimization problem is solvable by defining an objective function (such as maximization of biomass or minimization of the sum of fluxes) to be optimized and the use of linear or quadratic optimization algorithms to identify flux solutions that satisfy this objective function within the constraints of the network stoichiometry and defined inputs and outputs.

metabolic network fluxes have been described in detail for several plant species and tissue types. This work has provided an unprecedented view of the metabolic phenotype and afforded the opportunity to examine metabolic behavior *in vivo* in some detail. This has led to important new insights into how metabolism is configured in different systems and tissues. Flux analysis has also revised the view of the nature of metabolic processes: metabolic reactions can now be considered in the context of the broader network as opposed to being enshrined in specific pathway configurations. Depending on the conditions, reactions can operate in different directions and the connections between reactions can be reconfigured. This is most apparent in central hubs of metabolism, such as the tricarboxylic acid (TCA) cycle [13,20]. Nevertheless, because of the technical nature of flux analysis and the complexity of the networks being described, it is difficult to gain an overview of any general findings that have emerged from this work. Therefore, our aim here is to take a step back from the details and ask a more general question: what have we learnt from analysis of the metabolic flux phenotype?

Metabolic control by outputs?

Perhaps one of the more surprising findings is the extent to which FBA can accurately predict network fluxes in plant cells [21–23]. This is surprising because the method makes no reference to enzyme kinetics or regulation. Does this mean that enzyme kinetics and regulation are not important and that we should reconsider our view of metabolic regulation? Not at all, but there is an important implication that can be drawn from the predictive success of FBA: namely that enzyme regulation is not necessarily a key driver of the flux distribution across the network. Instead, enzyme regulation (by which we mean allosteric regulation and post-translational modifications that affect enzyme activity) should be viewed as a mechanism for maintaining metabolic steady state by preventing the accumulation or depletion of metabolite pools and not as a mechanism that sets the flux levels. A classic example of this is the change in TCA cycle activity in leaves between light and dark. It is well known that pyruvate dehydrogenase is inhibited in the light and this restricts entry of carbon into the TCA cycle. However, the important point is that the regulation of pyruvate dehydrogenase is a means of enabling the system to respond to the requirements of light metabolism. Specifically, the activation of photorespiration requires the TCA cycle to be reconfigured as an NADH-exporting metabolite shuttle to deal with the excess reductant generated by the mitochondrial steps of photorespiration. The trigger for altered pyruvate dehydrogenase activity is the increase of mitochondrial matrix concentrations of NH_4^+ and NADH from photorespiration, which leads to inactivation of the enzyme owing to their effects on the kinase and phosphatase that control its phosphorylation state [24].

Arguably, enzyme kinetic and regulatory features enable a metabolic steady state to be maintained within the constraints imposed on the metabolic system [25]. Because FBA is a constraints-based method, we can examine these constraints in more detail. Typically, FBA uses three main constraints: first, the inputs into the system, for example carbon source, nitrogen source, and light; second, the main outputs of metabolism, which generally are the end products of metabolism (the polymers and stored metabolites that constitute the new biomass of growing cells); third an 'objective function' to which the system is optimized. This constraint assumes that the system has been optimized by natural or artificial selection towards a specific goal. For a crop plant bred for yield, maximum synthesis of biomass per unit carbon input is a realistic objective function [26]. By contrast, for a wild plant such as *Arabidopsis* (*Arabidopsis thaliana*) adapted to nutrient-poor soils, minimization of the sum of fluxes in the network (a proxy for minimal investment in enzyme machinery) generates realistic flux predictions [23]. In fact, these objective functions are similar, with both leading towards optimal