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Modeling Plant Metabolism: From Network Reconstruction to Mechanistic Models

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Abstract

Mathematical modeling of plant metabolism enables the plant science community to understand the organization of plant metabolism, obtain quantitative insights into metabolic functions, and derive engineering strategies for manipulation of metabolism. Among the various modeling approaches, metabolic pathway analysis can dissect the basic functional modes of subsections of core metabolism, such as photorespiration, and reveal how classical definitions of metabolic pathways have overlapping functionality. In the many studies using constraint-based modeling in plants, numerous computational tools are currently available to analyze large-scale and genome-scale metabolic networks. For ^{13}C-metabolic flux analysis, principles of isotopic steady state have been used to study heterotrophic plant tissues, while nonstationary isotope labeling approaches are amenable to the study of photoautotrophic and secondary metabolism. Enzyme kinetic models explore pathways in mechanistic detail, and we discuss different approaches to determine or estimate kinetic parameters. In this review, we describe recent advances and challenges in modeling plant metabolism.

Contents

1. INTRODUCTION

Model: representation of a system or process by a mathematical formalism (e.g., algebraic equations), programming language, or both

Metabolic engineering: the directed modification of metabolism based on recombinant DNA technology toward the production of desired chemicals

Metabolic flux: rate at which matter is transported through the multiple reactions of a metabolic pathway

Metabolic network: series of linked biochemical or transport reactions that can achieve several distinct overall net biochemical transformations

With a growing global population, increasing demands for food and feed and a shortage of arable land have led to a need to enhance crop yields and improve resistance against abiotic and biotic stresses (105). At the same time, to shift away from society's dependence on fossil fuels, the development of carbon-neutral, renewable, plant-based resources for bioenergy and biomaterials is urgently needed (110). Plant sciences play an important role in addressing these challenges. There is a rapidly growing number of sequenced plant genomes (94), and owing to the recent applicability of precise, RNA-guided genome editing tools to model and crop plants (9, 17), the redesign of plant metabolism has become increasingly feasible. The metabolic engineering design-build-test cycle in crop plants and functional genomics research efforts in model and crop plants can be expected to benefit from metabolic modeling (41, 130). Metabolic modeling and metabolic flux analysis (MFA) often reveal robustness of metabolism against different environmental or genetic perturbations, which is why these approaches have proved to be important components during decades of metabolic engineering of industrial microbes (71, 74). With regard to plants, current efforts to improve photosynthetic carbon assimilation and crop yield include implementing C_4 photosynthesis and designing and testing synthetic pathways to reduce carbon loss during photorespiration (162).

In this review, we first describe the importance and challenges of defining the metabolic map of a plant cell. We then give an overview of frequently used methods for metabolic network and flux analysis in plants: (*a*) metabolic pathway analysis of smaller subsections of cellular metabolism, (*b*) constraint-based analysis of large-scale models, (*c*) isotope tracer–assisted in vivo flux analysis, and (*d*) models relying heavily on explicit enzyme kinetics.

2. DEFINING THE METABOLIC MAP

Plant metabolism is particularly complex in its multicellularity, subcellular compartmentation of metabolic pathways, and redundancy of pathway functions. For example, to generate the five-carbon building blocks used to synthesize the structurally diverse group of terpenoid metabolites, vascular plants use two distinct biosynthetic pathways: the acetate/mevalonate pathway and the 2-*C*-methyl-D-erythritol 4-phosphate pathway (86, 156). Other organism groups most often

ENTNER-DOUDOROFF PATHWAY IN PLANTS

The Entner-Doudoroff (ED) pathway is a variant of the glycolytic pathways long believed to occur only in several prokaryotic taxa. Recent evidence supports the presence of this pathway in cyanobacteria, several algal taxa, mosses, ferns, and flowering plants (21). The ED pathway intersects with both the Embden-Meyerhof-Parnas pathway of glycolysis and the oxidative pentose phosphate pathway but has two unique enzyme-catalyzed steps, transforming 6-phosphogluconate into glyceraldehyde 3-phosphate and pyruvate (phosphogluconate dehydratase, 2-keto-3-deoxygluconate-6-phosphate aldolase). To what extent and under which physiological conditions the ED pathway is used in cyanobacteria and flowering plants have not yet been explored, but the ED pathway might be particularly relevant under mixotrophy, i.e., when reduced carbon and light are available at the same time (21).

possess only one of these pathways (156), but in plants, these pathways reside in parallel in the cytosol and plastid compartments, respectively. Plants feature more of such parallelisms, for example, with regards to glycolysis. The presence of ATP- and pyrophosphate-dependent phosphofructokinase enzyme isoforms in plant cells (both allosterically regulated) is thought to contribute to plants' metabolic plasticity and ability to cope with various biotic and abiotic stresses (107). Plants seem to be unique in harboring both enzyme types at the same time (93). Such parallelism and redundancy might easily evade detection by biochemists. Therefore, core metabolism in plants likely still hides many surprises, such as the very recent detection of the Entner-Doudoroff pathway in cyanobacteria and barley (21) (see the sidebar titled Entner-Doudoroff Pathway in Plants).

Technological advances in DNA sequencing and the exponential decrease in sequencing cost have led to a genomic revolution. Currently, more than 200 angiosperm genomes are publicly accessible (19). High-quality genomes encode the complete metabolic inventory of an organism. The representation of all genome-encoded metabolic functions in a knowledge database can be understood as the metabolic potential of an organism and can be transformed into a computable format (84). What currently limits this approach is the lack of detailed experimental information available for genome annotation. Since it is clearly impossible to characterize all the genes that are identified in each newly sequenced genome, homology-based gene annotation is regularly relied upon (121, 150). Although much effort has been put into the development of advanced tools specifically for inference of enzyme function, such as the Ensemble Enzyme Prediction Pipeline (126), such approaches are ultimately dependent on a gold standard set of experimentally characterized proteins. Transfer of the full enzyme function (e.g., as defined by all four digits of the Enzyme Commission number) is required to unambiguously define reaction stoichiometry in metabolic models. For transfer of function via protein sequence homology, enzyme function may start to diverge quickly below 70% pairwise sequence identity (121). Even for intensely studied organisms such as yeast or humans, a substantial fraction of enzymes may have ambiguous or false annotations, a finding that is part of the problem of the "catalytic dark matter" (36). For *Arabidopsis thaliana*, an estimated 57% of the enzymes in AraCyc 6.0 are missing direct experimental evidence (174).

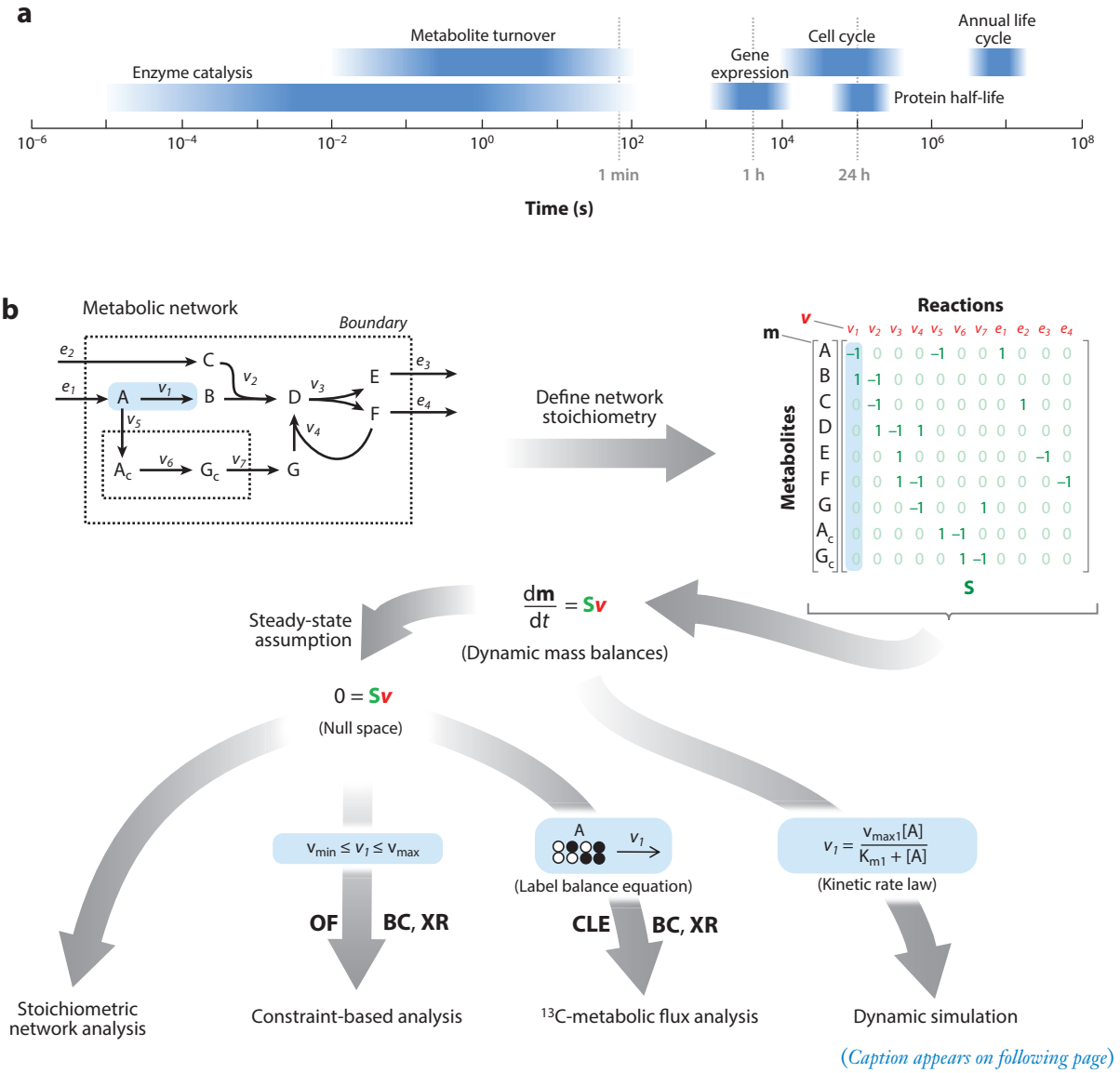
In addition to bona fide metabolic enzymes, a substantial part of the unexplored enzyme inventory may be associated with metabolite damage control. Alongside the intensely studied DNA damage and repair mechanisms, researchers are increasingly considering the harmful effects of the accumulation of pathway intermediates damaged by chemical instability (50, 63). Another challenge for metabolic modeling is enzyme promiscuity, which relates to relaxed substrate and reaction specificity (72). Enzyme promiscuity is prevalent in plant specialized (secondary) metabolism (164), making it hard to define accurately in stoichiometric metabolic networks.

Metabolic pathway: series of linked biochemical or transport reactions that cooperate to catalyze one overall net biochemical transformation; elementary flux modes, for example, are a mathematically strict definition of pathways

Reaction: biochemical transformation of small molecule substrates to one or several products, or transport of small molecules across a membrane

3. MODELING TECHNIQUES

An important principle underpinning mathematical modeling of biological systems is that of characteristic time hierarchy or timescale separations (47, 55). The cellular processes taking place in an organism and environmental influences operate at very different timescales (**Figure 1*a***). Metabolism and gene regulation are often considered different layers that are most often modeled separately, and integrative approaches to unify these layers are challenging (44). Metabolism can be simulated dynamically based on systems of ordinary differential equations that include enzyme kinetic rate laws (**Figure 1*b***). Since information on enzyme kinetic parameters, particularly for larger metabolic networks, is often limited, other approaches are followed. Many studies on metabolism invoke an assumption of steady state or quasi steady state. This simplification again



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Principles and approaches to metabolic modeling in plants. (*a*) A time hierarchy of biological processes can be defined based on their response times (i.e., the amount of time needed to adjust to a perturbation) (55). Shown are approximate response times (enzyme catalysis, metabolite turnover, gene expression, and protein half-life) in relation to the developmental and cyclic processes that are inherent to an organism and strongly influenced by its environment (cell cycle, diurnal cycle, and annual life cycle of an annual plant). Based on the ranges of response times, metabolism can adjust more quickly than changes in gene expression or protein levels occur in response to environmental changes (see also **Supplemental Table 1** for information on how these times were estimated). (*b*) The mathematical bases that are shared by or distinguish between the major modeling approaches discussed in this review are illustrated, starting from the structure of a metabolic network and its encoding into a stoichiometric matrix (**S**) and vectors of metabolite concentrations (**m**) and reaction rates (**v**). Expressions that are typical for each approach are exemplified by the reaction v_1. Based on the stoichiometric network, the mass balances around all metabolites can be augmented by kinetic rate laws for analysis and simulation of the system dynamics. For a constant physiological setting, metabolite levels and reaction rates can be assumed to be constant (steady-state assumption). **Sv** is a linear equation system describing a multitude of possible steady-state flux states (null space). Stoichiometric network analysis can be used to reveal all distinct pathways within the metabolic network. Constraint-based modeling and ^{13}C-metabolic flux analysis explore flux states, often by integrating additional information on cellular biomass composition (BC), measured rates of exchange with the environment (XR), objective function (OF), and carbon labeling experiments (CLE). Other abbreviations: A, concentration metabolite A; K_m, Michaelis binding constant; v_{max1}, maximal velocity for reaction v_1.

relates to timescale separation. If we assume that cell-internal metabolic processes change much faster than environmental conditions and the external signals affecting the cell (**Figure 1*a***), then metabolism can be viewed from a perspective of metabolic quasi steady state (154), a basic concept to stoichiometric modeling and the different derived MFA approaches (**Figure 1*b***).

Supplemental Material >

3.1. Stoichiometric Network Analysis

Methods of stoichiometric network analysis, also termed pathway analysis, are important to characterize a metabolic process at a structural or stoichiometric level by systematically inspecting all distinct overall network conversions (127). Methods of stoichiometric network analysis require only the definition of a network based on reaction stoichiometries and reaction directionality. Elementary flux modes (EFMs) are minimal and distinct sets of network reactions that can operate together at steady state and are independent from the rest of the network in catalyzing a net transformation between certain network inputs and outputs with a characteristic stoichiometry (125, 127). A mostly equivalent type of network analysis is extreme pathway analysis (123). In short, extreme pathways can essentially be understood as a subset of EFMs, and all possible steady states of a network can be defined by linear combinations of the extreme pathways (76, 123, 124).

3.1.1. Elementary flux modes. An example of the use of EFM analysis is in a study on the biochemical control of the accumulation of sucrose in sugar cane culm tissue (117). To devise metabolic engineering strategies to enhance sucrose content, the authors derived an enzyme kinetic model. In doing this, they first analyzed the stoichiometry of the considered 10-reaction network and found 14 EFMs, which were further categorized into (*a*) six modes resulting in synthesis of sucrose from glucose and fructose and storage in the vacuole, (*b*) five modes describing cyclic sucrose synthesis and degradation of sucrose without storage accumulation, and (*c*) three distinct modes of glycolytic degradation of glucose or fructose. Since multiple modes that perform the same overall conversion can be recognized, EFMs give an understanding of pathway redundancy in a system. Also, as all possible conversions in the system are explored, EFMs give a quantitative measure of network flexibility or robustness. With increasing size of a network under investigation, the enumeration of all possible EFMs can quickly become a challenge due to a combinatorial explosion of the number of identified pathways and the limitation given by computer memory. To address this problem, various extensions of the EFM concept to large networks have been developed that are not further discussed here (146).

While the comprehensive analysis of cellular stoichiometric networks became a focus in plant modeling in recent years (see Section 3.2), the characterization of the flexibility of subsections of central metabolism by EFM analysis is still relevant. For example, the tricarboxylic acid (TCA) cycle is known as a central hub between catabolism and anabolism. Apart from the commonly understood role of the TCA cycle in oxidative degradation of substrates to drive mitochondrial ATP synthesis, several noncyclic modes of the TCA cycle are part of metabolic conversions required under different physiological conditions (142), which can be exhaustively enumerated by EFM analysis (13, 140). Besides the TCA cycle, the oxidative pentose phosphate pathway (OPPP) has been recognized as another versatile subsection of central metabolism. Schuster et al. (128) analyzed a 19-reaction network combining OPPP with glycolysis, resulting in seven functional modes. EFM analysis has also been applied, in parallel with ^{13}C tracer experiments, to identify pathways with optimal conversion of sugars to fatty acids in developing seeds (132). In this work, some aspects of the integrated operation of glycolysis, OPPP, and the Calvin cycle were demonstrated.

3.1.2. Applications of stoichiometric network analysis. EFM analysis has also been used to systematically dissect functional modes of the photorespiratory cycle, which is an integral part of C_3 photosynthesis that deals with the consequences of RubisCO oxygenation activity. Reactions of photorespiration stretch across four subcellular compartments, and the cycle is intertwined with nitrogen assimilation and other processes (10, 59). In a recent study, EFM analysis was used to inspect a network of 90 reactions, comprising the photorespiratory cycle and associated processes in the plastid, peroxisome, mitochondrion, and cytosol as well as metabolite exchanges between these compartments (64). Fifty-six flux modes were identified with the RubisCO oxygenase reaction being active, revealing the complex coupling of photorespiratory activity with the reduction of nitrate to ammonia, mitochondrial metabolism, cytosolic ATP production, and the glutathione-ascorbate cycle. To further confirm how the 90-reaction network represents the process of photorespiration, the authors compared gene essentiality within the network to known photorespiratory mutants. There are 43 reactions that are active in all 56 photorespiratory flux modes. The authors found an overall good agreement between the corresponding genes and mutants known to negatively affect photorespiration (64).

3.2. Constraint-Based Modeling

Constraint-based modeling (CBM) is concerned with metabolic networks at the cellular scale and beyond. A complete set of biochemical reactions of an organism, as defined by its genome, is known as a metabolic reconstruction (84), and its mathematical representation is referred to as a genome-scale model (GEM) (84). Methods of CBM explore the range of possible flux states of a GEM, given the requirements of maintaining steady state (reaction stoichiometry) and staying within the limits assigned to the rates of individual exchange reactions, such as measured maximum substrate uptake rates (109, 112). Here, it is important to realize that the mathematical constraints used in CBM typically allow a larger space of achievable cellular flux states (solution space) (103). Flux balance analysis (FBA), the most basic and widely used method, finds flux states that are optimal with respect to a presumed optimality criterion (102). The optimality criterion, or objective function, may state that an organism is optimized to grow with a maximal possible growth rate, to produce cellular energy most efficiently (maximal ATP yield), or to distribute flux load across the network to use enzyme capacity most efficiently (137). In addition to FBA, a substantial repertoire of other computational methods has been developed, including analysis of gene deletions and methods for adding constraints based on various types of omics data (75, 84). For further reading on general

concepts and applications of CBM, the primer by O'Brien et al. (103) is recommended. Detailed protocols for network reconstruction and simulation are recommended as well (57, 149).

Simulation: various kinds of numerical calculations that are performed with a model

3.2.1. Challenges in the construction of plant genome-scale models. Reconstruction of a metabolic network starts from genomic, biochemical, and literature data. Model reconstruction is an arduous task. In some cases, the reconstruction of microbial genome-scale networks has been driven by community efforts (54, 148). **Supplemental Table 2** lists 35 published GEMs for more than 10 seed plant species. Of these GEMs, 11 are of the model plant species *A. thaliana*. Yuan et al. (171) detected substantial discrepancies in model-predicted fluxes between three of the *A. thaliana* models when the same modeling constraints and objective functions were applied. This might reflect varying approaches in network reconstruction. Most often, the primary source of information for *A. thaliana*–specific biochemical reactions and pathways is AraCyc (174), and less frequently, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (69). Models for other plant species listed in **Supplemental Table 2** mostly rely on pathway reconstructions from the Plant Metabolic Network (PMN) database (126). PMN and KEGG plant pathway resources mostly lack information on subcellular localization of enzymes and on transmembrane transport functions. While genome-wide computational predictions and experimental data on subcellular localization of *A. thaliana* gene products are available online (60, 70), not all localization predictions are of high confidence, and some are ambiguous. Therefore, comprehensive resolution of subcellular compartmentation of genome-scale metabolic plant networks remains challenging. A part of the challenge is the limited knowledge on transmembrane transport reactions, which in many cases are needed to connect pathway modules across membranes. An estimated 1,800 *A. thaliana* genes could encode membrane proteins with transport functions (129). Lacking specific knowledge on intracellular transport, such reactions can be added by computational inference based on algorithms that minimize the number of transmembrane transports that would be required to render a network functional (96).

Supplemental Material >

Genome-scale plant models typically contain more than 1,000 reactions (**Supplemental Table 2**). Information on reaction direction and irreversibility is essential to flux modeling. In a strict sense, derivation of these properties requires knowledge of the Gibbs free energy of a biochemical reaction and of the reactant concentrations (79). In practice, directionality information is often adopted from existing models and databases, derived by a standardized set of heuristic decision rules (52), or, at best, could be defined using group contribution methods for estimating the standard Gibbs free energies for metabolites and reactions (67).

3.2.2. Defining cellular maintenance energy requirements. Metabolic pathways and processes that consume energy cofactors [e.g., ATP, NAD(P)H] put a demand on cofactor producing processes; however, not all cellular energy–consuming processes are explicitly defined by the biochemical reaction network. A substantial part of a plant cell's energy budget is allocated to cell maintenance processes (34). Cellular maintenance is defined as the energy expenditures [e.g., ATP, NAD(P)H] required for functions that do not directly fuel cellular growth and includes costs due to protein turnover, transport, membrane leakage, or ATP dissipation in substrate cycles (144). Such additional costs are typically added to the model as a fixed flux through a generic ATPase reaction. In experimental settings where tissues or cell cultures grow heterotrophically and substrate uptakes can be measured accurately, maintenance ATP cost can be assessed by increasing the flux of the generic ATPase in the model until the model-predicted uptake of carbon substrates matches the measured uptake (i.e., until a measured carbon conversion efficiency is reached) (52, 108). However, modeling cellular maintenance costs solely as ATP consumption might be unrealistic, since oxidative stress can also add redox expenditures, which have to be paid in production

of NADPH that is above the cellular demands for biosynthetic purposes (144). In the case of *A. thaliana* cell cultures, flux through the OPPP and accompanied NADPH production was measured by in vivo flux analysis (^{13}C-MFA). The NADPH production was about 50% higher than the cellular biosynthetic demands, and the surplus was accounted for by a generic NADPH oxidase reaction (22).

3.2.3. Modeling of multicell interactions. Since plants are multicellular organisms, models representing different cell types and their interactions are important. A primary metabolic reconstruction represents the general metabolic potential of the plant. A cell- or tissue-type-specific model can be derived by defining the subsets of reactions present in that particular cell or tissue based on transcriptome and proteome data (75, 89). Various plant models that are generated by connecting cellular models representing different cell types have been reported. For example, interactions between mesophyll and bundle sheath cells in C_4 photosynthesis (14, 32, 91) and between different tissues in developing seeds (15, 118) have been studied by CBM. Other studies have explored whole-plant systems represented by interconnected leaf, stem, and root cell models (33, 136, 173). Some of these studies have also taken account of the pronounced differences between day and night metabolism, in that starch accumulation in leaves during the daytime serves as energy storage for the nighttime (33, 136). Such whole-plant diel models are entirely simulated based on a steady-state approach as two replicate plant modules, representing day and night metabolism, which balance each other.

Another example of multicellular models is rhizobial symbiosis in legumes. Pfau et al. (106) reconstructed a general genome-scale metabolic network for *Medicago truncatula*, a model plant for legume-rhizobia mutualism. The rhizobia bacteria reside in root nodules, where they fix atmospheric nitrogen with an oxygen-sensitive nitrogenase and trade the nitrogen to the plant in exchange for carbon resources. Although nitrogen fixation in the bacterial symbiont requires a low-oxygen environment, oxygen is needed for the efficient generation of ATP by oxidative phosphorylation, so the nodule is unlikely to be a completely oxygen-free environment. To discern how oxygen levels can affect rhizobia metabolism and the mutualistic benefits of carbon-nitrogen trade, Pfau et al. (106) developed models for *Sinorhizobium meliloti* rhizobia as well as *M. truncatula* root- and shoot-tissue-specific models. Here, the tissue-specific models were created using the gene-protein-reaction associations in the general network reconstruction and either permitting reactions to be on or constraining them to be off based on differential expression of the associated genes (12). Together, the three models simulated the transport of photosynthetically fixed carbon to the roots, where it was then transported into the nodules in exchange for a nodule metabolite. The identity of the metabolite that entered the nodule was significantly affected by the oxygen concentration (106). When oxygen was plentiful, the rhizobia would import succinate and use it via the TCA cycle for ATP generation (oxidative phosphorylation). However, as oxygen levels decreased, the metabolite imported was more likely to be malate, which produces less NADH in the TCA cycle. Consequently, this study reveals how carbon use in rhizobia is jointly affected by metabolic needs and the nodule environment.

3.3. In Vivo Flux Analysis

Like stoichiometric network analysis (Section 3.1) and CBM (Section 3.2), in vivo flux analysis is built around the key concept of a metabolic network and its mathematical representations (**Figure 1**). However, instead of invoking an optimality principle to calculate fluxes, in vivo flux analysis integrates isotope tracer experiments with network stoichiometry of central metabolism to measure metabolic flux in a living cell (165). ^{13}C-MFA studies uncover the integrated

functioning of central metabolism and can reveal regulatory circuitry if flux maps derived under different physiological conditions are compared. Plant central metabolism tends to be recalcitrant to attempts of redirecting fluxes (143). Studying this idea of rigidity in plant central metabolism has been part of the motivation behind numerous plant ^{13}C-MFA studies over the past 20 years (**Supplemental Table 3**).

Supplemental Material >

Important mathematical and theoretical concepts for ^{13}C-MFA were originally developed in the field of microbial metabolic engineering, and a great diversity of analytical protocols, computational methods, and software tools has emerged (101). Among the five major isotope tracer–based flux analysis approaches that were distinguished by Niedenführ et al. (101), the isotopic stationary and nonstationary ^{13}C-MFA approaches have been of major relevance in recent plant studies. Upon feeding of the labeled precursor, redistribution of substrate label through the metabolic network can be traced in a dynamic way or after an isotopic steady state is reached (101) (**Figure 2*a***). The flux distribution in the central metabolism biochemical network is then indirectly observable based on a best fit between the empirical data (i.e., labeling signatures, measured fluxes such as substrate uptake rates) and computationally predicted labeling states and fluxes (165). State-of-the-art algorithms used in this process represent labeling states of metabolites as cumulative isotopomers (cumomer) fractions or are based on elementary metabolic unit network decomposition (**Figure 2*b***).

3.3.1. Steady-state ^{13}C-metabolic flux analysis. The stationary approach has been applied quite frequently to plant cell or tissue cultures if they can be grown heterotrophically while being fed a ^{13}C-labeled substrate. While not all of the significant work can be discussed here, 18 of the studies surveyed in **Supplemental Table 3** applied the steady-state ^{13}C-MFA approach. Typically, a culture grows for a prolonged time on a labeled substrate until the cell mass has doubled multiple times and an isotopic steady state is reached (**Figure 2*a***). The duration of the culture can be between 5 and 20 days (**Supplemental Table 3**). Critical to the stationary approach is awareness that during such prolonged incubation, the physiological and metabolic state of a culture may shift significantly. Also, substantial cellular heterogeneity might be present in a cultured tissue. In both cases, the flux modeling process might give a distorted picture of cellular metabolism. More detailed discussion on this issue can be found elsewhere (111, 119). Also, for the best resolution of a flux map in ^{13}C-MFA, the choice of isotope label is important, and it is critical to obtain as much labeling information as possible from metabolites relevant to the studied metabolic network (100). Isotope label can be quantified by techniques based on ^{13}C-nuclear magnetic resonance or mass spectrometry (**Figure 2*a***). The information readout differs between the two analysis types, and combining both may better resolve the isotope labeling state of a molecule (2, 100, 111). **Figure 2*b*** shows the relationship between a mass spectrometry readout (mass isotopomer fractions) and the carbon isotope labeling state that is fully described by isotopomer or cumomer fractions. In extension of established mass spectrometry analytical protocols (2), the use of tandem mass spectrometry has recently been explored (8). Also, the flux modeling process can benefit substantially from a priori design of the isotope labeling experiments, in which mixtures of labeled substrates can be computationally evaluated for optimal flux identifiability prior to the real experiment (100). Furthermore, the resolution of flux maps in plant studies has been enhanced by the use of parallel labeling experiments, in which different substrate labels are used in a series of parallel experiments and the resulting complementary labeling information is integrated into one simulation (29, 85, 134). This approach has been applied to flux studies on central metabolism flux in developing seeds of *Brassica napus* (68, 134), *Glycine max*, and *Helianthus annuus* (1, 4–6) as well as *Zea mays* kernels and root tips (6, 7). Another feature that is useful with the steady-state ^{13}C-MFA approach is the readout of compartment-specific labeling information if amino acids are analyzed. For example,

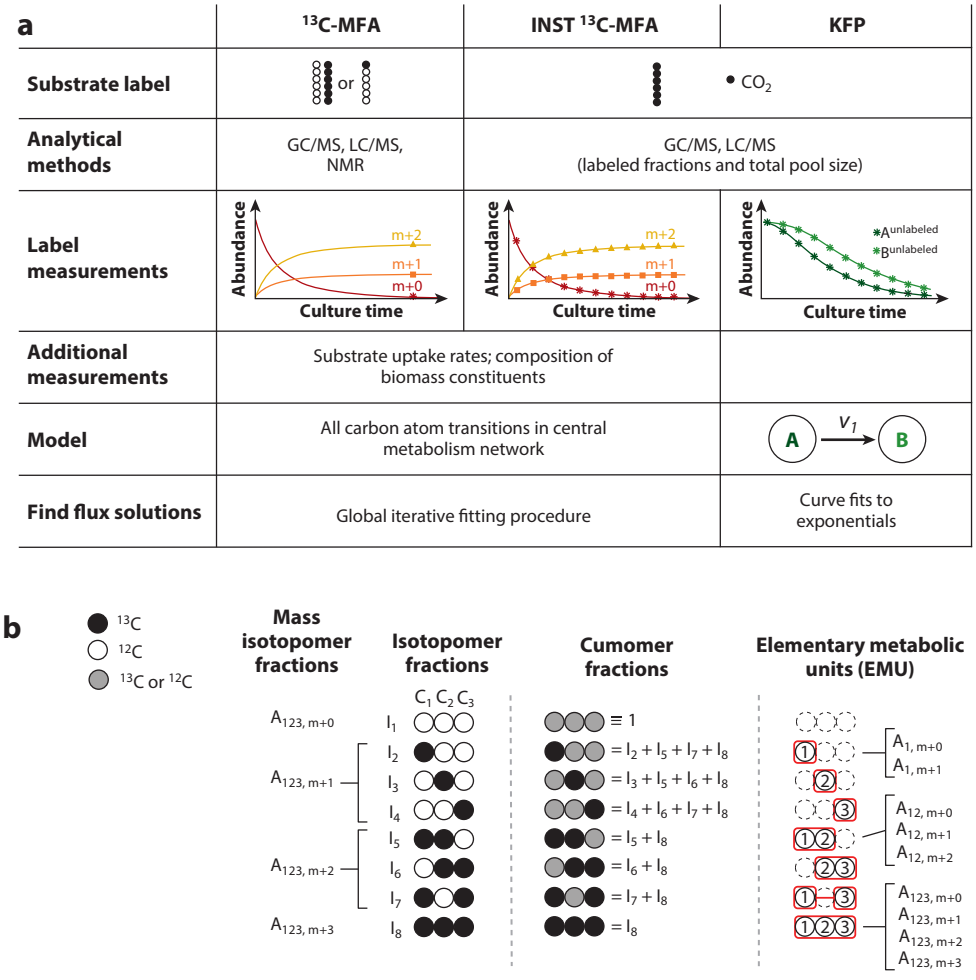


Figure 2

Flux modeling using ^{13}C labeling experiments. (*a*) In traditional ^{13}C-metabolic flux analysis (^{13}C-MFA), substrates of mixed isotopic composition are fed to the biological cells or organism, such as a mixture of labeled (*black circles*) and unlabeled (*white circles*) substrates or substrates that are positionally labeled, while uniformly labeled substrates are often fed in isotopically nonstationary ^{13}C-MFA (INST ^{13}C-MFA) and kinetic flux profiling (KFP). Analytical methods are applied to the variants of ^{13}C-MFA to determine mass isotopomer or positional label information based on gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), or nuclear magnetic resonance (NMR) spectroscopy. KFP also uses GC/MS or LC/MS measurements, but the subsequent analysis uses the decay kinetics of unlabeled molecular species without considering positional labeling information; thus, the labeling isotope could be ^{15}N or ^{2}H instead of ^{13}C. (*b*) Different isotopomer systems used in ^{13}C-MFA to represent labeling states in the network. For a three-carbon compound (A), eight isotopomer fractions (I) fully describe any possible labeling state. The same information can be represented as cumomer fractions or as mass weights in elementary metabolic units (EMU) to reduce the computational time necessary for simulations of isotope balanced networks (see 169 for more information on these mathematical representations). Red boxes denote the atoms that are included in an EMU.

valine is synthesized from pyruvate in the chloroplast. Therefore, the valine labeling signature yields information about the chloroplast-specific pool of pyruvate (3, 131). This principle aids in resolution of subcellular compartmentation in flux maps.

Recent studies have shown how flux measurements in primary metabolism can be combined with other biochemical data to advance understanding in pathway regulation (16, 133). In studying variation in seed composition among nine genotypes of oilseed rape (*B. napus*), Schwender et al. (133) measured metabolite levels, flux, and enzyme activity profiles in central metabolism as well as proteome data in parallel. While it formerly had been shown that in oilseed rape, a substantial part of the carbon flux from sugars into lipids can proceed via the carbon-efficient RubisCO shunt (132), the correlative multilevel analysis of flux, metabolome, and enzyme data revealed potential regulatory mechanisms with regards to control of glycolytic flux and a lipid/starch trade-off. The findings suggest that plastid pyruvate kinase activity controls flux through the phosphofructokinase step of glycolysis and ADP-glucose pyrophosphorylase via an allosteric feedback mediated via phosphoenolpyruvate and 3-phosphoglycerate, respectively. **Figure 3** shows results from simulations of an enzyme kinetic model that incorporates these regulatory features. It illustrates how the flux ratio at the glucose 6-phosphate branch point can be controlled only by the level of pyruvate kinase activity (parameter v_{max}), while the model simulations also recapitulate the major changes in metabolite levels (**Figure 3*b***).

3.3.2. Nonstationary ^{13}C-metabolic flux analysis. In addition to stationary ^{13}C-MFA, transient labeling approaches with more complex workflows have been developed for plants over the past decade (18, 167). In isotopically nonstationary ^{13}C-MFA (INST ^{13}C-MFA) and kinetic flux profiling (KFP), isotope labeling of metabolic intermediates is measured in a time series to capture the transient of the system toward approaching isotopic steady state. Only transient labeling approaches are amenable to exploration of photoautotrophic systems with CO_2 as the sole carbon source because when reaching steady state, every carbon comes from $^{13}CO_2$, and hence labeling patterns become uninformative (135). For central metabolism, transient labeling approaches require direct measurement of intermediates of glycolysis, the Calvin or TCA cycle, or other pathways, and their respective pool sizes need to be measured or estimated for the modeling process. A comprehensive flux map of 76 fluxes of photoautotrophic central metabolism, based on the INST ^{13}C-MFA approach and feeding $^{13}CO_2$, was first achieved using the unicellular cyanobacterium *Synechocystis* (168), followed by a similar study resolving fluxes in photosynthesizing *A. thaliana* rosettes (88). Ma et al. (88) used $^{13}CO_2$ labeling and INST ^{13}C-MFA to study how primary photosynthetic metabolism in *A. thaliana* adjusts if plants grown at low light (200 μmol $m^{-2}s^{-1}$) are acclimated for a prolonged time to 500 μmol $m^{-2}s^{-1}$. As expected by measurements of the net photosynthetic rate, RubisCO carboxylation flux was increased in the acclimated plants. In addition, in acclimated plants, a relatively higher proportion of carbon was lost to photorespiration and more was diverted to sucrose export, while the relative flux toward starch synthesis was decreased under high light. This study highlights that the ability of plants to adapt to different light levels is important for their survival and biomass productivity. It also points out the need to obtain flux maps under various environmental conditions.

3.3.3. Other nonstationary labeling approaches. While INST ^{13}C-MFA is based on network-wide isotopomer balancing, nonstationary labeling is frequently being used in other, less generalized modeling frameworks. For example, KFP has been used to explore autotrophic metabolism (58, 145, 172). The approach results in more localized flux information (**Figure 2*a***). Also, there are limitations for determination of flux through reversible reaction steps. In what is referred to as gross flux in KFP (172), the distinction between net flux and exchange flux is not as

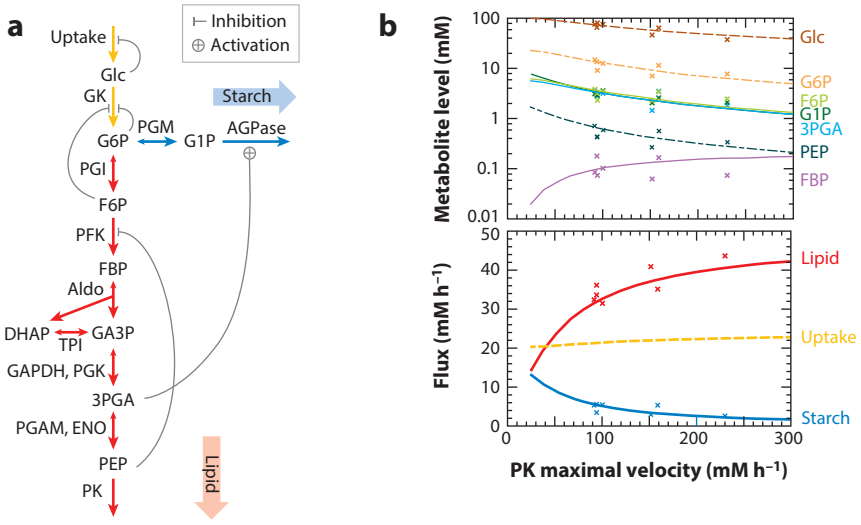


Figure 3

Simulations of an enzyme kinetic model illustrating the feedback control of carbon partitioning as suggested by analysis of flux, metabolite levels, and enzyme activities. Developing embryos of *Brassica napus* from different genotypes were cultured in vitro for ^{13}C-metabolic flux analysis and targeted metabolomics, proteomics, and enzyme activity profiling (133). (*a*) Based on the study, an enzyme kinetic model was developed (J. Schwender, unpublished data) with 11 reactions defined as kinetic rate laws (*single-headed arrow*: irreversible rate law; *double-headed arrow*: reversible rate law). At the G6P (glucose 6-phosphate) branchpoint, glucose uptake flux (*yellow arrows*) is partitioned toward lipid biosynthesis (*red arrows*) and starch biosynthesis (*blue arrows*). (*b*) Model simulations (*lines*) are shown along with experimental data (*x*'s) from the study. The model was fit to the average of two data sets corresponding to flux values close to 100 mM h^{-1} for chloroplast PK activity. Then the model was simulated for a range of PK activity between 25 and 300 mM h^{-1} to predict steady-state metabolite levels and fluxes in the biochemical network. Flux units shown are in millimole per hour per liter of metabolically active volume. Model simulations were performed using COPASI (61). Abbreviations: 3PGA, 3-phosphoglycerate; AGPase, ADP-glucose pyrophosphorylase; Aldo, aldolase; DHAP, dihydroxyacetone phosphate; ENO, enolase; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GK, glucose kinase; Glc, glucose; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PGAM, phosphoglycerate mutase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PGM, phosphoglucose mutase; PK, plastidic pyruvate kinase; TPI, triose phosphate isomerase.

clearly resolved as it is in ^{13}C-MFA. In ^{13}C-MFA, reversible reactions are assigned an exchange flux, a component of mass flow that goes in both directions at the same time (166). In addition to KFP, various studies integrate kinetic labeling data with kinetic models (**Supplemental Table 3**). For example, labeling transients obtained from feeding [$^2H_5$]phenylalanine to petunia flowers, producing volatile phenylpropanoid compounds, allowed researchers to determine in vivo kinetic parameters for a kinetic model of the benzenoid network (28). Metabolic control analysis then allowed major control steps to be identified (28).

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Problems for both INST ^{13}C-MFA and KFP arise from isotopic distortion effects due to cellular and subcellular heterogeneity. This means the labeling transient of a metabolite can be distorted if parts of what is measured as a metabolite come from a metabolically inactive subpool. These effects have to be corrected for in the modeling process based on fitting of dilution parameters or by inclusion of nonaqueous fractionation techniques into the workflow (58).

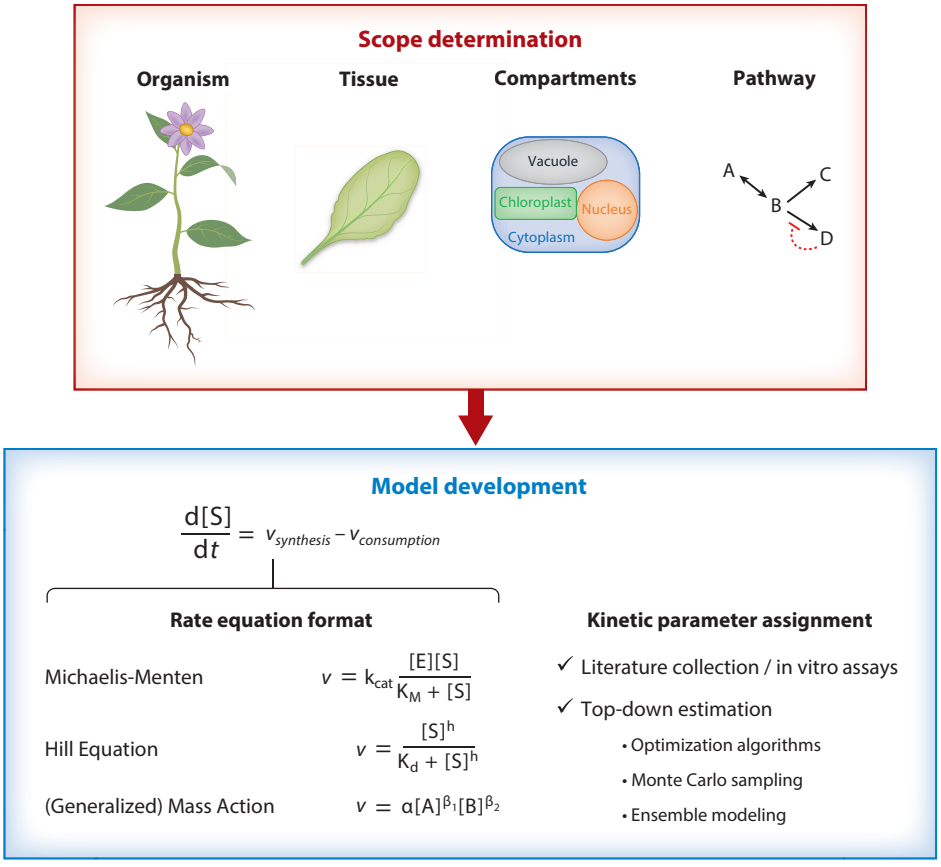

Figure 4

Workflow of kinetic modeling in plants. Kinetic models typically target a specific metabolic process and specific aspects of its regulation or responses to the environment that might be of interest. This determines which reactions are to be represented in the model and which types of regulatory features, such as specific allosteric mechanisms, need to be represented in the kinetic rate equations. Abbreviations: α, rate constant; β, kinetic order; E, enzyme concentration; h, Hill coefficient; k_{cat}, kinetic rate constant; K_d, effector binding constant; K_M, Michaelis binding constant; S, substrate concentration; v, rate of reaction.

3.4. Enzyme Kinetic Models

Kinetic modeling is a mathematical approach to describe cellular metabolism in a mechanistic manner. Most of its applications in plants are focused on a target pathway in a specified tissue (97, 116) (**Figure 4**; **Supplemental Table 4**). Similar with MFA, it uses mass balance equations to describe the metabolic dynamics within a pathway. In contrast to MFA, the reaction velocity is no longer represented by a fixed parameter, but it is simulated as a rate equation, which is a function of the concentrations of substrates, the catalyzing enzyme, and other metabolites as allosteric regulators if necessary. Michaelis-Menten kinetics are usually the default choices for the rate equations (67a). However, other kinds of functions are also widely used in different kinetic models, including the Hill equation (163), mass action (46), and generalized mass action (62, 155) (**Figure 4**; **Supplemental Table 4**). Specifically, the Hill equation is used when substrate cooperativity is observed for the corresponding enzyme kinetics (30, 175). And if no mechanistic kinetic

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information is available for the reactions, generalized mass action is a good choice to empirically fit the data (38, 39, 82, 83).

3.4.1. Parameter determination. If most enzymes within the target pathway have already been characterized with in vitro assays, the easiest way to parameterize the kinetic model is to apply those kinetic parameters to the model (30, 153, 158, 160, 175). Since enzyme amounts vary between different species and tissues, the v_{max} for each enzyme needs to be quantified from the studied organism instead of the in vitro systems. In plants, this approach has been taken to study sucrose metabolism (153), photosynthesis (159, 160, 175), the aspartate-derived amino acid pathway (30), lignin biosynthesis (158), and others. Care must be taken when one implements these models, as the in vitro assays' conditions might be distinct from the physiological ones (147). The best practice is thus to perform any needed in vitro assays under conditions that best mimic the intracellular environment. Ideally, kinetic models built in this way have good agreement with the in vivo dynamics (30). While this bottom-up approach is straightforward, a top-down parameter estimation is necessary when some or all parameters are missing. Indeed, the situation of missing literature values for enzyme parameters is frequently encountered, especially when it comes to secondary metabolism (28, 38, 39, 48, 56, 82, 83, 87, 104). In this case, this top-down approach is essentially an optimization problem, in which the objective function is to minimize the differences between the model predicted dynamics and available measurements by adjusting the values of unknown kinetic parameters. Various optimization algorithms are applied to solve the parameter estimation problem (27, 28, 87, 99), yet because of the nonconvexity of the problem, none of them guarantee they can identify the global minimum. This limitation makes the model's fidelity questionable, and careful validation with independent data sets is indispensable to verify that the model trained in this way correctly reflects in vivo behaviors. Some successful examples using this optimization framework include the parameterization of phenylpropanoid metabolism (56), fenclorim metabolism (87), the flavonoid pathway (104), and the benzenoid network (28).

Other methodologies for parameter estimation partially overcome the limitation of the current optimization algorithms. Ensemble modeling increases the reliability of the modeling output by summarizing outputs from thousands or even millions of individual models (38, 39, 82, 83, 151). Each individual model is parameterized with a sample from a prior parameter distribution, which is usually a uniform distribution spanning the possible physiological range. One model is selected into the ensemble only when it passes the prespecified criteria, which are the minimum agreement it needs to match with the known facts. Therefore, although each model might fit the data up to a suboptimal degree, the sum of many models could end up with an output range covering the reality with a high confidence. The limitation of ensemble modeling is that the choices of sample number and filtering criteria are somewhat arbitrary. Moreover, when the parameter distribution matching the observation is significantly narrower than the prior distribution, the valid samples are very difficult to obtain. To improve the efficiency of ensemble modeling, various frameworks have been developed by combining it with optimization (38, 83), FBA (39, 82, 83), dimension reduction (39), and other methods. A series of models for lignin biosynthesis in different plant species were developed using ensemble modeling (38, 39, 82, 83), and hypotheses of metabolic channeling by multiple enzymes were proposed based on comparison between models with different structures.

Monte Carlo sampling is another methodology to infer parameter values by estimating the distribution of parameters that allow the model to fit given experimental data (157). While a vanilla Monte Carlo sampling method requires the knowledge to calculate the probability given a sample, Markov chain Monte Carlo (MCMC) methods eliminate the need for such knowledge with a carefully designed sample proposal and accepting mechanisms (157). An advanced MCMC

sampling strategy (49) has been successfully applied for the parameter estimation of a kinetic model of phenylpropanoid metabolism (48). The advantage of MCMC is that it guarantees the global minimum can be reached given a sufficient number of samples, which can be achieved in a reasonable computation time with a moderate-size model. However, when it comes to a high-dimensional problem, even MCMC could suffer from the extremely high computation demands for parameter estimation.

3.4.2. Applications of kinetic models. Once the kinetic model is developed, it can be used in different ways. One of the most common applications is metabolic control analysis (40). As is suggested by its name, metabolic control analysis is performed to calculate the changes of the target flux when each network component is slightly perturbed. For instance, the flux control coefficient for individual enzymes within the pathway can be obtained with this analysis, and instead of only a single enzyme being the sole limiting step determining the pathway flux, the control of flux is spread to multiple enzymes in most cases (11, 20, 28, 30, 117, 153, 160). Other applications of kinetic models include flux estimation; simulation of metabolic responses to genetic or environmental changes (25, 26, 28, 38, 39, 48, 80, 83, 87, 98, 158); parameter scanning, in which one adjusts the value of a given parameter in a specified range to see the model's sensitivity to the parameter (11, 30, 31, 87, 113, 158, 160); pathway modulation, in which one monitors the significance of each kinetic mechanism by removing it from the model and observing the effect (11, 30, 39, 48, 77, 82, 114); and pathway optimization, in which one simulates the optimal adjustments to the pathway to achieve a given objective, which can be a metabolic engineering goal (83, 160, 175). The kinetic model can also help with the optimization of bioprocesses in bioreactors for plant cell cultures, where better control strategies can be developed for higher product yields with a model-aided systems approach (25, 26).

3.4.3. Limitations of current kinetic models. While kinetic modeling has improved our understanding of cellular dynamics in plants, most studies have focused only on targeted pathways (**Supplemental Table 4**). Such limitations in the modeling scope constrain the model's ability to describe the dynamic interactions across multiple pathways. Development of large-scale kinetic models is currently limited by multiple factors, including data availability and amount and computational hurdles (152). In addition, the presence of different cell types along changing developmental stages makes it more challenging to model plants, while large-scale kinetic models are indispensable to the mechanistic interpretation of omics data (116, 139).

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4. FUTURE PERSPECTIVES

In this review, we have described the major modeling approaches used to better understand and predict plant metabolic behavior. Having highlighted the separate approaches and their advantages and shortcomings, the need to develop integrated modeling approaches is obvious.

4.1. Whole-Plant Models

While ^{13}C-MFA provides in vivo flux maps, this empirical information comes with limitations in the resolution of the cellular network (131). Transfer of flux information from ^{13}C-MFA–derived flux maps via flux ratio constraints into large-scale FBA models helps to shrink the solution space, resulting in more realistic FBA flux predictions (53). In addition, more generalized frameworks for the integration of ^{13}C tracer experiments into large-scale models or GEMs have emerged (92, 141).

4.2. Plant Development

Metabolism drives growth and ultimately sustains plant development through its life cycle. At the whole-plant level, functional–structural plant modeling is a widely used approach that integrates the environment, plant architecture, and organ-level carbon and nitrogen partitioning (138). Whole-plant models that link down to the molecular level are still rare (23, 45). One study addressed changing source-sink relationships during growth of the barley plant (45). Metabolism of shoots, stems, and roots was simulated by cellular FBA models, and the static FBA modeling was extended by a dynamic approach (90) into a dynamic whole-plant model. Such multiscale modeling approaches need quantitative inputs on growth dynamics. In the future, quantitative phenotyping data obtained at the tissue, plant, or canopy level (24, 42) could be integrated into such whole-plant models.

4.3. Cell-Type Specificity

One recurring issue in plant metabolic and modeling studies is the presence of different cell types that create physiological and metabolic heterogeneity across a studied tissue. It would be desirable to study and model the metabolic phenotype with higher spatial resolution. Experimental techniques such as single-cell metabolomics (35) are definitely needed to provide high-quality data sets for model development. In conventional ^{13}C-MFA, labeling measurements obtained from a heterogenous cell population are averaged over multiple cell types, resulting in a distorted flux map. This problem could potentially be addressed by peptide-based ^{13}C-MFA, which allows for a label readout from proteins that are uniquely present in a certain cell type (43). Similarly, reporter protein–based ^{13}C-labeling techniques have been proposed to resolve subpopulation-specific flux in microbial communities (122) and have recently been extended to cell type–specific ^{13}C-labeling approaches in plants (120). In another study, the central metabolism of mesophyll and guard cells of *A. thaliana* leaves was explored based on CBM and by ^{13}C-labeling (115). In this study, guard cell–specific flux was estimated by ^{13}C-labeling transients after isolation of guard cells from leaf epidermis.

4.4. Scales of Kinetic Models

To address the limitation in size of kinetic models due to limited information on enzyme kinetics and other data, inputs from other modeling strategies such as FBA and ^{13}C-MFA can help with kinetic model development at genome scale. For instance, Khodayari & Maranas (73) have successfully developed a genome-scale kinetic model of *Escherichia coli* using fluxomic data from a previous ^{13}C-MFA approach (66). The model was parameterized with an ensemble modeling approach, integrating with a genetic algorithm-based optimization. Besides the expansion of the kinetic models to larger scales, another promising direction is to combine kinetic models for cellular metabolism with models that capture other regulation, such as gene regulatory networks (51). Such combinations should greatly expand the applicable scopes of the models, as they incorporate the regulatory controls over enzymes under different conditions and timescales. Many studies have shown the benefits of integrating transcriptomic or proteomic data into FBA models (37, 65, 81, 161), and similar successes are expected for kinetic modeling in the near future as well.

4.5. Design and Integration of Synthetic Pathways into Plant Metabolism

The stoichiometry-based modeling approaches discussed in this review are important tools for the design of new (synthetic) metabolic routes within the context of the plant metabolic network (78). Various recently reported synthetic biology approaches aim at increasing the efficiency of

pathways by which plants use light to convert CO_2 into biomass (162). This includes various synthetic photorespiratory bypass routes that reduce loss of carbon relative to the native photorespiratory cycle (162). The malyl-CoA-glycerate pathway is an example of a synthetic pathway for more efficient generation of acetyl-CoA (170), a precursor of energy-dense fatty acids and storage oils. Acetyl-CoA is conventionally derived from pyruvate by pyruvate dehydrogenase at a cost of one CO_2 released per acetyl-CoA unit produced. In cyanobacteria the malyl-CoA-glycerate pathway can generate acetyl-CoA from photosynthetically generated C_3 sugars or from glycolate, the second intermediate of photorespiration, without loss of carbon (170). This route might therefore help to increase yields of energy-dense oils in bioenergy crops.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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