Life Science Informatics Publications
Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences
Journal Home page http://www.rjlbpcs.com/
RJLBPCS
ISSN 2454-6348

Original Review Article **DOI: 10.26479/2019.0502.26**

METABOLIC FLUX ANALYSIS IN PLANT METABOLIC NETWORK

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ABSTRACT: Theoretical and experimental observations in metabolic engineering both indicate that metabolism operates at the level of networks. In plants, metabolic complexity attains a high degree because of compartmentation and the synthesis of a very wide variety of secondary metabolites. Metabolic flux analysis (MFA) gives tools to measure and model the operation of metabolism and is making important contributions to understand the metabolic complexity. This review gives an overview of different MFA approaches, the experimental measurements needed to apply them to get the flux information across the metabolic network.

KEYWORDS: Metabolic flux analysis (MFA), Gene-protein reaction (GPR), Genome-scale metabolic models (GEMs), Flux balance analysis (FBA), Compartmentation.

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1.INTRODUCTION

The metabolic networks of plants are extensively complicated over different organisms. Because of its diverse interlinked form of plant life, like being immobile, autotroph and poikilothermic and devouring huge biochemical collections and an extensive level for subcellular segmentations. Thus, the low success rate of the outcome of, particularly the primary metabolism of metabolic engineering is not shocking, as individual gene changes generally cause a minute change in the aspired traits. Likewise, the correlation among the phenotype and genotype is also integrally complex since the working of distinct proteins relies on the active state of the complex metabolic network [1,2]. Metabolic flux analysis (MFA) provides such equipment's which has a purpose to

distinguish fluxes via the network and shed light into their handling [3]. Through metabolism, MFA measures the flux of constituents via metabolism, flux maps yielding which can assist in elucidating the phenotypes in details. From the previous studies it was revealed that, understanding towards metabolism has improved through MFA, which depicts the routes of fluxes via metabolic network [4,5], besides that it reveals novel paths and cycles [6,7]. We can make a hypothesis about metabolic mechanism with the help of flux maps achieved under different circumstances of growth as well as from mutants [8,9,10,11]. For microbial systems: describing the structure and stoichiometry of the network is the prime requirement in MFA, this key step can be achieved from the fully annotated genome along with the datasets of metabolite, transcript, and proteins. The network assembly can be used to describe the probable range of flux maps that a network is able to maintain and to define the finest specific cellular targets like (evolutionary selection pressures), just as exploiting production [12,13,14,15]. The contrast between the results of fluxes expected from analytically established MFA and those measured from finest meet evolutionary impetus may be a valued device to assess the validity of the proposed impetus. Fluxes between cells and tissues are measured through experimentally based approaches which are established on either steady-state or kinetic isotopic classified tests and their analysis by computer-assisted/based modeling. Those MFA approaches of interest have given due importance which has evaluations and models of multiple fluxes via a metabolic network or generally, a sub-network, and comprise systems in the steady state along with that one whose flux may be varying. MFA approaches might be separated into numerous groups that vary in the vital data information, the types of models and the variety of information gained. Different approaches of MFA were proposed through the state of the biological systems, whether it assessed in steady state or sub-network state. Flux analysis is started with sets of network reactions depiction, which stoichiometrically reveal the substrates of respective reactions to its results. Several means are given through the network, the stoichiometric depiction is signified the full range of possible metabolic natures, Extreme pathway analysis (EPA) [16] and elementary mode analysis (EMA) [17,18,19] are essential approaches, which are used to examine this range and outline the bounds of the possible steady-state flux allocations. Although steady-state MFA does not operate a prognostic model, it is pleasing as it produces flux maps without involving a measure of metabolite pool sizes, or the approximation of kinetic limits, that are needed for dynamic MFA, but generally hard to get. There is no need of constant flux in case of unsteady-state or dynamic/kinetic method, so in this case, we can change the pool size and flux are recognized from time course analysis of pool sizes and classification. The use of single flux estimations of dynamic classification and for pathway revelation is well flourished in plants biochemistry and made massive contributions. Numerous autonomous limitations are used in the dynamic MFA because distinct enzymatic and transports stages are modeled, each containing several concentrations and rate constant values. Labeling experimental steady-state (^{13}C-MFA) systems should be in a metabolic steady-state

sufficiently so that it reaches isotopic steady state. But several plants tissues don't sight steady-state metabolism or can't be considered to the isotopic steady state under physiologically significant circumstances. So, in this situation, dynamic MFA is required to measured multiple fluxes through networks, and these types of methods have the additional benefit of passive models this can be used to guess the consequence of genetic or other fluctuations on pool sizes and metabolic fluxes [20,21]. Metabolic control analysis (MCA) is needed for identification and inspects pathways monitoring points, so dynamic MFA is also essential for the same case [1, 22]. Diverse enzymes are assigned quantitatively to control the flux along the pathways in MCA [23,24,25]. In Top-Down Control Analysis, it is a powerful projecting guide for metabolic engineering, which acknowledges the enzymes that preserve utmost govern over fluxes [26], blocks in which enzymatic reactions are grouped, information on control of flux between blocks are yielded from MCA analysis, however not within these blocks. Modern latest tools like mass spectroscopic and nuclear magnetic resonance (NMR) made successes in MFA approaches recently, positionally categorized range of substrates with stable isotopes, and key advancement of modeling concept and computational approaches. Comprehensive explanations regarding the performance of various MFA analyses are stated in the literature; broad outline [27] are, FBA [28, 29], EMA [17, 18, 29], EPA [16], dynamic and steady-state MFA [30,31,32,33,34,35] and MCA [36, 37].

Systems Biology

Current genome sequence technology generates thousands of genome sequences which allow us to resolve the biological mechanism and its constituents related to the cell those create organism cells. Systems biology inspects the interaction between different components (proteins, metabolites, genes and regulatory elements) of the network and also finds out that how these components change the phenotype of the cell [38, 39]. Systems biology emphasizes compiling of large data sets and checks their regularity [40]. The application of systems biology in plants leads to "*In silico plant*" concept [41]. Previous studies revealed that available upgrading in the systems biology made it more convincing towards accession for not only model plants but other major vital plants like *Oryza sativa* [42]. Previous studies reviewed the recent successes in the field of systems biology which contain, exploration of abiotic stress responses [43], the interaction between host and pathogen [44, 45], analysis of nitrogen nourishment [46], and review of common metabolism [47]. The metabolic model can estimate the flux yield based on the inserted data and the type of model, different paths expected by which fluxes can transfer, and possible unique paths [48].

Genome-scale metabolic models (GEMs)

Genome-scale metabolic models consist of every identified metabolic reaction, which arises within an organism, cells, tissues or tissue-compartment. The model comprises three vital aspects: the network reaction, gene-protein-reaction (GPR) interactions and the reaction of biomass. Foremost, the model consists of all internal reactions and metabolite carriers, that comprising the intake and

transfer of metabolites and their related metabolic charge. A stoichiometric matrix, also known as a coefficient matrix that symbolizes the stoichiometric coefficient of every metabolite within every metabolic reaction. Besides that, a substitute biomass creation reaction is added that represents the accretion of all biomass constituents. Lastly, gene-protein-reaction (GPR) relationships are established to relate the genes that are connected with a metabolic reaction. Boolean Logic gates are used to develop GPR relationships, where a gene (a subunit of a protein) is represented as "and" relationship and an isozyme is represented as "or" relationship [49]. Genome-scale metabolic models (GEMs) constructions are increased to meet with the pace of current genome sequence annotation technology which produces thousands of sequenced genomes [50]. An ideal genome-scale metabolic model interconnects the biological components like gene, protein, and enzymes for every metabolic reaction [51]. GEMs wrap up the recognized metabolic knowledge of organisms in a mathematically characterized reaction network. The core target of the GEMs is to improve the yields of bioproducts via giving genetic intermediate approaches regarding genes up and down regulations and gene knockouts [52]. A metabolic network should be constructed before the implementation of FBA so it can provide the stoichiometric matrix [53]. Numerous metabolic networks are established from current databases of administered genomic data and biochemical database, like as, The Kyoto Encyclopedia of Genes and Genomes (KEGG) database [54], BRENDA [55], Gene Ontology (GO) [56], MetaCyc [57] and as well as genome databases [58], biochemical databases [59,60,61], organism-defined databases [62], protein localization databases [63], reconstruction packages [64], simulation environments [65,66,67,68], and packages for visualization [69]. A finite number of packages and software tools are accessible freely and paid, which purpose is helping the modeling reconstruction development (Table 1). A decade ago, genome-scale metabolic reconstruction for microorganism had successfully constructed, which created exclusive visions into the metabolomics mechanism [74,75]. Genome-scale metabolic models of prokaryotes have been analyzed with metabolic engineered based optimization and algorithms tools. Tools like OptKnock, OptORF, and OptFlux are very popular, which are widely used to simulate the synchronous (knockout) or down or up-regulation of numerous genes [50], [76]. In the past due to lack of genomic information and metabolic pathways complexity, application of genome-scale modeling was not well flourished in plants in spite of this first genome-scale modeling for *A. thaliana* was developed [77,78,79]. Later, the metabolic models were also constructed for other plants like Arabidopsis and rice [77,78,80,81,82] and many other plants species given in the Table (2).

Table 1: Generally used databases for metabolic models reconstruction

Usable Sites	References	Remarks
KEGG pathways	[59]	Biochemical databases
KEGG rice pathways		Genome research database for rice
IUBMB Enzyme nomenclature		Universal information on enzyme nomenclature
Brenda Enzyme database	[55]	Comprehensive enzyme repositories
BioCyc	[57]	Pathway/Genome Databases (PGDBs)
BiGG Models	[70]	Knowledgebase of GEMs
MetaCyc	[57]	Metabolic pathway database
TheSeed	[71]	Comparative genomic analysis tool
Entrez Gene	[72]	Gene-specific information database NCBI
The Genomes Online Database (GOLD)	[73]	Information hub for genome and metagenome
COBRA Toolbox	[102]	MATLAB needed

Table 2: Major plants metabolic pathways databases

Pathway Database	Species	Pathways	Genes	Enzymatic Reactions	Enzymes	Compounds	Reference
RiceCyc ver 3.3	*Oryza sativa (Japonica)*	308	47886	2103	6040	1543	[83]
SorghumCyc ver 6.0	*Sorghum bicolor*	478	5986	2948	5988	2222	[83]
MaizeCyc ver 2.2	*Zea mays*	424	39655	2132	8887	1453	[83]
BrachyCyc ver 2.0	*Brachypodium distachyon*	321	26672	2057	7723	1641	[83]
AraCyc ver 16.0	*Arabidopsis thaliana*	627	5229	3585	5451	2820	[83]
MedicCyc ver 1.0.1.1	*Medicago trunculata*	219	4010	1498	3426	1215	[84]
LycoCyc ver 3.3	*Solanum lycopersicum*	456	34727	2616	8033	1867	[83]
PotatoCyc ver 1.0.1.1	*Solanum tuberosum*	201	20713	1079	1317	849	[83]
CoffeaCyc ver 2.4	*Coffea canephora*	312	8226	1780	2223	1343	[83]
EcoCyc ver 22.5	*Escherichia coli*	350	4500	2023	1611	2936	[85]
MetaCyc ver 22.5		2666	12313	15198	12006	15089	[86]
PlantCyc ver 13.0		1013	2890	4630	3475	4544	[83]
PetuniaCyc ver 2.4	*Petunia x hybrid*	130	3428	775	294	619	[83]
NtabacumCyc	*Nicotiana tabacum*	569	69211	3309	19517	2424	[87]
NbenthamianaCyc	*Nicotiana benthamiana*	541	57139	3008	12506	2163	[87]
NsylvestrisCyc	*Nicotiana sylvestris*	449	35533	2659	6346	1981	[87]
NtomentosiformisCyc	*Nicotiana tomentosiformis*	517	34378	2907	9257	2100	[87]
SolanaCyc	Solanaceae family	199	209	835	257	1441	[87]

Overall, these existing plant GEMs has potentially proved to be precise, robust, and efficient in analysis and prediction of conditional changes in particular expression of central carbon metabolism [51,88]. Regarding metabolic engineering, there are some issues related to the application of GEMs to microorganisms and plants. Apart from microorganisms, usually plants are unable to grow under an organized environmental system. In general, some attention is to be required when applying the network flux analysis outcomes of microorganisms to plant metabolic engineering analysis [89]. In this concern, Shachar-Hill delivered a descriptive report of lysine production, common in microbes and plants [90]. In *Corynebacterium glutamicum*, lysine productivity was enhanced via the application of mathematical modeling system and with the help of these tools major metabolic obstacles were identified which leads to tremendous improvement in productivity of lysine [91]. A similar approach was made in the endosperm of maize, and no limitation was found. Previously, GEMs was unable to be constructed for the secondary metabolites [51], however, the secondary metabolism was included in the exceptional Arabidopsis metabolic model [78]. Other challenges include the incomplete annotation of plant genomes and its subcellular localization reactions, which becomes a hurdle during plant genome reconstruction [92]. Methodologies have been proposed for tackling such challenges, like subcellular localizing metabolic reactions, predicting software and gene annotating tools for comparative genomics [93, 94]. Integration of proteomic or transcriptomic datasets and genome-scale modeling is another initiative which can be applied for exploring the complex metabolic activities of plants [95,96]. A combinatory approach was implemented to guess the metabolic reaction of Arabidopsis at different conditions, and it was observed that the metabolic data along with the transcriptomic data enhanced the predictions of metabolism even the transcripts levels do not correlate with the fluxes [94]. Additional exploration has revealed that GEMs effectively connects the gap between metabolite-centric and flux means [96]. Constraint-based models (CBMs) are generally well known for metabolic network reactions studies. Historically they are employed to explore metabolic networks reactions. CBM was constructed in the framework of metabolic engineering with the objective to enhance the biomass production of chemicals via optimizing metabolic pathways [97]. It is the systems biology which connects to an encoded genome with phenotypic whole-cell flux states [98].

Genome-scale metabolic modeling in plants

The growing availability of whole genome sequence database has opened new horizon towards the development of metabolic network reconstruction. Genome-scale reconstruction is the predecessor of all genome-scale metabolic models. Today numerous databases and tools are available for metabolic reconstruction, such as AraCyc for Arabidopsis [79] and RiceCyc for rice [81], PlantCyc for plants [99], Plant Metabolic Network PMN (13.0) [100] and MetaCyc [57], along with these for compartmentalization reactions AraPerox [101], SUBA [102], and PPDB [103] were used. All these resources are useful in modeling reconstruction. Metabolic network reconstruction can address

innumerable biological functions like a prediction of cells culture conditions [104]; understanding the plant metabolic mechanisms, its regulations, and behaviors [105]; and integrate experimental data with phenotype and its prediction regarding the phenotypes. The plant's metabolic function is based on the interaction between different subcellular compartments, cells, tissues, and organs. And this interaction helps in the reconstruction of organ-specific models, and these models support in understanding the complex plant metabolic processes on a whole plant genome scale [106]. In spite of all these challenges, useful plant metabolic networks formed for *Arabidopsis thaliana* [77,78,79], maize [82], barley [105], rice [81] and the biofuels crops like sorghum and sugarcane [80]. The process of preparing the genome-scale metabolic reconstruction consists of five major steps which have been broken down into several sub-steps [107] (Fig 1).

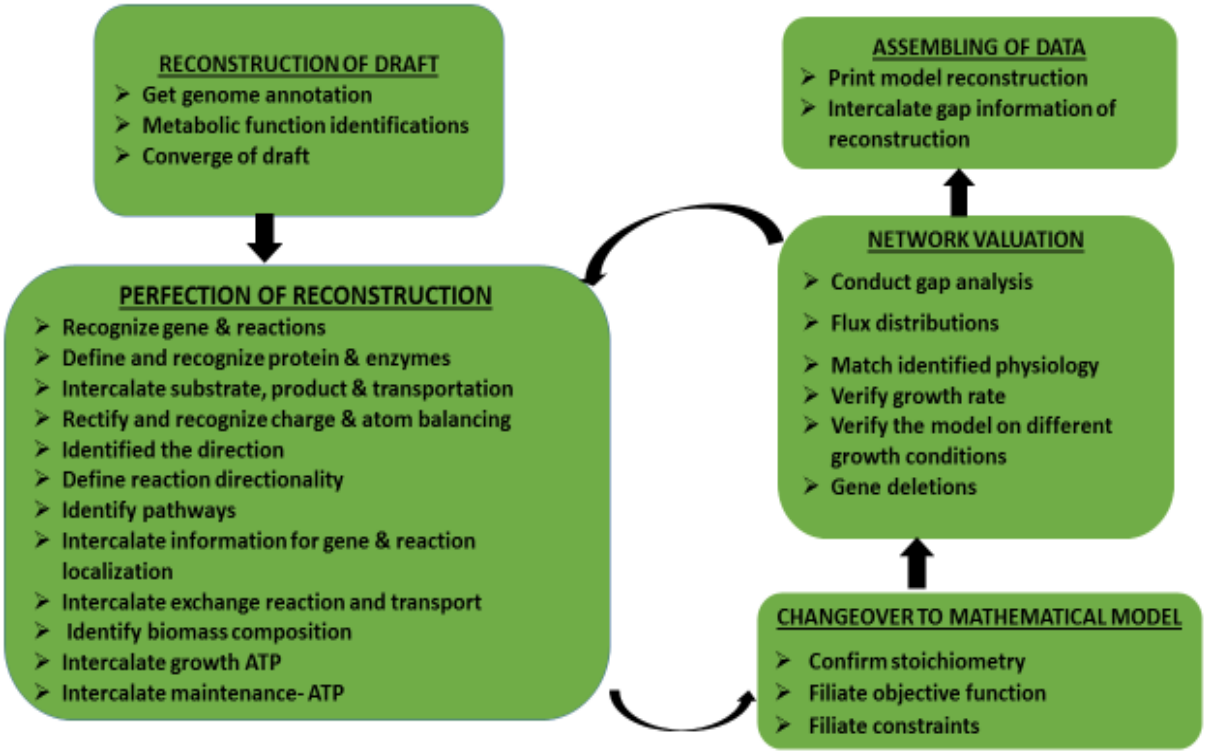

Fig 1: Flowchart of the metabolic network reconstructions steps

Operating system accessible for publicized metabolic model

GEMs are crucial approaches for metabolic engineering study and systems biology because they have the potential to simulating complex steady-state behavior. As the area of bioinformatics research is expanding day by day, so as the computational tools becomes massive. It extends the vast range of software platforms comprising the COBRA toolbox for Matlab [108], COBRApy (a python set that assists basic COBRA approach), KBase (Web-based US Department of Energy Systems Biology Knowledgebase) [109] and ScrumPy in python [110,111], beside that further tools and libraries like OptFlux in Java [112], Cameo [113], and SurreyFBA [114]. The COBRApy software assists the next generation of metabolic modeling and utilizes Parallel Python to divide

simulations through multiple CPUs permitting faster FVA simulations, which might be time-saving because of the large complex nature of plant metabolic models [111]. COBRApy is user-friendly software, which let the handlers to create their own constraints and goals [111]. The KBase web-based software permits users to generate their own workflows which can universal among researchers permitting other public domain to generate the simulations [109]. Through KBase, approach users can execute standard FBA, pFBA, beside that gene and reaction knockouts [109].

Challenging task in plants: Subcellular compartmentation

Metabolic flexibility increases due to subcellular compartmentation, specialization as well as regulation. Subcellular compartmentation demonstrates defiance to metabolic analyses, along with MFA. Compartmentation leads sophisticate the structure of the metabolic network in MFA, the determination of metabolite labeling and the measurement and localization of metabolic levels that might vary for the similar metabolite in diverse compartments. The occurrence of big vacuoles and metabolically alive plastids in plants makes further defiance analogized with few fungal and animals systems. Kruger, Le Lay & Ratcliffe 2007 revealed in their study that fails to interpret accurately for compartmentation, can possibly result to different flux maps, models and assumptions, as was revealed in case of useless cycling related with sucrose and glucose yield [115]. MFA analysis has the ability to define the relative contributions of diverse compartments to metabolic fluxes if the compartmentation might be determined. Beside that MFA possess the ability to disclose the presence of multiple pools of the similar metabolite as in case of choline in leaves [116]. Location of enzymes and transporters decide the structure of a metabolic network, and the position of those proteins can be driven with different level of determination by microscopic immunohistochemistry also fluorescence tagging, by proteomics and organelle fractionation, also by targeting projection on the basis of sequence. Insufficient, imprecise also ambiguous data on network structure is inappropriately modeled in plant metabolism, and precaution must be taken to find a crucial hypothesis. Such testing might be computational, to screen either model depend on various structures of the network can check evenly well for recognized data, and also perfectly experimental data, by pursuing evidence about the position of significant proteins. In dynamic MFA metabolite concentrations are used, and it is determined by dividing the total levels calculated after extraction by the volume occupied by them, information of their compartmentation is also required. In both dynamic as well as steady-state MFA measurement of metabolite labeling is used, and that can be distinct for the same metabolite in various compartments [117].

Three-dimensional complication of plant systems

Flux model based on isotopic labeling observations are simplism of authenticity. The numeral of the factors that might be perceptively determined are restricted by the presented experimental dimensions that report on metabolic construction at the cellular and subcellular levels [117,118]. Therefore, the key factor for MFA is to improve the analytical techniques so that models are further

standardized to other 'omics' accounts. For flux analysis in some cases, tissues might be cultured separately or in couplets [119, 120], yet when an individual tissue is mixed its diverse surface can't survive. In cell population of seeds potential imaging equipment with fluorescence [121,122] or magnetic resonance [123] have specified the scope of heterogeneity. When such a method along with MFA might retrieve the potential of light to re-introduce carbon in evolving embryos and characterize gradients in lipid density [124]. Metabolic flux was influenced by the penetration of light in Brassica embryos and it promotes to alterations in the tropic state among outer and inner cells of the tissue. Identifying a desirable phenotype like as better lipid density in a subsection of cells from individual tissue amid flux evaluation beside with other data from transcripts or proteins might signify the best comparison to pinpoint the relevant changes required for engineering [125].
Furthermore, plant cells have significant subcellular characters with essential pathways such as the oxidative reactions in pentose phosphate metabolism [126] and glycolysis [127,128] alive in somewhat three spatially diverse positions and together with channeling mechanisms [129]. Numerous systematic methods have been related to flux analysis based on either information about pool size or metabolite labeling was of concern. Subcellular fractionation approaches can deliver pool size evaluations to constrain transient isotopic labeling models [130,131]. In, flux analysis explanations of subcellular labeling based on metabolic yields that are precisely synthesized in a recognized compartment. Approaches to measuring plastidic origin starches besides with cell wall, sucrose or protein glycosylation [8,117] which are extra-plastidial have been essential for deciding the degree of equilibration of hexose pools and the capability to compartmentalize models [117]. Different pools of acetyl-CoA precursors are used in the biosynthesis of plastidic and cytosolic origin of fatty acid along with carbohydrates, so the origin of acetyl-CoA might be deduced by suitable labeling and examination of the subsequent fatty acids [132,133]. Isotopic labeling of proteins is highly motivated in the previous works. Proteins act as an admirable communicator since they are decoded in compartments stable with genome location. Therefore labeled proteins afford a way to evaluate the isotopic equilibration of amino acids among organelles [134]. This idea was improved latterly by evaluating the labeling in peptides with high-resolution MS [135,136] and then exploiting the peptide-based labeling information to computationally rectify flux values [137].

Purification methods for organelles

By approaching organelle purification procedures, we can partially meet the knowledge about compartments on proteins as well as metabolites [138,139,140]. In non-aqueous bifurcation, tissues are rapidly frozen and then lyophilized under situations in which the position and levels of metabolites are as less disturbed as possible [141]. Due to incomplete separation of organelles, estimates of the compartmentalized level of the metabolite by this method is depending on deconvolution techniques using a marker of a known protein in different compartments [142]. How much success is a deconvolution strategy for determining differences in an unknown labeling

between compartments is not clear, although lesser amounts of pure organelle fractions would be required for label determination by sensitive mass spectrometric techniques? Aqueous fractionation methods lead in better separation of organelle fractions [143] that is separated by density-gradient centrifugation, also this is necessary for protein localization exertion, though it is rare to preserve metabolites position except for less mobile end products.

Compartment-peculiar info metabolites

An alternative approach was implemented to resolve the frim metabolite labeling in diverse compartments which comprises the less usage of reporter or read-out metabolites that are particular to subcellular positions. For instance, a vital metabolic intermediate, acetyl-CoA that performs various roles in different compartments, which is not transported across membranes [144,145]. Acetyl-CoA (a precursor for lipid synthesis) for the *de novo* synthesis of fatty acid, formed by plastidic pyruvate dehydrogenase [146], while for extension of fatty acid, acetyl-CoA is produced in the cytosol [147,148,149]. In these pools, labeling can be resolved by examining fatty acids which are synthesized in the plastid as well as extended in the cytosol [132,150]. So, the labeling calculated in 16-18 carbon fatty acids classified as the labeling of plastidic acetyl-CoA, besides that labeling in the longer fatty acids classified as the labeling of acetyl-CoA in the cytosol. Diverse metabolites readout may be used to differentiate the labeling the vital sugar phosphate pools situated in the cytosol and plastid. In the plastid sucrose and starch, labeling characterized the isotopic state of its precursors, glycans protein and cell walls are traced with the labeling forms of the cytosolic carbohydrate from where they produced. Though labeling in sucrose might be calculated instantly, chemical breakdown or enzymatic is channelized to assist the study of polymer-associated carbohydrates. Hence, for instance, acid hydrolysis of starch and protein glycans produces levulinic acid, whom labeling might be examined through NMR [151]. This approach has also potential to examine positional enhancement and long array coupling between carbons that gives important information for flux analysis [151].

Predictive metabolic flux analysis

There are few methods have been developed for prediction of fluxes through the metabolic networks for wild-type and other phenotypes.

Flux balance analysis (FBA)

FBA is a mathematical strategy for studying the movement of metabolites via a metabolic network. FBA primarily makes use of the stoichiometry matrix **S** of size m x r, where m is the number of metabolites in the system and r is the number of reactions. Every row of **S** specifies for a specific metabolite in what quantity it participates in each reaction. Therefore, each element ('j') of **S** contains the stoichiometric coefficient of metabolite i^{th} in reaction j. The word flux is used to describe a reaction rate at steady state. In FBA the aim is to, find a flux distribution of the network that fulfill (1) the steady-state condition **S.v =0** (this uses the phenomenon that metabolism occurs

on a fast time-scale compared to gene regulatory events and thus that accumulation rates of metabolites are zero effectively [152], (2) thermodynamic feasibility (some reactions are known to be irreversible), (3) maximal flux constraints when these are known and (4) a linear objective function to be maximal. This objective function is typically one of the fluxes in the model, such as biomass synthesis, or a linear combination of several fluxes, such as biomass production plus a product of our desire. Because the objective function is linear in the fluxes, this technique is an application of linear programming. Mathematically, FBA can be summarized as:

$$\begin{aligned} &\text{Max/Min } \mathbf{Z} = \mathbf{c}^{\mathbf{T}}\mathbf{v}, \text{ such that} \\ &\mathbf{Sv} = \mathbf{0} \\ &\alpha_k \leq v_k \leq \beta_k \end{aligned}$$

where c and v are column vectors of length *r*, the number of reactions. This can be thought of as first constraining all possible solutions to the ones that allow a steady state and satisfy the bounds (this results in a multi-dimensional cone within the null space of S) and then finding the optimal solution among the remaining degrees of freedom.

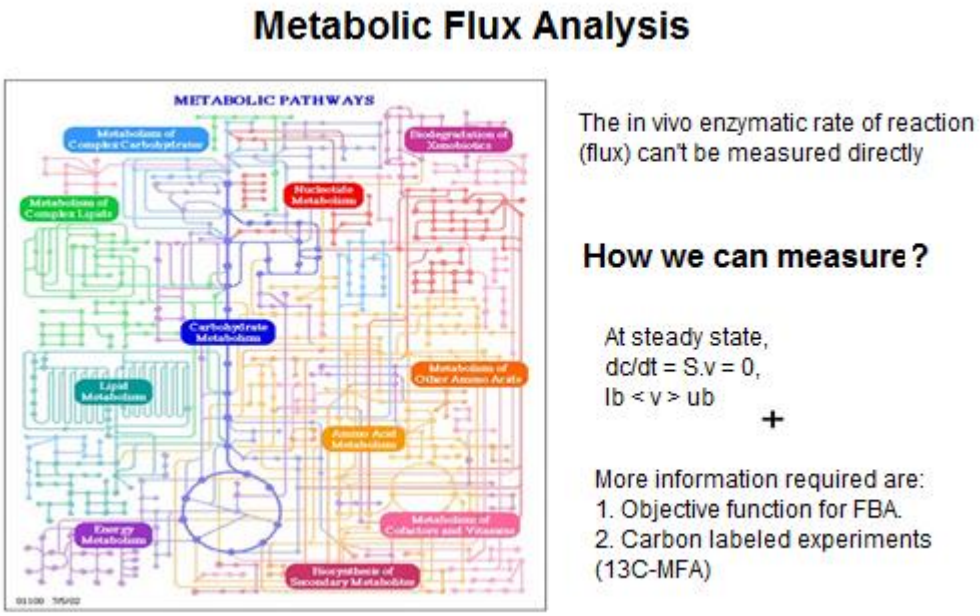

Fig 2. Minimal information required for the metabolic flux analysis. Metabolic reference pathway adopted from KEGG pathways database (https://www.genome.jp/kegg-bin/show_pathway?map01100)

Metabolic flux remains in quasi-steady state concerning growth and typical process transients because the metabolism has transient lower than few minutes in comparison to the cellular growth rate and dynamic changes in the organism's surrounding. Two things of metabolic information are required to formulate the mathematical flux balance model: first, metabolic stoichiometry is needed to note down all the chemical reactions that occur in the metabolic network of concern, and the second requirement is the materials required in the metabolic system, which includes maintenance

requirements, biomass synthesis and in certain cases significant product secretion [15]. Stoichiometric model is formulated by acquiring the metabolic information and putting the data into a suitable mathematical framework by supposing that the cell is struggling to encounter a specific objective [15]. The genome-scale metabolic network reconstructions give information about the biochemical reactions ongoing in an organism and also about the enzymes that carry out those reactions. These enzymes get encoded by the genes, and hence through the genome-scale metabolic network, we come to know about the genes that regulate particular metabolic reactions. FBA calculates the flow of metabolites through these metabolic reactions, thus making it feasible to guess the rate of production of essential biotechnologically metabolite that causes the organism growth [53]. Currently, 178 organisms with metabolic models are accessible at http://systemsbiology.ucsd.edu/InSilicoOrganisms/OtherOrganisms, and by current sequencing technology enables the metabolic models can be generated at the high pace each year [107,153]. FBA is the critical tool for coupling the knowledge encoded in these models [53]. FBA is mostly convenient tool for understanding the functions of the metabolic network system, and it is based on linear programming optimization [15,154]. For FBA the only information we need to know is the stoichiometric network, which gets generated from genomic information of the metabolic pathways of the organisms. All the activities or reactions occurring in the system related to the metabolites, like inputs and outputs transport activities and enzymatic reactions are all represents the stoichiometric network. Based on the information gathered from the experiment and literature, the knowledge of biomass formation in the respective reactions can also be obtained. FBA can explain the limits of system production and not this only but also act as a basis for evaluation with experimental flux data to propose perfection to the system [155]. In plants limited access of experimental data is there, specifically in leaves where CO_2 is the substrate, but in case of sink tissues, ample data sets are there in which sucrose and amino acids act as a substrate [7,156]. Fluxes produced by FBA might also beneficial even in absence of experimental data for evaluation, as it might emphasize the curial reactions, that in some circumstances will unable to conclude without experimental data. When a set of fluxes generated through FBA model, to know about the flow of carbon one may not only see the fluxes in the figure but also have to explain all the metabolite sources and sinks. This might be specifically crucial for energy sources like NADPH, ATP, CO_2, as these energy sources paths disclose the background of the simulated cell activities [155]. A better insight of maximized yield might be gained by leading chains of constraint sets whose resolution can be matched. For instance, one can measure not only outcome number but also metabolic regulation under diverse circumstances such as intensity of light or nutrient accessibility, one can compare metabolic activity in different sources of nutrients like nitrogen sources, nitrate (least reduced) between ammonia (highly reduced) to examine either the preserving in reducing equals

can be practical, if so at what limit. One can explore the capabilities of diverse metabolic types, like as C3 between C4 carbon fixation [157].

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

Many computational methods have been developed for the prediction of metabolic fluxes. Two approaches have been established for that purpose to forecast flux adaptation in reaction to a given variation in a specific flux, like as in knockout mutation. The first methodology is founded on MOMA. This is established through minimization of the sum of the squared differences between the initial and oriented flux maps. MOMA forecast is in general close contract with experimental results, and its feasible usage makes MOMA an important tool for metabolic engineering [158]. The other methodology, ROOM, is established on the minimization of the number of flux varies [159]. The development of this technique ROOM is based on the examination that gene expression drastically changes after the organism undergoes an affective disorder in development quickly after a metabolic perturbation, but after a period of acclimation, gene expression returns to their initial period near to the one prior to perturbation. ROOM forecast is in general close contract with experimental results for bacteria, predictions are also in closely similar with experimental data for bacteria, and ROOM predictions surpass MOMA forecast in experiments where an acclimation period has been involved. In comparison to MOMA, ROOM generally finds several comparable solutions, which makes the applied application of ROOM for metabolic engineering least unambiguous, exclusively for the large complex networks of plant metabolism. ROOM, which is established on the biological examination, does give an understanding of how metabolic maps are controlled [117].

Distinct knowledge about the functioning of plant systems provide by MFA studies

Different MFA approaches are utilized to explore diverse plant tissues like cell suspension, microalgae, developing seeds, stems, root tips, transformed root cultures, flowers, leaves, trichomes, and tubers. Numerous studies are conducted in the past to examine the capability for, and also elucidate the difficulties in executing MFA in entire plants [160,161,162]. In recent years work towards applying MFA with plants under physiologically normal states has taken different directions. Although the steady-state MFA methodology has addressed vital queries, comprising the role of Rubisco in evolving seeds and the oilseed metabolism regulation [163], despite that they face challenge towards their application to higher organisms like mammals and plants because of their mosaic media preparation, subcellular localization, and gradual labeling dynamics [117]. So far, the primary application of MFA has done on confined cells or tissues, in which frequently fifty to hundred reactions are, checked [117]. Alternate technical approaches are encouraged due to a technical obstacle in prolonging the analysis to plant networks [154], like as the union MFA/EMA [7] and MFA/FVA, which have been practiced to study evolving *B. napus* embryos [164,165]. To acquire the isotopic steady state MFA needed a long duration of time, so to avoid that isotopically

nonstationary MFA (INST-MFA) technique came into existence. INST-MFA explores the metabolite labeling formats acquired during the transient labeling period earlier to isotopic steady state. Photosynthesis and human cells are successfully studied through this technique [131,166,167]. $^{13}CO_2$ labeling gives rise to the regular labeling of all metabolites in the steady state, due to this steady state MFA is unsuitable to photoautotrophic tissues [168]. Thus, steady state MFA is a legitimate technique for exploring mixotrophic and heterotrophic plant tissues, so it can't applicable in photosynthesis studies. Sweetlove *et al*. revealed the uncertainty of the isotopic steady state in leaves because of complexity emerged from the light-dark cycle and the slowdown ratio of metabolic pools [169]. Young *et al*., acknowledge this problem through the INST-MFA technique in *cyanobacterium Synechocystis* [167]. Comprehensive flux map was acquired for entire Calvin-Benson cycle reactions along with few side reactions, besides that it consists of a malic enzyme, photorespiratory pathway and that catalyze by Glucose-6-phosphate dehydrogenase. The metabolic pool sizes remain suite as free parameters in this analysis, while in the kinetic flux balancing, a similar formulation in application, in Arabidopsis, model was constrained with measured pool sizes acquired by mass spectrometry and non-aqueous fractionation to deliver acquaintance on subcellular pool sizes. Szecowka *et al*., derived a set of intracellular fluxes in integral irradiated Arabidopsis rosettes [131]. They examined the dynamic reallocation of the label from $^{13}CO_2$ delivered to leaves, and from that, a minor set of fluxes were measured. This technique endorsed to resolve kinetic fluctuations in isotope configurations of 40 metabolites of major carbon metabolism and to standardize them alongside four classically resolved flux signatures of photosynthesis [131].

Non-destructive methods for metabolite analysis

In vivo non-destructive approaches such as NMR spectroscopy and imaging that might give knowledge on the levels and discovered labeling metabolites [170,171]. Generally, NMR is restricted by subtlety to coverage on the large adequate metabolites, besides that in suitable circumstances, it provides methodologies to the circulation of those metabolites through subcellular compartments, commonly among the vacuole and the remnant of the cell. A substance which is positioned in multiple intracellular environments might provide specific signals based on either the signal are sensitive to any change in pH, ionic composition or viscosity among those compartments [171,172]. The pH reliance of NMR signals is mainly used for phosphorylated compounds as well as organic acids [173,174], also information about compartment has been getting on amino acids [175,176] and ammonium [177]. Steady-state fluxes directly projected by *in vivo* NMR spectroscopy by magnetization transfer; besides that, it is so interpretive in heterotrophic plant tissues about the turnover of phosphorylated compounds [171]. For high-quality time course measurements of labeling can also be obtained by *in vivo* NMR reveal in by Troufflard *et al*, that reflects fluxes directly [178]. Protein reporters of fluorescent along with fluorescence microscopy gives further non-destructive *in vivo* technique to analyze metabolite levels in various compartments

[179,180]. For numerous metabolites, sensitive reporters have been generated mostly for amino acids and sugars, so that might be particularly aimed at numerous diverse intracellular compartments. By making the calculation of subcellular metabolites density and their fluctuations in response to perturbations [180].

Kinetic models

In FBA of Genome-Scale Metabolic models, it was presumed that there is no any alteration in the density of metabolites over time. However, in kinetic models, it has the capability to simulate the dynamic alterations in concentration over time by comprising enzyme limits [181,182]. Besides that it might directly integrate substrate concentrations, substrate-level constrain barriers and enzyme levels [181,183,184]. It is the utmost comprehensive and analytically mathematical depiction; it needs dynamic behavior of enzymes as input and usually practiced to least proportion of metabolic network, ranged from 10 to 50 reactions [185]. In contrast to the approach of steady state stoichiometric, dynamic models measure both fluxes and metabolite concentration within a time-dependent manner of the system [186]. In this method, each reaction is distinct as an enzyme which catalyzes the transformation of its substrate into product and reactions are displayed using diverse equations. A kinetic model might be both predictive and inclusive if there is sufficient decisive data [187,188]. Wang *et al*. developed a kinetic model of monolignol biosynthesis in *Populus trichocarpa* by carrying an inclusive study to get the reaction and kinetic parameters of all the related enzymes form on functional recombinant proteins. Because of the obstacle faced in procurement the needed information, yet limited inclusive model has been existing in plants metabolism [188]. This deficiency might be overcome with the structural-kinetic model that can deliver a potential way. This technique signifies an intermediate bond between the several dynamic kinetic models and the stoichiometric methods. Despite it unable to describe exact dynamic behavior, it defines the constancy and vitality of the precise metabolic state and rectifies associated interactions and parameters leading the system's dynamic features. Steuer *et al*., gives the precise mathematical description, along with the projected workflow for modeling [189]. A structural kinetic model was constructed to examine the Calvin-Benson cycle which comprising of 18 metabolites and 20 reactions. The model effectively educes dynamic characteristic of the system without depending on any specific postulation about the active form of the kinetic rate equations [189]. Steuer *et al*., implemented the same method to the TCA cycle in plants to identify and evaluate the dynamic behavior [190]. To negotiate the difficulties another method has been implemented to gather the kinetic model in a "top-down" fashion, amounting to fitting the model to the observed metabolite concentrations and fluxes. This methodology was practiced to model the benzoic system in the *Petunia hybrida* flower that leads to effective recognition of the vital flux-controlling steps [191]. In sugarcane (*Saccharum officinarum*) a "bottom-up" kinetic modeling methodology has been

designated in modeling phloem flow in the form of a convection-diffusion reaction framework [192].

2. CONCLUSION

Genome-scale metabolic modeling is explicated quickly and might soon attain the point where it might commence to make an influence on plant metabolic modeling fashion. Even so, severe difficulties are there which have to address before to implement it for plant metabolic models at genome-scale level. New modeling technique like INST-MFA and flux profiling approach likewise along with sophisticated labeling techniques will overcome the hindrances and uplifts the accuracy of modeling results. Newly progress in INST-MFA strategies will bring rapid accuracy and new occasions to the understanding of complicated metabolic networks and pathways and better interpretation of the cellular community like subcellular compartmentation which is the more complicated issue in plants.

ACKNOWLEDGEMENT

The authors acknowledge all researchers who have contributed in the for metabolic flux analysis studies in plants by modeling, simulation tools and analytical techniques and also apologize to them whose work in this area was not directly cited in this review due to space confinement. The authors also appreciate the support received from the CSIR India for fellowship funding to Villayat Ali, and Research Seed Grant to Dr. Malkhey Verma from Central University of Punjab, Bathinda, India.

CONFLICT OF INTEREST

Authors have no any conflict of interest.

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