Progress in Lipid Research 58 (2015) 97–120

Contents lists available at ScienceDirect

Progress in Lipid Research

journal homepage: www.elsevier.com/locate/plipres

Review

Tracking the metabolic pulse of plant lipid production with isotopic labeling and flux analyses: Past, present and future

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ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form 30 January 2015
Accepted 11 February 2015
Available online 13 March 2015

Keywords:
Metabolic flux analysis
Isotopic labeling
Acyl editing
Central metabolism
Mass spectrometry

ABSTRACT

Metabolism is comprised of networks of chemical transformations, organized into integrated biochemical pathways that are the basis of cellular operation, and function to sustain life. Metabolism, and thus life, is not static. The rate of metabolites transitioning through biochemical pathways (i.e., flux) determines cellular phenotypes, and is constantly changing in response to genetic or environmental perturbations. Each change evokes a response in metabolic pathway flow, and the quantification of fluxes under varied conditions helps to elucidate major and minor routes, and regulatory aspects of metabolism. To measure fluxes requires experimental methods that assess the movements and transformations of metabolites without creating artifacts. Isotopic labeling fills this role and is a long-standing experimental approach to identify pathways and quantify their metabolic relevance in different tissues or under different conditions. The application of labeling techniques to plant science is however far from reaching it potential. In light of advances in genetics and molecular biology that provide a means to alter metabolism, and given recent improvements in instrumentation, computational tools and available isotopes, the use of isotopic labeling to probe metabolism is becoming more and more powerful. We review the principal analytical methods for isotopic labeling with a focus on seminal studies of pathways and fluxes in lipid metabolism and carbon partitioning through central metabolism. Central carbon metabolic steps are directly linked to lipid production by serving to generate the precursors for fatty acid biosynthesis and lipid assembly. Additionally some of the ideas for labeling techniques that may be most applicable for lipid metabolism in the future were originally developed to investigate other aspects of central metabolism. We conclude by describing recent advances that will play an important future role in quantifying flux and metabolic operation in plant tissues.

Contents

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http://dx.doi.org/10.1016/j.plipres.2015.02.002
0163-7827/Published by Elsevier Ltd.

1. Introduction

1.1. Network fluxes define cellular phenotype and are measured with isotope labeling

Plant cellular function is defined by networks of enzymatic reactions with substrates and products that are linked by mass and energy balances. Through developmental or environmental cues the expression of genes change the operating network and underscore the dynamic nature of metabolism. Fluxes establish the products of metabolism and can be measured through the rate of accumulation for end products (i.e., compounds that are not turned over such as storage protein or oil in a seed). However at metabolic steady state cellular intermediates do not accumulate and are both produced and consumed at rates that cannot be readily inferred from metabolite concentrations alone (i.e., metabolomics). Thus as a metabolic attribute, fluxes must be assessed through other means. Isotopes can serve as "tracers" that describe the rate of conversion between pools over time, by providing a change in metabolite molecular mass that occurs without perturbing metabolism. Therefore the isotopic "labeling" indicates formation and turnover through movement of an isotope from one metabolite to the next, leading to flux descriptions that are important to studies on metabolic operation, regulation and control [1].

1.2. Cellular roles for lipids in metabolism are diverse and complex

In biology few types of molecules serve as many diverse roles or are as poorly characterized as lipids. The Arabidopsis genome contains over 600 genes annotated with functions putatively tied to lipid and/or fatty acid catabolism and anabolism, however less than half of the genes have been characterized with *in vivo* studies of mutants that demonstrate a clear role in lipid metabolism [2,3]. In the most rigorously studied pathways many enzymes have multiple isoforms that conduct identical or very similar biochemical reactions, but reflect specialized cellular, subcellular or developmental activities for different aspects of lipid metabolism. Whereas some mechanisms of lipid metabolism are conserved between organisms, the subcellular descriptions of biosynthesis and degradation in plants are distinct from other species [4]. Thus textbook descriptions of lipid metabolism are not universal, and the operational network of metabolic reactions can vary between species, tissues, and cells; as well as across developmental and environmental conditions. Fluxes change to accommodate the different cellular demands for lipid production (e.g., membrane, surface, or storage lipids), as well as turnover (e.g., storage oil breakdown during germination) necessary to produce other metabolic precursors, energy, and to maintain homeostasis [5,6].

Lipids comprise membranes that are a defining feature of cell biology. Membrane lipids separate cells from their environment (i.e., plasma membranes) and establish subcellular organelles that compartmentalize metabolism in eukaryotes. Layered on surfaces, cutin and suberin provide a resistive, protective barrier to natural elements, while other lipids perform signaling functions or serve in an energy storage capacity. Triacylglycerols (TAG) in the seeds (e.g., soybean, rapeseed, sunflower) or fruit (e.g., olive, avocado, palm) of many plants are an energy dense storage form of biomass. Apart from pericarp tissues, stored oils are remobilized at germination providing carbon and ATP for plant growth until autotrophic metabolism can be sustained [7]. The biochemistry and genetics of storage lipid accumulation have received extensive attention, and this area of research remains an important focus in part because acyl chains are one of the most highly reduced forms of carbon (i.e., approximately twice the energy content per gram of dry weight than carbohydrates or storage protein) and can be used to supplant non-renewable petroleum in many applications. Our dependence on TAGs for food, fuel and chemicals contributes to an industry currently estimated at over $120 billion per year (http://lipidlibrary.aocs.org/market/prices.htm). Despite the many roles for lipids and intense research in plant lipid biochemistry and genetics over the past half century, significant gaps in our understanding remain, foremost among these are the identity and *in vivo* function of genes and enzymatic reaction networks involved in lipid metabolism [2,3].

1.3. The scope of opportunities for lipid production and scientific discovery

Given that oil content varies in plants from less than 1% of dry weight (e.g., lentils, potatoes) to approximately 70% (pecans, walnuts) and can exceed 88% in mesocarps (e.g., palm); there exists a large difference in metabolic operation among various cells that is an inviting prospect for engineering increased oil accumulation in plants. Tissues such as leaves are usually less than 5% lipid, however the abundance of leafy biomass *vs.* seed/mesocarp tissue in most plants has led to significant recent efforts to engineer lipid accumulation into vegetative tissues for biofuels [8–14]. To

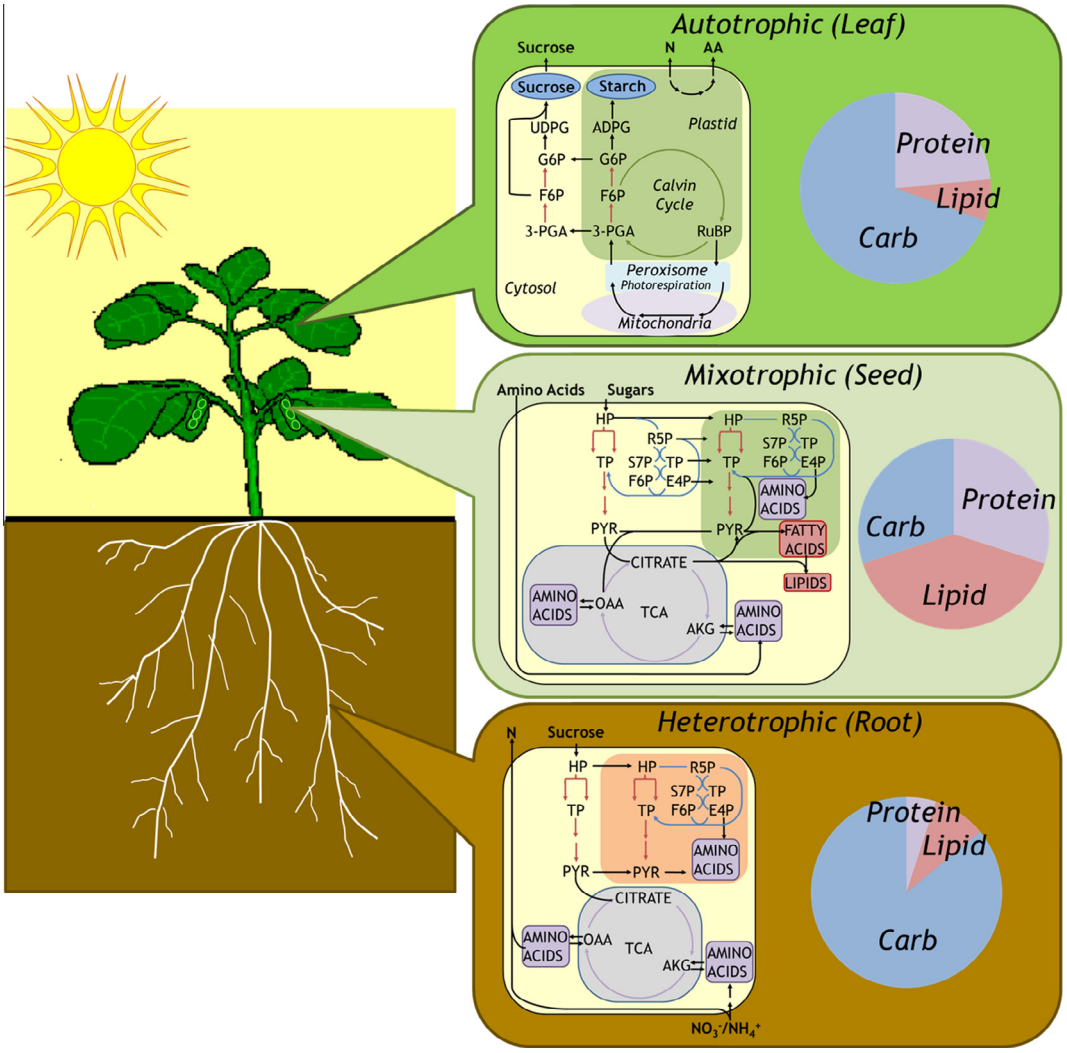

Fig. 1. Plants operate at a systems level with different cellular metabolic activities and biomass compositions in different tissues. Simplified networks of metabolism are shown. Leaves that are primarily autotrophic assimilate CO_2 through the Calvin cycle (green arrows) and glycolytic enzymes (red arrows) to make organic compounds, mostly sucrose, with energy from sunlight. Seeds can operate hetero- or mixotrophically converting sugars and amino acids into storage reserves that can include significant amounts of storage lipid. Seeds may have duplicated pentose phosphate (blue arrows) and glycolytic pathway activities along with TCA cycle (purple arrows) and significant amino acid biosynthetic flux necessary to make storage reserves. In some instances green seeds also possess the capacity to reassimilate respired CO_2. Roots receive carbon, predominantly as sucrose from other plant tissues and function heterotrophically with significant TCA cycle activities and oxidative pentose phosphate metabolism to assimilate nitrogen and meet cellular demands. Together the cellular activities characterize the plant as a system. (Abbreviations: ADPG, adenosine diphosphoglucose; AKG, alpha-ketoglutarate; AA, amino acid; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; HP, hexose phosphate; N, nitrogen; OAA, oxaloacetate; PYR, pyruvate; R5P, ribose-5-phosphate; RuBP, ribulose 1,5-bisphosphate; S7P, sedoheptulose-7-phosphate; TCA, tricarboxylic acid cycle; TP, triose phosphate; UDPG, uridine diphosphoglucose; 3-PGA, 3-phosphoglyceric acid).

date there have been a few examples of significantly altered oil levels in crop plants [15–17], and other engineering efforts that demonstrate the potential to change fatty acid composition for health or industrial applications, though mechanisms that control FA synthesis, FA modification, and accumulation in plant tissues are still not well understood [18–20]. Future progress will require a more complete understanding of central carbon metabolism including further annotation of genes, networks and enzyme function, regulation of central carbon flux with FA biosynthesis, and the coordination of FA modification with lipid assembly and turnover to produce more oil with specific FA compositions. Advances in genetics and omic-level analyses could be complemented by isotopic labeling of targeted pathways as well as larger network flux analyses; providing the rationale to revisit what has been learned through prior isotope investigations.

In this review we first provide a description of the analytical methods including a description of isotopes, instrumentation, and different types of experiments. Next some of the seminal discoveries in metabolism that were established through labeling analyses with substrates commonly used to explore lipids are reviewed. After some general considerations on computational metabolic flux analysis, we describe what insights about regulation and control have been learned from experiments that perturbed metabolism. Finally, the most recent developments in the application of isotope labeling, novel instrumentation and flux analysis strategies are described that represent a significant opportunity for future explorations in lipid metabolism and that aim to define plant function at the systems level (e.g., the coordinated auto-, mixo- and heterotrophic carbon metabolism and regulation across cells and tissues, Fig. 1).

2. General considerations

2.1. Elemental isotopes used in biochemical studies

Isotopes of low natural abundance can serve as tracers and are used to describe metabolic pathways, enzymatic reaction mechanisms and movement of atoms within plant cells. The isotopes for an element contain differing numbers of neutrons resulting in unique masses. Isotopes can be stable or radioactive based upon their half-lives, requiring different detection techniques and contributing to their use in scientific explorations. In most labeling experiments, a portion of the most abundant isotope of an element (e.g., ^{12}C, ^{16}O, ^{1}H, ^{14}N) is substituted with either a different stable (^{13}C, ^{2}H, ^{15}N, ^{17}O or ^{18}O), or long-lived radioactive (^{14}C, ^{3}H, ^{35}S, ^{32}P, ^{33}P) form. Other radioactive tracers with very short half-lives (e.g., ^{11}C, half-life ~20 min relative to ^{14}C, half-life 5730 years) can be synthesized just prior to use and assessed by positron emission tomography (PET) analysis [21–23]. Such short-lived radioisotope investigations are more specialized and will not be further considered though their application in photosynthesis, long distance transport and other plant processes are becoming more common [22,24–26].

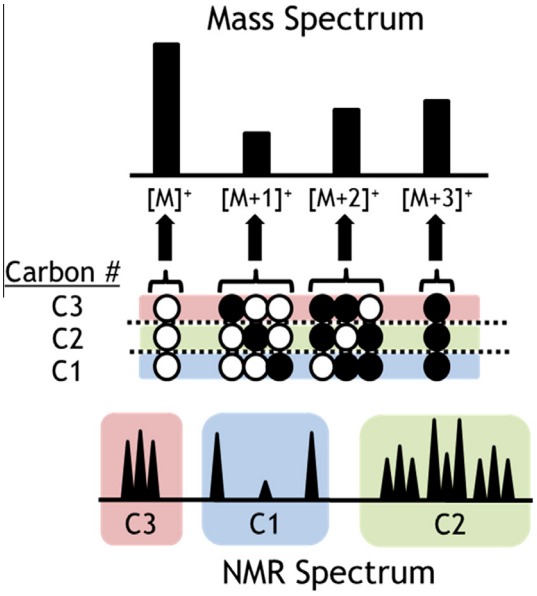

Fig. 2. NMR and mass spectra. A hypothetical set of NMR and mass spectra from analysis of a three carbon molecule that contains unlabeled (12-carbon; open circles) and isotopically labeled (13-carbon; closed circles) in various combinations known as isotopomers. For a 3 carbon molecule there can be up to $2^3 = 8$ isotopomer labeling descriptions because each of the 3 carbons can be either labeled or unlabeled. The bar graph is a typical description provided by mass spectrometry, where the intensity of the $[M]^+$ to $[M+3]^+$ peaks represent the relative abundance of labeled species within the population for the compound. The isotopic composition is measured based upon mass and does not resolve the location of the isotope within the metabolite, rather the amount of labeling is distinguished only by the differences in weight detected through mass spectrometry, thus "mass isotopomers". For the three carbon molecule there are four mass isotopomers. The interpretation of NMR spectra is often more complex and indicates the amount of labeling within a particular position in the molecule as well as its relation to other labeled molecules within the carbon backbone. In carbon-13 spectra the higher degree of labeled carbons bonded to each other creates more complex peak splitting patterns, thus the pattern of peaks for carbon 2 (C2) is more complicated because it can be connected to zero, one or two ^{13}C atoms. Each connected ^{13}C atom resulting in splitting of the peaks. NMR spectra provide "positional isotopomer" information indicating the enrichment of a particular atom and also describe bond-connectedness to other ^{13}C atoms.

The type of information obtained from stable or radioactive isotopic enrichment is distinct. Stable isotopic enrichment can be assessed within individual atoms of the molecule (e.g., using NMR), whereas radioactivity generally describes the overall labeling of the molecule unless additional techniques (e.g., a lipolytic cleavage) are employed. Assaying the incorporation of radioisotope into biomass does not require prior separation of metabolites per se and sensitive detection of radioactivity through liquid scintillation counting or autoradiography is not influenced by the presence of unlabeled metabolites. This can be of benefit because the kinetics of labeling can be established using very low 'tracer' amounts of radioisotope for short labeling durations (a few seconds to a few hours) presuming that the endogenous substrate pool labels quickly (i.e., little to no lag time). By definition isotopes contain the same number of protons per atom and maintain very similar biophysical properties; allowing investigations to probe metabolism with minimal perturbation from label introduction. Nonetheless, care is necessary to avoid enzymatic discrimination between labeled and unlabeled elements, (i.e., isotope discrimination or kinetic isotope effects) which is well-known and can lead to artifacts [27–30]. In some cases these same distinctions have been used to constructively characterize physiology as described elsewhere [31,32].

With stable isotopes there is less of an enzymatic preference [33], and in addition stable isotopes do not require special handling precautions. However, possibly the greatest benefit is that the analysis of stable isotopes by mass spectrometry and NMR can provide additional positional and compositional information at the atom and bond levels of metabolites without laborious preparations. The description of isotopic composition for a compound is referred to as the set of isotopologues whereas isomers with equivalent numbers of each isotopic atom but at different positions in the molecule are referred to as isotopomers (i.e., isotopic isomers; [34]). The terms "mass" or "positional isotopomers" are often used to describe molecule composition that varies by the number of

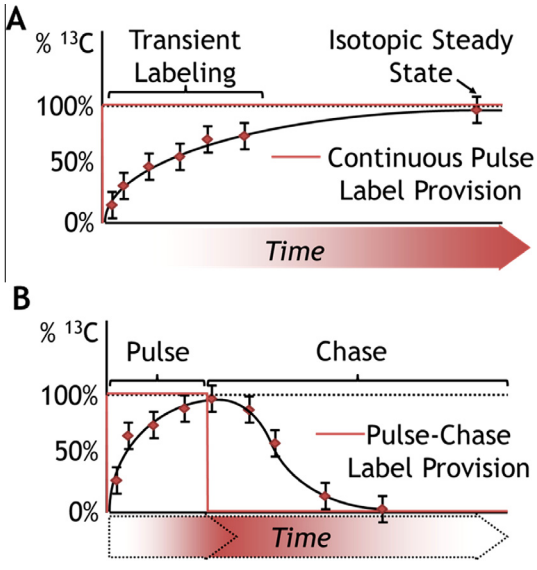

Fig. 3. Transient and steady state isotopic (e.g., ^{13}C) incorporation from pulse or pulse-chase labeling experiments. Isotopic labeling experiments display the incorporation into or dilution of isotope from metabolites that accompanies the metabolic operation of the biological system. (A) Providing a continuous source or "pulse" of ^{13}C to metabolism results in an increasing amount of isotopic labeling in metabolites that eventually approaches a steady state value. If the source of carbon is completely ^{13}C, the metabolite will eventually approach 100% labeled, presuming it is an intermediate that is completely turned over. (B) In a pulse-chase experiment the isotope is provided for only a limited duration resulting in labeling for a period of time followed by dilution when the source of carbon is switched from ^{13}C to ^{12}C.

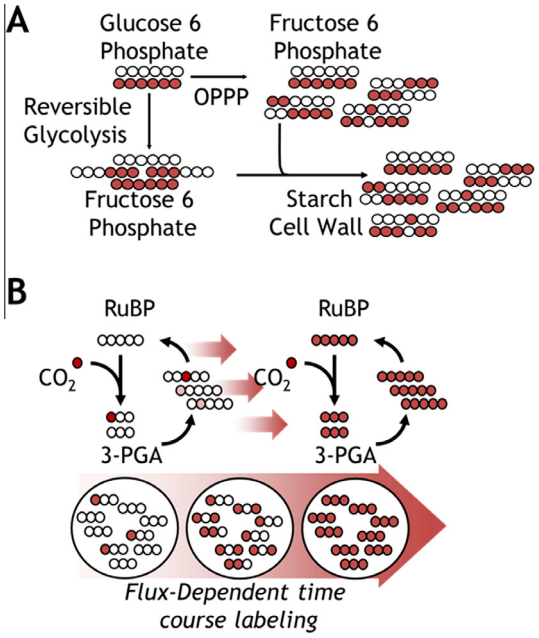

Fig. 4. Isotope distribution in heterotrophic and autotrophic pathways. Bond breaking and reforming reactions of metabolism alter the metabolic labeling of metabolites. (A) Provision of both unlabeled (open circles) and isotopically labeled (closed circles) glucose to cells utilizing the oxidative pentose phosphate pathway (OPPP) and glycolytic steps will result in different labeling descriptions in intermediates such as fructose-6-phosphate as well as derived end products such as starch or cell wall. (B) In autotrophic metabolism the provision of isotopically labeled carbon as $[^{13}C]O_2$ results in complete labeling of metabolites over time, therefore information on the fluxes through metabolism must be obtained by measuring the transient incorporation of isotopes.

each isotope (i.e., causing changes in mass) or that varies by the location within the molecular structure (i.e., position). The analysis of relative labeling with stable isotopes within a molecule was coined Mass Isotopomer Distribution Analysis (MIDA) by Hellerstein and colleagues [35]. Along with Kelleher and Masterson [36], these authors established frameworks to describe FA biosynthesis and applied the concepts with $[^{13}C]$glucose, $[^{13}C]$acetate, and ^2H_2O experiments to inspect aspects of metabolism [37,38]. As methods to correct for natural abundance levels of isotopes were developed [39,40], the labeling description became highly quantitative and well-positioned for computational modeling developments such as metabolic flux analysis [MFA, see Section 4.1; [41–43]] that is frequently used to study aspects of central metabolism.

2.2. Measuring isotopic enrichment with MS and NMR

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are complementary techniques to assess stable isotope incorporation (Fig. 2). Mass spectrometry "weighs" the composition of isotopes that result in altered mass. Through interpretation of fragmentation products, MS can assess the degree of labeling in molecular substituents and in theory the positional location of the isotope within a molecule. NMR is an atomic property that distinguishes isotopes with non-zero nuclear magnetic moments and is well-suited for positional labeling analysis. General principles of NMR spectroscopy emphasize its versatility to assess metabolism in entire tissues or through the analysis of extracted metabolites [44–50]. NMR requires more material, additional transient scans, reduced temperature or special tubes to accommodate the reduced sensitivity of instruments. Multi-dimensional NMR experiments or modification of the solution or metabolites chemically are also

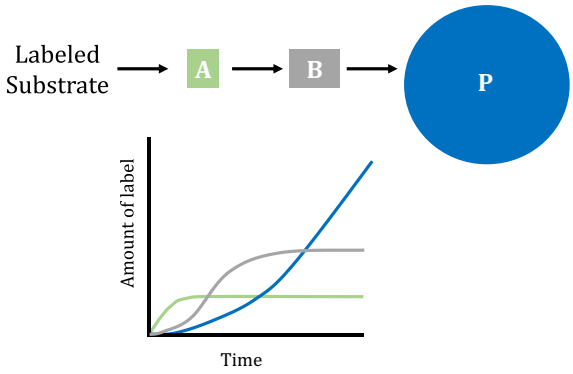

Fig. 5. Labeling kinetics in a linear metabolic pathway. Labeling of network intermediates in series from a continuous pulse of isotope resulting in intermediates that approach a steady state labeling value and a product pool that accumulates label over time throughout the experimental duration.

common techniques used to improve resolution and sensitivity [51–56]. Though the extra effort and lower throughput of NMR has limited its use to more specialized investigations, quantification of positional enrichment with NMR can lead to more intuitive assessments of metabolic pathways (relative to mass isotopomer descriptions) that may be especially important when the network is not well-characterized a priori. Recent advances in NMR are discussed further elsewhere [49,53].

2.3. Steady state and transient labeling

Isotope studies take one of several forms based on whether steady state, pulse, or pulse-chase experiments will be most informative. Steady state labeling analyses imply that the isotope is provided in excess for sufficient time (usually minutes to hours) to achieve an unchanging description of labeling in metabolic intermediates (Fig. 3). In developing seeds or cell-culture suspensions metabolism operates at a pseudo steady state during periods of development, therefore isotopically labeling these tissues with a mixture of labeled and unlabeled substrates will result in redistribution of the isotope that reflects pathway use and flux. Such studies have been regularly performed with metabolic flux analysis to document the partitioning of carbon, energy and reducing equivalents to oil, protein and carbohydrate in seeds or cell cultures [57–64]. However steady state labeling analyses are limited to assessments of pathways that enzymatically rearrange the distribution of isotope. For example, in central metabolism of developing seeds the provision of $[^{13}C]$labeled glucose will result in fructose-6-phosphate (F6P) that is labeled at different positions depending on the degree of reversibility of glycolysis and the use of the oxidative pentose pathway (Fig. 4A). The labeling in F6P can be measured directly with LC-MS/MS or inferred from measurements of plant storage products such as starch and cell wall.

More challenging is the study of autotrophic metabolism which depends upon CO_2 as a source of carbon. CO_2 does not contain carbon–carbon bonds that can be rearranged and the incorporation of $[^{13}C]O_2$ will lead to complete enrichment of metabolites at steady state which are no more informative for pathway flux than the initial unlabeled description (Fig. 4B, [65]). Thus the study of leaves and other autotrophic cells may be best suited to transient or "nonstationary" isotopic analysis [66–70]. Pathways that have limited carbon bond rearrangements or network branching such as specialized metabolite production or pathways based on a single precursor like fatty acid biosynthesis that utilizes acetyl-CoA units have typically been investigated utilizing the kinetic incorporation of radioisotopes over time (i.e., pulse labeling at metabolic steady state as described below). However these pathways may also be

prime candidates for nonstationary metabolic flux analysis with stable isotopes in the future (see Section 4).

The incorporation of isotopes over time provides a transient, dynamic description of pathway activity and is referred to as a pulse labeling experiment (Fig. 3A). If the experiment is extended after the labeled substrate has been removed and the change in label accumulation or depletion in metabolites is measured during this "chase" period, then the experiment is referred to as a "pulse-chase" experiment (Fig. 3B). Pulse and pulse-chase experiments utilize the kinetics of isotopic labeling over time to determine the precursor–product relationships of intermediates within a metabolic pathway or network [71]. In a pulse experiment the individual pools become labeled in the order which they are generated by metabolism (Fig. 5). Assuming there are no diffusion or uptake issues for the substrate, the lag time for labeling in the first metabolite will be very small and the incorporation of label over time into the first metabolite pool will reflect a hyperbolic pattern. If diffusion is an issue the curve will exhibit a lag period and become sigmoidal. The first metabolite in a sequence incorporates label at a linear rate but then starts to level off as steady state enrichment is achieved. The metabolic intermediates downstream will exhibit sigmoidal curves with an initial lag period followed by more rapid linear labeling then approach a maximal value. The lag reflects the time needed to fill precursor pools. Products of metabolism can lag significantly because in the network they are often far from the source of label. As products are not turned over into other pools they will continue to accumulate label during the course of the experiment (Fig. 5). Thus, a pulse labeling experiment with short time points (typically seconds, minutes, to only a few hours) can be used to assess the labeling of each intermediate in the pathway and establish network flow. Following the labeling with a chase period (i.e., pulse-chase experiment) enables the tracking of isotopic enrichment as well as dilution and is potentially more informative because reactions that create as well as consume a pool can both be elucidated. Though conceptually straightforward, the movement through intermediates can be challenging to establish because many metabolic steps operate reversibly with forward and backward reaction rates near-equilibrium. As a consequence isotope flow between successive intermediates can exceed the net flux of molecules transferred from one pool to the next. Thus as with any experiment the information obtained will reflect the biological system and its measureable attributes.

Together these considerations contribute to the design of the experimental labeling strategy that minimally addresses: (a) the time scales of metabolism (i.e., both rate of metabolism, and the practical experimental duration including the viability of excised tissue during incubation), (b) the pathway reactions including atoms and bonds that are altered, (c) the sufficient uptake of the labeled substrate and its impact on metabolism, and (d) the capacity to measure label incorporation in relevant metabolites. Given the number of considerations and the time and resources necessary to do labeling studies, *in silico* methods that can optimize the experimental design have received considerable attention [72–79].

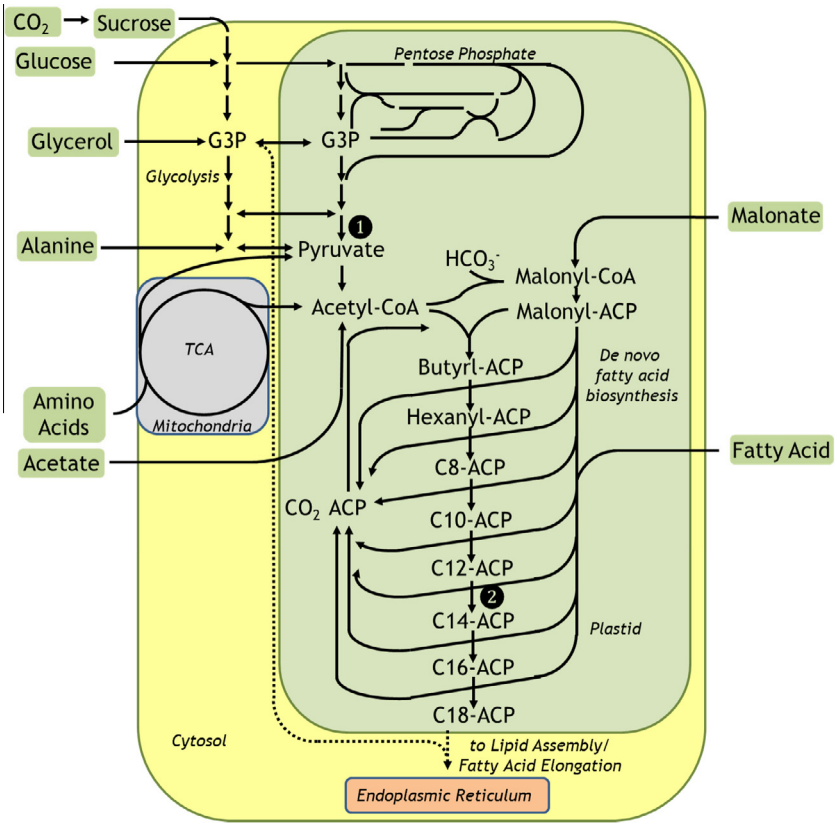

Fig. 6. Label introduction into central carbon and fatty acid biosynthetic pathways for lipid analysis. Isotopes can be supplied through substrates that are involved in different bond breaking and reforming reactions and therefore provide complementary information about network operation and pathway flux. A number of commonly used exogenous isotopically labeled sources are indicated by green boxes. Sugars and CO_2 are incorporated through production of sugar phosphates by the Calvin cycle, pentose phosphate pathways, and glycolysis and are therefore augmented significantly in the production of acetyl-CoA. Labeled acetate is frequently used to assess fatty acid and lipid metabolism because it is directly converted to acetyl-CoA and is cheap relative to other labeled substrates. Fatty acid biosynthesis repeatedly adds an acetyl group to the acyl-ACP chain until fatty acids are 16–18 carbons long (i.e., for long chain fatty acids). The acyl chains are then released from ACP and used for lipid production that takes place in the plastid or the endoplasmic reticulum. Closed circles with numbers refer to specific enzymatic steps including: ❶ plastidic pyruvate kinase and ❷ medium chain acyl-ACP thioesterase that are specifically referenced in text.

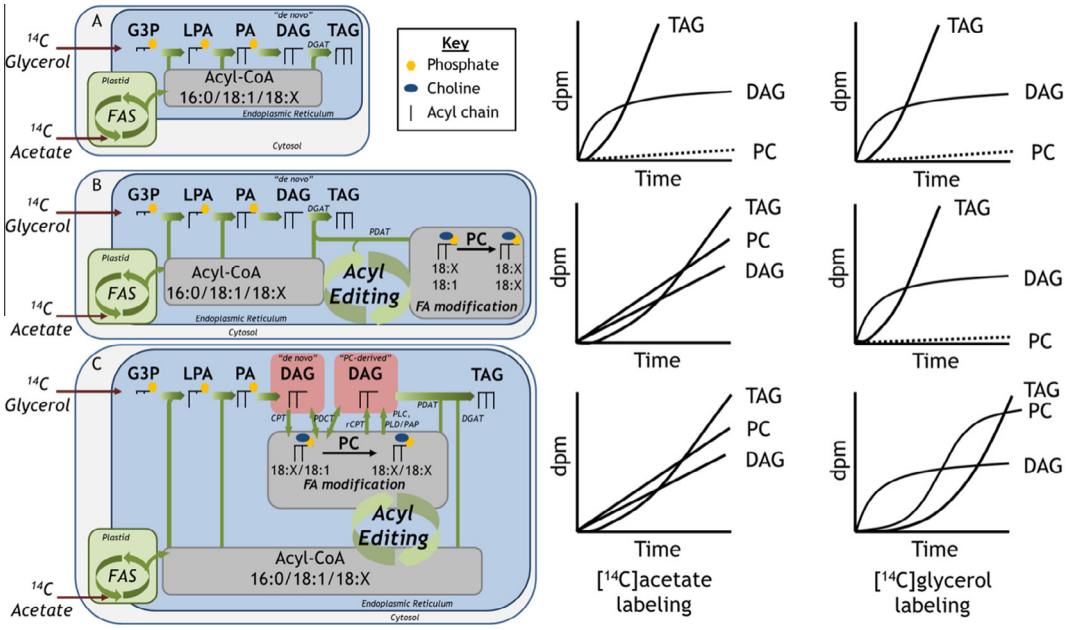

Fig. 7. Network descriptions and precursor–product relationships for lipid assembly. (A) The Kennedy pathway with linear incorporation of acyl chains into glycerol-backbone to generate TAG. (B) Additional mechanism of acyl editing that exchanges FA between the acyl-CoA pool and PC prior to TAG biosynthesis by the Kennedy pathway. (C) Acyl editing with multiple DAG pools that further emphasize PC involvement in TAG production that may not involve the Kennedy pathway. The precursor–product panels to the right of each figure indicate the different qualitative labeling patterns for each of three lipid groups. The patterns are dependent on the network relationships including the pathway sequence to TAG production. DAG labeling from [^{14}C]acetate prior to PC or TAG is indicative of *de novo* glycerolipid biosynthesis, whereas acyl editing results in faster labeling of PC relative to DAG and TAG when [^{14}C]acetate is provided. If the production of TAG requires PC as an intermediate between two DAG pools (shown in red) then the [^{14}C]glycerol labeling will result in more complicated labeling trajectories. The metabolic labeling patterns are subject to the number and size of individual pools, net and reversible fluxes and the network that splits and joins reactions for metabolites. Therefore the panels can vary extensively from the most basic description of a precursor–product relationship presented in Fig. 5. (Abbreviations: G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; FAS, fatty acid biosynthesis; DGAT, acyl-CoA:diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; CPT, CDP-choline:diacylglycerol cholinephosphotransferase; PDCT, phosphatydlcholine:diacylglycerol cholinephosphotransferase; rCPT, reverse-CPT; PLD, phospholipase D; PAP, phosphatidic acid phosphatase; PLC, phospholipase C; dpm, disintegrations per minute).

3. Pioneering investigations in lipid labeling to modern day

3.1. Enzymatic steps elucidated by tracking isotopes

Before the advent of modern day molecular biology techniques, isotopic labeling studies were used to elucidate biochemical pathways including substrates and products of individual reactions. In the 1940's Ruben and Kamen established that water not carbon dioxide is the source of evolved oxygen using H_2[^{18}O] [80]. The discovery and implementation of carbon-14 by the same investigators revealed that the 'dark' reactions of carbon fixation need not take place in light [81] and provided inspiration for the work of Benson and Calvin that eventually led to descriptions of the Calvin-Benson cycle [82] as well as C4 metabolism [83]. The early examples made clear the value offered by tracers, but methodological complexities and the laborious nature of the analytical biochemistry, along with the limited access to tracers suppressed a more widespread application. Cholesterol and fatty acid measurements based on ^{2}H incorporation, for example [84,85], involved tedious measurements of the density of heavy water after combustion of organic compounds. Therefore radioactivity-based approaches gained favor because they could be used with routine lipid extraction methods [86] and could be sensitively measured.

Fatty acid biosynthesis and lipid metabolism have been studied extensively using labeled acetate [reviewed in [87,88]]. While not the endogenous substrate for fatty acid metabolism [89], acetate is readily taken up and incorporated into both the cytosolic and plastidic acetyl-CoA pools and subsequently utilized to make newly synthesized fatty acids [90,91] and also track the incorporation of newly synthesized FAs into glycerolipids [e.g., [92,93]]. Inorganic carbon [e.g., [89,91,93,94]] isotopically enriched water [e.g., [95]], glycerol [e.g., [96,97]], fatty acids [e.g., [98–101]] as well as other substrates [e.g., [27,97,102–104]] have all been used to probe lipid metabolism depending upon the tissue or reaction(s) of interest (Fig. 6). The selection of substrates remains a critical aspect to experimental design but was particularly important in early studies that required biosynthesis of the radiolabeled substrate from inorganic compounds (e.g., [^{32}P]inorganic phosphate or [^{14}C]bicarbonate) followed by extensive purification steps prior to the labeling experiment.

3.1.1. Elucidation of lipid assembly and the Kennedy pathway

The use of radioactivity with microsomal preparations and cell free extracts has provided descriptions of the *in vitro* enzymatic esterification of FA to make membrane and storage glycerolipids, and was used to elucidate the Kennedy pathway (Fig. 7A; [105,106]) comprising a series of four enzymatic steps. The process involves two consecutive acyl-CoA dependent acylations of G3P by *sn*-1 glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), producing LPA and PA, respectively. Subsequently, the phosphate is removed by phosphatidic acid phosphatase (PAP) producing *de novo* assembled DAG that can be further acylated by the acyl-CoA dependent diacylglycerol acyltransferase (DGAT) to produce TAG. Phospholipid and TAG biosynthesis from glycerol-3-phosphate (Fig. 7) were demonstrated utilizing [^{32}P]glycerol-3-phosphate and [^{14}C]palmitate or oleoyl-CoA with microsomes and cell free extracts from animal livers [107–109] as well as plant tissues [106,110]. Pertaining to

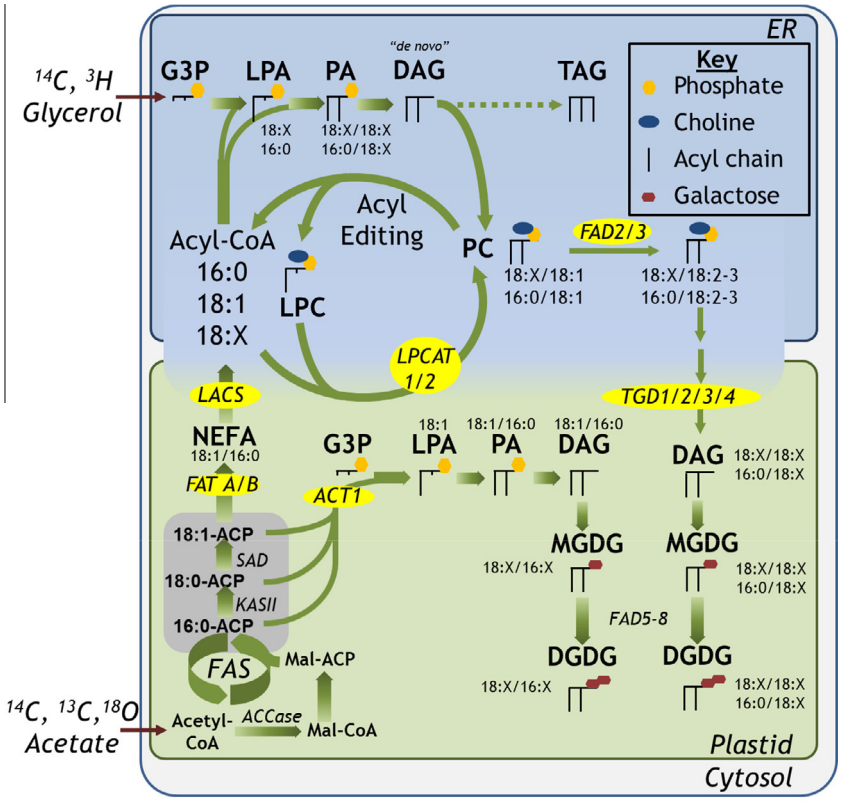

Fig. 8. Fatty acid biosynthesis and lipid assembly in the plastid and endoplasmic reticulum. Acyl chains are produced on an ACP backbone through fatty acid biosynthesis (FAS) in the plastid where the acyl-ACP can be utilized for prokaryotic membrane lipid assembly in the plastid, or hydrolyzed to fatty acids before attachment to coenzyme A (CoA) by long chain acyl-CoA synthetase (LACS) outside of the plastid. The transport mechanisms to and from the ER are unknown though studies suggest a lipid such as PC, LPC, PA, or DAG may be involved, possibly through a channeling mechanism. LPCAT association with the plastid envelope [152] supports the involvement of PC and possibly is directly tied to acyl editing. Acyl groups are processed with acyl editing and modified on PC for the production of lipids in the ER. Others have suggested that LPCAT in the chloroplast envelope supports a mechanism for LPC transport in both directions that need not be protein mediated [163,335] and LPC can partition into membranes as well as the aqueous phase. The return of lipids to the plastid for MGDG and DGDG production utilizes trigalactosyldiacylglycerol (TGD) protein mutants in the transport process [145,156]. At least PA, PC, DAG, and LPC have been implicated as acyl transport lipids; however the movement of fatty acids between the plastid and ER remains an active area of research thus the figure shows a gradient between blue and green organelles in this region. The figure was inspired by [136] with modifications to address the text. Enzymes specifically discussed are presented in yellow ovals. The pathways for phosphatidylglycerol, other phospholipids, and sulfolipid production are not shown. (Additional Abbreviations: ACCase, acetyl-CoA carboxylase; Mal-CoA, malonyl-CoA; Mal-ACP, malonyl-ACP; KASII, ketoacyl synthase II; SAD, stearic acid desaturase; FAT A/B, fatty acid thioesterase A/B; NEFA, non-esterified fatty acid; ACT1, genetic mutant from *act1* locus of Arabidopsis; LPCAT, lyso-phosphatidylcholine acyltransferase; LPC, lyso-phosphatidylcholine; TGD, trigalactosyldiacylglycerol mutant; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol).

the preferred acyl substrate, palmitoyl-CoA, synthesized chemically, could be used in place of ATP, CoA and fatty acids to esterify G3P providing direct proof that acyl-CoA is used in lipid assembly. PC biosynthesis from phosphocholine was similarly outlined using [^{14}C]- and [^{32}P]phosphocholine [109], however repeating some of the basic isotope labeling and dilution studies in plants proved challenging [111,112] in part because little was known at the time about the organelle location of lipid production. Eventually a number of aspects of the Kennedy pathway that were analogous to mammalian tissues were confirmed [97,106,113,114]. More recent studies have leveraged commercially available labeled substrates with or without [115,116], the aid of modern genetics, genetic engineering, and homology-based gene identification to demonstrate enzyme function and activity in cell extracts, microsomes and transgenic plants [116–121]. Regular discovery of new enzymatic reactions [122,123] indicates that not all the enzymatic steps (and their associated genes) within lipid metabolism have been elucidated. Differences among species emphasize the role of *in vitro* kinetic activity measurements in isolated microsomes to provide considerable insight for network elucidation and operation [124]. Thus efforts to identify and describe the "parts list" and the range of operation of enzymes in biochemical networks are critical to models that link enzymatic steps by precursor–product labeling relationships and describe coordinated network operation.

3.1.2. Desaturation substrates and pathways

Plant lipid metabolism differs significantly from mammalian systems in several aspects including distinct substrates for desaturation and multiple subcellular locations for lipid assembly that were elucidated by a combination of *in vitro* and *in vivo* labeling experiments. Seminal *in vitro* studies with [^{14}C]palmitate [125] and *in vivo* pulse chase studies with [^{14}C]sucrose [126] or [^{14}C]O_2 [127] first described the mechanisms for desaturation in plants. Oleic acid was labeled rapidly followed by linoleic and linolenic fatty acids; however during the chase the linoleic and linolenic acids continued to increase at a rate that was elevated relative to the oleic acid indicating that oleate was a precursor for desaturation to linoleic and linolenic acid [126,128]. Unlike animal systems that utilize acyl-CoA substrates for desaturation, in plants most FAs are bound to plastidic or ER membrane lipids for this process [129–134] with the exception of stearoyl-ACP desaturation. Early work with safflower labeling showed [^{14}C]oleoyl-CoA was esterified to PC leading to [^{14}C]linoleoyl-PC but without the formation of detectable [^{14}C]linoleoyl-CoA [129] supporting PC-based

modification. Desaturation of acyl-CoA's directly in plants was more definitively ruled out through the use of [^{14}C]non-hydrolysable ether analogs of PC [135].

At nearly the same time as initial descriptions of desaturation, the assembly of glycerolipids and the production of polyunsaturated fatty acids were ascribed to two separate biochemical pathways present in different organelles. The prokaryotic pathway that takes place in the plastid and the eukaryotic pathway in the ER [87,136] (Fig. 8) are distinguishable because of the attachment of hexadecanoic groups (16 carbon fatty acids) at the sn-2 position of glycerolipids – a result of the acyl-specificity of LPAAT of the prokaryotic pathway [137]. Utilization of prokaryotic pathway diacylglycerol (DAG) for phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) synthesis and the subsequent repeated desaturation of the sn-2 hexadecanoic to hexadecatrienoic group in only certain plants has led to the name "16:3 plants" [138]. Plants which assemble galactolipids primarily from a DAG-backbone imported from the ER and containing a sn-2 octadecatrienoic acyl chain are termed "18:3 plants" [139]. Thus, the fatty acid composition of galactolipids is commonly used to determine if the prokaryotic or eukaryotic pathway is the dominant path for galactolipid synthesis in plants [140].

Galactolipid assembly through both routes was described using a combination of continuous pulse, and pulse-chase metabolic labeling experiments with isotopically labeled acetate, glycerol, fatty acids and CO_2 supplied to leaf tissue, isolated chloroplasts and seedlings [99,133,137,139,141]. The transit of molecules between the ER and plastid, as required in the eukaryotic pathway, was observed when label that accumulated in PC during a pulse, was traced to subsequent increases in galactolipid labeling during the chase period. The pattern indicated a precursor–product relationship between ER localized PC and plastid localized MGDG and DGDG [132,141]. Further dual labeling with acetate and glycerol demonstrated the same ratio of fatty acid to backbone enrichment in PC as in MGDG of the chase experiment. This led Slack and colleagues [96] to conclude that the entire DAG moiety was transferred from the ER to the plastid; thus revealing the modern day description of the extra-plastidial or eukaryotic pathway of galactolipid synthesis. The identification of Arabidopsis mutants with changes in FA desaturation or membrane lipid composition by Browse, Somerville, Benning and co-workers combined with altered metabolic labeling of the two pathway system has subsequently allowed specific genes in lipid metabolism (i.e., both lipid assembly and FA desaturation) to be correlated with these pathways (Fig. 8) [142–147].

3.1.3. Acyl chain formation and trafficking between the chloroplast and ER in leaves

Though the basic paths and substrates for acyl chain and lipid formation are known, a number of details about their transport remain unclear. Fatty acid chain lengths of up to 18 carbons are made through a series of condensing enzymes that pass an acyl-ACP group and elongate it with the addition of two carbon acetyl groups repeatedly (Fig. 6). The central role of ACP was established through labeling experiments with [^{14}C]malonyl-CoA, purified ACP and ACP antibodies [104]. Ohlrogge, Kuhn and Stump varied the ACP levels to establish dependency of *de novo* fatty acid biosynthesis with this protein and also the plastidic location of fatty acid production. Export of newly synthesized FA into the eukaryotic pathway starts with release of the FA from ACP by a thioesterase [2]. Mechanisms for acyl chain export from the plastid have remained enigmatic, and may not require dedicated fatty acid transporters because non-esterified fatty acids (NEFA) that flip-flop diffuse within phospholipid membranes are immediately converted to CoA esters after export by long chain CoA synthetases [LACS; [148]] (Fig. 8). Acyl-CoAs cannot diffuse across membranes, thus their re-entry into the plastid is prevented and the overall process results in uni-directional transport. However, whereas *lacs9* mutants resulted in approximately 10-fold less acyl-CoA produced from exogenously supplied [^{14}C]NEFA [149] the mutants did not give a distinct phenotype possibly indicating redundancy with other LACS genes that are involved in the process [150].

The underlying mechanisms of fatty acid export have been difficult to fully describe in part because the activation and movement of fatty acids may be a channeled process as suggested from [^{14}C]acetate labeling experiments using spinach, Arabidopsis leaves and T87 Arabidopsis cells [27,151,152]. Arabidopsis suspension cells labeled with [^{14}C]acetate produced [^{14}C]PC very quickly with an estimated lag time of approximately 5.4 s. The short lag is significantly less than the time needed for bulk acyl group exchange and may implicate the involvement of PC in shuttling acyl groups to the ER [152]. A form of channeled transport could also be supported by the known physical interactions between the endoplasmic reticulum and the mitochondria, plasma membrane, trans-golgi and chloroplasts [153,154]. To assess the genes involved the PC-MGDG precursor–product relationship, FA and glycerol backbone labeling of leaf lipids was characterized using mutants (Fig. 8). Arabidopsis *act1* mutants (from the *act1* locus in Arabidopsis and also referred to as *ats1*, Fig. 8) pulse-chase labeled with [^{14}C]acetate demonstrated the role of plastidial acyl-ACP dependent glycerol-3-phosphate acyltransferase (GPAT) activity in partitioning FA between prokaryotic and eukaryotic pathways. Specifically, the lack of plastidial GPAT activity in the *act1* mutant resulted in diverting the flux of acyl groups from the prokaryotic pathway into the eukaryotic pathway, enhanced production of MGDG from PC, and effectively converted a 16:3 plant into an 18:3 plant [146,147]. A second set of lines with altered trigalactosyldiacylglycerol (TGD) proteins that facilitate the transfer of lipids, presumably either PA or DAG derived from PC, into chloroplasts [155–161] were characterized using [^{14}C]acetate pulse-chase experiments [145] (Fig. 8). Whereas wild-type Arabidopsis leaves showed the precursor–product relationship between MGDG and PC that is characteristic of eukaryotic galactolipid production, *tgd-1* lines did not exhibit a similar precursor–product pattern. Also the *tgd* phenotype is unlike *act1* lines that have reduced plastidic pathway biosynthesis [146,147]. Instead the *tgd*-based MGDG pool exhibited strong initial labeling presumably from the plastidic pathway, but without a second labeling peak in MGDG labeling that would signify the conversion of labeled PC into MGDG at later time points. The authors concluded that *tgd-1* lines had an impaired ability to transfer acyl groups from PC to MGDG and that the TDG1 protein was involved in this process [145]. Other investigations specifically interested in the form of lipid exported, suggest that partial hydrolysis of PC to LPC is a prominent step in trafficking acyl groups back to the chloroplast [162–164] based on acyl labeling assessments of *sn*-1 and *sn*-2 positions. Together, these studies highlight a recurring theme – the central role of PC in donating and accepting acyl groups, and emphasize the need for further mechanistic investigations of the lipid carrier(s) that import and export acyl groups between chloroplast and ER. In particular, additional labeling studies with mutants that fail to interconvert phospholipids could help resolve the pathway attributes.

3.1.4. Metabolic labeling reveals alternative fluxes of acyl groups into membrane lipids and TAG

The flux of FA from the plastid through the Kennedy pathway is minimally required to produce TAG composed of newly synthesized fatty acids exported from the plastid (e.g., 16:0, 18:0, 18:1); however, many plants accumulate TAG containing fatty acids that have been further modified (e.g., desaturation, hydroxylation).

Because PC is the substrate for most of these modifications [165,166], acyl fluxes into and out of PC are essential for the production of TAG, and the rate of PC labeling (Fig. 7) will reflect its participation. A combination of at least three mechanisms allow the flux of fatty acids into/out of PC for modification and eventual TAG synthesis (Fig. 7B–C): (1) TAG synthesis from phospholipid:diacylglycerol acyltransferase [PDAT; [123]]; (2) derivation of the DAG substrate for TAG synthesis from PC; and (3) the exchange of acyl groups between PC and the acyl-CoA pool by a PC deacylation and lyso-PC reacylation cycle coined "acyl editing" [93,94]. For each of the three mechanisms no net synthesis of PC is required for TAG biosynthesis. Acyl editing proceeds by the forward (and probably reverse) action of LPCAT [116,119,120,167,168], and incorporates nascent 18:1-CoA into PC for modification and PC-modified fatty acids can re-enter the acyl-CoA pool for incorporation into the sn-1, -2, -3 positions of TAG by Kennedy pathway enzymes [Fig. 7B; [169]]. Alternatively the use of PDAT results in the transfer of fatty acids from the *sn*-2 position of PC to the *sn*-3 hydroxyl group of DAG resulting in the production of TAG and lyso-PC. The lyso-PC is subsequently reacylated by LPCAT as part of the acyl editing cycle (Fig. 7B). If DAG used for TAG biosynthesis is derived from PC, then DAG is in essence synthesized twice because it is also the precursor for PC production (Fig. 7C). First, the production of *de novo* DAG occurs through the Kennedy pathway for PC synthesis and second, the removal of phosphocholine from PC produces a PC-derived DAG that can be used to make TAG. Mechanisms that are potentially involved in the *de novo* DAG → PC → PC-derived DAG pathway of TAG production include: (a) both the forward and reverse reactions of CDP-choline:diacylglycerol cholinephosphotransferase (CPT) [170,171], (b) CPT to produce PC and phospholipase C (or phospholipase D and PAP) for the subsequent production of PC-derived DAG, and (c) phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) [121] which transfers the phosphocholine head group from PC to DAG, creating a new PC molecular species and a PC-derived DAG (Fig. 7C). Of the possibilities, only PDCT has been experimentally shown to deliver PC-derived DAG for TAG biosynthesis based on evaluation of Arabidopsis mutants with *in vivo* metabolic labeling [121]. Proposed further modifications to the acyl editing scheme have been suggested by Lager and coworkers [122] with the production of PC from two molecules of lysophosphatidylcholine (LPC) using a lysophosphatidylcholine transacylase (LPCT). The other product of this reaction, glycerophosphocholine (GPC) could be converted back to LPC using a recently discovered acyl-CoA:glycerophosphocholine acyltransferase (GPCAT). This previously undescribed set of reactions does not require regeneration of CDP-choline and will likely impact future interpretations of acyl editing; however the enzymes catalyzing these steps have not been identified.

The use of isotopic labels has been essential in the characterization of the flux of acyl groups through acyl editing in plants. The exchange of labeled fatty acids from acyl-CoAs into and out of PC by LPCAT enzymes was initially characterized in microsomes from oilseeds [167,168]. However, the involvement of acyl editing as a major component of lipid metabolism was indicated through *in vivo* metabolic labeling of leaves tracing the precursor–product relationships of nascent fatty acid incorporation into membrane lipids. Traditional descriptions of glycerolipid assembly hypothesized that newly synthesized fatty acids were initially esterified to glycerol-3-phosphate to produce mostly 18:1/16:0 and 18:1/18:1 (*sn*-1/*sn*-2) molecular species of PA in the plastid and ER, respectively. Then, the molecular species were desaturated while linked to membrane lipids in the plastid (e.g., MGDG) or ER (e.g., PC). These concepts were brought into question when five minute [^{14}C]O_2 pulse labeling of *Brassica napus* leaves followed by one hour and twenty-four hour chases demonstrated labeled lipid molecular species that fit the hypothesis for prokaryotic MGDG, but not eukaryotic pathway derived PC [94]. Subsequent pulse labeling experiments with [^{14}C]glycerol for less than ten minutes revealed the traditional PA → DAG → PC precursor–product relationship of the eukaryotic pathway in Pea leaves; however [^{14}C]acetate labeling indicated that PA and DAG were not the primary precursors for incorporation of nascent fatty acids into PC [93]. Further stereochemical and molecular species analysis of the labeled products indicated newly synthesized fatty acids were first incorporated into eukaryotic pathway lipids by esterification to mostly the *sn*-2 hydroxyl of lyso-PC (but also *sn*-1 acylation of 1-lyso-2-acyl-PC) creating a new PC molecular species containing one nascent fatty acid and one previously synthesized fatty acid. The relative amount of sn-1 *vs*. sn-2 acylation depends on species and tissue [93,172]. Additionally, the pool of acyl-CoA utilized for Kennedy pathway reactions included a significant amount of unlabeled fatty acids that were released from PC during the recycling to lyso-PC. This acyl editing cycle allowed for direct incorporation of newly synthesized 18:1 into PC for desaturation and subsequent production of an acyl-CoA pool containing a mixture of nascent (16:0, 18:1) and further desaturated (18:2 and 18:3) fatty acids for *de novo* membrane lipid production. The rate of acyl editing was estimated to be up to 20 times that of fatty acid synthesis in rapidly expanding pea leaves, indicating that even if *de novo* glycerolipid assembly in the ER utilizes the same acyl-CoA pool as acyl editing, the much higher rate of the acyl editing cycle ensures that most nascent fatty acids exported from the plastid first enter PC. Apart from leaves, the acyl editing mechanism has also been observed in developing seeds [120,172,173] and Arabidopsis cell cultures [152], and may be a more general mechanism for the incorporation of newly synthesized fatty acids into the extra-plastidial lipids.

Similar to the characterization of the acyl editing cycle, *in vivo* pulse labeling of tissues accumulating TAG has helped decipher when TAG is synthesized from *de novo* DAG or alternatively from PC-derived DAG. When TAG is synthesized directly from nascent fatty acids using the Kennedy pathway, there is little PC labeling from [^{14}C]acetate or [^{14}C]glycerol (Fig. 7A, graphs). The addition of acyl editing activity results in more significant labeling of PC from [^{14}C]acetate but not [^{14}C]glycerol (Fig. 7B; graphs); however the glycerol labeling is useful in helping distinguish the flux of TAG synthesis from *de novo*- versus PC-derived DAG (Fig. 7A, C; [120,133,172,174,175]). During oil accumulation in developing seeds the vast majority of lipid metabolism is for TAG biosynthesis, therefore significant labeling in non-Kennedy pathway intermediates describes a more complicated path of acyl flux that produces TAG. When TAG is produced from PC-derived DAG (Fig. 7C); labeling from [^{14}C]glycerol results in a significant incorporation into DAG followed by PC and finally TAG after a significant lag. Fig. 7C indicates this through a precursor–product labeling description.

The amount of acyl flux through PC by the above mechanisms varies with plant species and tissues accumulating TAG. It is tempting to assume that the amount of PC-modified fatty acids that accumulate in TAG correlates with acyl flux through PC, however this is an underestimate because fatty acids move through PC regardless of whether they are further modified or not [5,172–174]. Thus the pathway of TAG synthesis must be elucidated by *in vivo* [^{14}C]acetate and [^{14}C]- or [^{3}H]glycerol labeling and by measuring the precursor–product relationships between DAG, PC and TAG. For example, tissue slices of developing cocoa (*Theobroma cacao*) cotyledons cultured with [^{14}C]acetate or [^{14}C]glycerol produced labeled TAG but had limited incorporation into PC from either substrate during the stage of rapid TAG accumulation, suggesting the classical Kennedy pathway is the major route of TAG synthesis (Fig. 7A; [176]). Cocoa TAG contains less than 2% PC-modified fatty acids [176], and thus did not require extensive flux

through PC (Fig. 7A). In coriander (*Coriandrum sativum*) a precursor–product relationship was observed between PC and TAG for [^{14}C]acetate but not glycerol labeling. Thus coriander lipid metabolism is more analogous to Fig. 7B where fatty acids participate in acyl editing cycle, but TAG is synthesized from *de novo* DAG with the Kennedy pathway [177]. As coriander mostly accumulates 18:1Δ6 fatty acids produced in the plastid, the substantial flux of nascent fatty acids through PC may in fact be part of the mechanism of fatty acid export to the ER [152] and not for fatty acid modification. Soybean (*Glycine max*), safflower (*Carthamus tinctorius*), flax (*Linum usitatissimum*), and *Arabidopsis thaliana*; [120,133,172,174,175] each demonstrate the PC to TAG relationship from both [^{14}C]acetate and [^{14}C]glycerol labeling. In these species acyl editing and the use of PC-derived DAG for TAG synthesis both contribute to fatty acid flux through PC and result in high levels of polyunsaturated-fatty acids in TAG (Fig. 7C). Though these examples indicate preferences for TAG synthesis, the paths are likely in simultaneous operation to varied extents, (e.g., the relative use of PDCT or CPT/lipase for PC-derived DAG production, and the use of DGAT or PDAT for the final acyl acylation of TAG [115]). In the case of DGAT and PDAT, DGAT may be used in any of the situations described in Fig. 7A–C, however, PDAT activity requires the acyl editing cycle to reacylate the lyso-PC co-product of TAG synthesis, and thus cannot be a part of a classical Kennedy pathway (Fig. 7A).

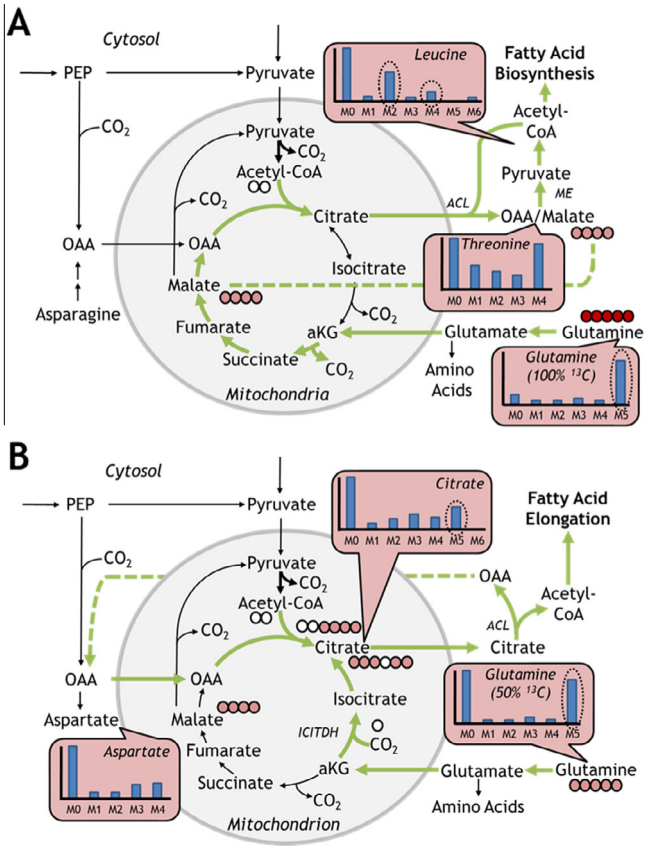

Fig. 9. Glutamine labeling studies indicate flow through organic acids to acetyl-CoA. Studies with [^{13}C]glutamine indicated that carbon from amino acids was used in the biosynthesis or elongation of fatty acids in oilseeds (figure inspired by [57,207]). Labeling in metabolites was measured by mass spectrometry resulting in mass isotopomers (i.e., M1, M2, M3 etc) that reflected the increase in molecular weight by 1, 2, 3 etc with incorporation of ^{13}C in place of ^{12}C. (A) Glutamine label in soybeans was transferred to amino acids derived from pyruvate and acetyl-CoA most likely through a combination of malic enzyme (ME) and ATP citrate lyase (ACL). The high labeling in glutamine (i.e., M5) was transferred to four carbon organic and amino acids such as threonine. Leucine that is synthesized through a combination of acetyl groups was more highly labeled in even mass isotopomers (i.e., M2, M4) indicating that a significant amount of acetyl-CoA was fully labeled. (B) The reversibility of isocitrate dehydrogenase (ICITDH) in brassica resulted in a high M5 labeling in citrate which would not otherwise occur from respiratory steps of TCA. Uniformly labeled glutamine (i.e., M5) was decarboxylated by aerobic TCA metabolism to produce M4 labeled succinate, fumarate, malate and oxaloacetate. When combined with unlabeled acetyl-CoA, the resulting citrate and isocitrate would contain four or fewer ^{13}C atoms (i.e., <M5). Thus M5 in citrate and isocitrate signified a reversible isocitrate dehydrogenase activity and with ACL provided additional acetyl groups for fatty acid elongation.

4. Extending isotopic labeling interpretation

4.1. Computational metabolic flux analysis using stable isotopes

The interpretation of labeling is often not straightforward and has been significantly aided by computational assessments of multiple pathways referred to as Metabolic Flux Analysis (MFA). These methods were initially developed to study steady state metabolism in other systems [e.g., [178–183]] but extensive resources for application in plants have now been described in books [184–186] and special issues of Phytochemistry [187] and the Journal of Experimental Botany edited by Kruger and Ratcliffe [188]. In each case the isotopic labeling experiment is assessed with a computer model that describes mass-balanced atom transitions of the biochemical reactions [43,189–191]. The model is a mathematical description of network branching, bond-breaking and reforming reactions, substrates and flux estimates that are used to simulate the label incorporation which can then be compared to the experimentally measured values through least squares regression. The process is repeated with different flux values until an optimized fit is achieved resulting in "best estimates" of flux and confidence intervals and statistics can then be assessed using Monte Carlo or techniques described elsewhere [192–194]. Progress in the quantification of label [195,196] has increased the number and precision of measurements resulting in more information than is minimally required to establish the flux values computationally (known as an 'overdetermined' system). The least squares comparison results in the best fit of all measurements and produces the lowest overall residuum (i.e., lowest residual error between *in silico* and experimental measurements). A more in depth survey of computational considerations including elegant mathematical representations are beyond the scope of this review and are described elsewhere [197–201].

4.1.1. Central carbon metabolism supplies carbon, energy and reducing equivalents for FA biosynthesis

In developing seeds, the carbon, energy and reducing equivalents necessary for fatty acid biosynthesis are derived from multiple sources requiring coordination between catabolic and anabolic metabolic pathways. Developing seeds receive organic carbon and nitrogen in the form of sugars and amino acids apoplastically from the maternal plant [202,203]. The supply of carbon and nitrogen constrains the potential metabolic operation; but the final seed composition is a consequence of the fluxes through biochemical pathways within the tissue. Branch points in the metabolic network establish the distribution of carbon, energy and reduced cofactors, thus the diversity in lipid and protein content in seeds is a consequence of differences in flux through these steps. Metabolic flux analysis has enabled the identification and quantification of the endogenous carbon sources for fatty acid biosynthesis and aided descriptions of the balance of ATP and nucleotide cofactors.

Some seeds like pea and soybean produce significant amounts of protein and therefore require a large amount of amino acid nitrogen. Most of the nitrogen supplied to the seed is in the form

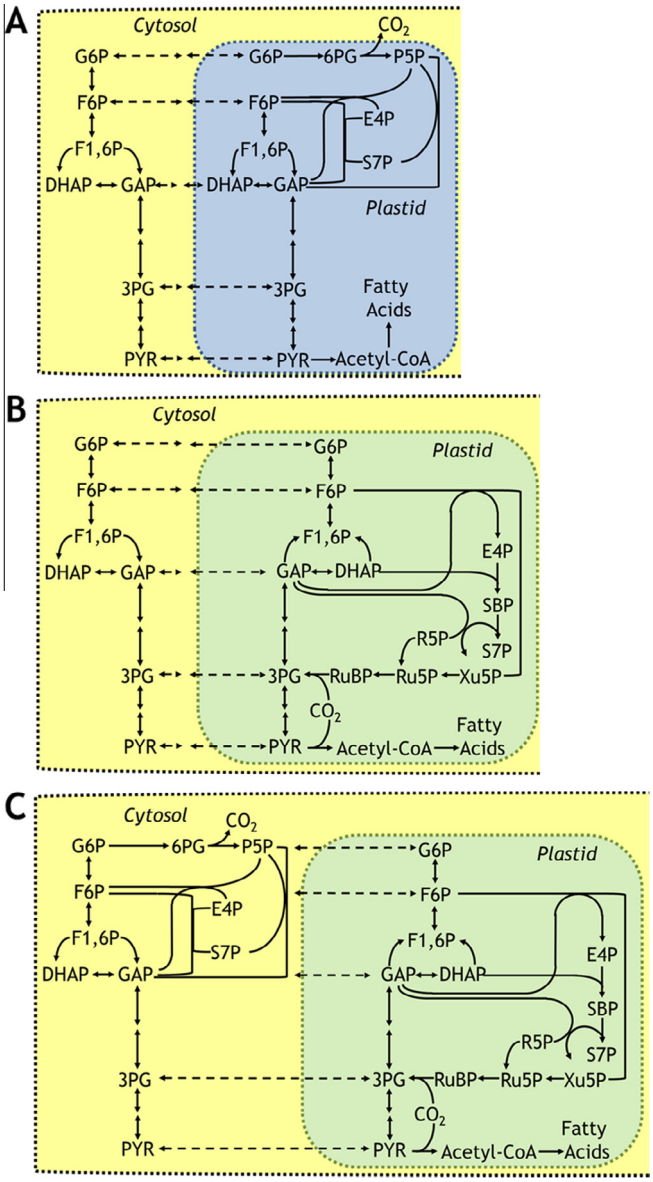

Fig. 10. Oxidative and non-oxidative pentose phosphate pathways have multiple subcellular locations. The elucidation of active pathways in central metabolism has remained difficult to assess because oilseeds have pentose phosphate pathway enzymes targeted to multiple locations which may be involved in (A) oxidative, (B) reductive or (C) both types of metabolism. Carbon, reducing equivalents and energy necessary for fatty acid biosynthesis can come from metabolic networks operating in different ways dependent upon the conditions and cell or tissue. For emphasis the plastids in (B) and (C) are green symbolizing that they make use of light and operate differently than (A).

of glutamine and to a lesser extent asparagine or alanine [202]. The nitrogen is redistributed by aminotransferases to other carbon skeletons for the production of all twenty amino acids and also results in the generation of organic acids. Radioisotopic studies [204,205] and stable isotope investigations with metabolic flux analyses in soybeans [57,206] indicate that organic and amino acid carbon is partially repartitioned into oil (approximately 10–20% of carbon in oil originates from glutamine) through conversion of malate by malic enzyme (ME) or in combination with other steps including ATP-citrate lyase (ACL; Fig. 9A). First, glutamine serves as a donor of nitrogen in transamidation reactions to produce glutamate. Glutamate aminotransferase reactions further reallocate nitrogen and result in alpha-ketoglutarate which can be converted to other organic acids (e.g., malate, citrate) using reactions commonly associated with, though not limited to the TCA cycle. Then malic enzyme can convert malate to pyruvate for acetyl-CoA production or citrate can be cleaved using ATP-citrate lyase resulting in acetyl-CoA (Fig. 9). Organic acids also serve as precursors for acetyl-CoA production in *Brassica* ([207]; Fig. 9B) and maize [208] embryos.

Acetyl-CoA can be derived from multiple sources in different subcellular locations though it cannot readily diffuse through membranes causing speculation about its origin and movement in plant cells (e.g., [209]). As a result the enzymatic paths of acetyl-CoA biosynthesis from hexose catabolism or amino acid metabolism have differing consequences on carbon and energy utilization. For example the production of two acetyl-CoA molecules from glucose-6-phosphate using glycolysis and pyruvate dehydrogenase produces: two CO_2, four NAD(P)H, and two ATP. Since fatty acid biosynthesis can be sustained with two NAD(P)H and one ATP per acetyl group, the supply and utilization of carbon, energy and reducing equivalents are stoichiometrically balanced by glycolytic reactions. Other pathways such as the oxidative pentose phosphate pathway (OPPP) can also play an important role in generating reducing equivalents for fatty acid biosynthesis however there is no strict requirement for a particular catabolic pathway's involvement. Estimates of the amount of OPPP in plant tissues such as seeds are particularly difficult to establish because isoforms for these enzymatic steps are found in both the plastid and cytosol [210] (Fig. 10) and can be further complicated by CO_2 reassimilatory mechanisms that improve the seed carbon economy [211].

Production of acetyl-CoA from pyruvate involves decarboxylation and creates one mole of CO_2 for every two moles of carbon dedicated to FA biosynthesis. RuBisCO in developing rapeseed and soybeans [212] and high internal concentrations of CO_2 [213] led to a description of RuBisCO-aided assimilation that was confirmed by [^{13}C]labeling studies [214]. [1-^{13}C]alanine taken up by rapeseed embryos was converted to pyruvate then acetyl-CoA with decarboxylation by pyruvate dehydrogenase producing [^{13}C]O_2. Reassimilation of the [^{13}C]O_2 resulted in labeling in glycolytic products that were upstream of pyruvate supporting the role for RuBisCO. It was further shown through ^{14}C investigations that the carbon reassimilation was light-dependent in green seeds [215]. Rapeseed and other green oilseeds such as soybean appear to have developed mechanisms to utilize sunlight for metabolic efficiency though the extent and descriptions remain unclear and may involve subcellular, cellular or tissue-level coordination [206,216–221]. The biological diversity and challenges associated with quantification of CO_2 respiring and assimilating steps in mixotrophic metabolism (Fig. 1) has led to wide-ranging estimates for flux through central metabolic pathways. Recent descriptions of plant respiration including an *in silico* study with label design considerations and modeling have summarized some of these challenges and opportunities [222,223].

Conversion of glutamine carbon to acetyl-CoA entails a different set of reactions that start with the donation of nitrogen from glutamine. Alpha ketoglutarate can be converted to acetyl-CoA by way of sequential production of succinate, fumarate, malate and pyruvate through enzymatic steps commonly ascribed to the TCA cycle along with malic enzyme and pyruvate dehydrogenase (Fig. 9). This series of steps will result in 4 NAD(P)H, an $FADH_2$ and one ATP equivalent, though respiring multiple carbons in the process. The additional reducing equivalents may supply electron transport for oxidative phosphorylation needed for protein biosynthesis as approximately 4.3 mol of ATP are required for each additional amino acid added to an elongating peptide [41]. Other seeds such as rapeseed utilize acetyl-CoA originating from the organic acid citrate as a carbon source for fatty acid elongation (i.e., cytosolic addition of acetyl groups for acyl lengths of twenty carbons or more). [^{13}C]glutamine labeling experiments allowed Schwender

and colleagues [207] to show that fully labeled alpha-ketoglutarate resulted in citrate with a significant fraction of highly labeled carbons (i.e., M5) – a result that would not be anticipated with traditional TCA activity including carbon respiration (Fig. 9B). The authors reasoned that isocitrate dehydrogenase (ICITDH) may act reversibly to more efficiently conserve carbon and produce citrate that can be exported and cleaved by ATP citrate lyase to produce acetyl-CoA for fatty acid elongation. Labeling in the elongated fatty acids at the terminal acetate as detected by mass spectrometry helped confirm the result [207]. These divergent descriptions in carbon handling emphasize that seeds are not merely passive collection receptacles [224] but function according to their operational metabolic networks and its physiological context [225].

4.2. Assessing resource partitioning through perturbation-response experiments

Given that plant metabolic operation is not static, the use of labeling with or without computational analyses can help describe cellular phenotypes and lead to hypotheses about regulation through perturbation experiments. At the biochemical level these studies have provided insight to wild-type metabolic operation as well as the response to genetic or environmental conditions. By changing the temperature [226], oxygen [62,227] nutrient status [57,61,63,64,228] or through genetic manipulation of central metabolism [59,229,230], or lipid metabolism [120] plant cells are forced to rebalance resource utilization resulting in altered phenotypes.

4.2.1. Alterations in carbon and nitrogen supply to fatty acids

The supply of carbon for fatty acid biosynthesis was examined with [^{13}C]labeling in genetically altered Arabidopsis lines [59] containing mutations in the *WRINKLED1* transcription factor [231–233] or plastidic pyruvate kinase genes [234]. In the *wri1-1* mutant allele seed oil content was reduced by 80% and resulted in an observable wrinkling appearance. Double mutants in plastidic pyruvate kinase $pkp\beta_1 pkp\alpha$ (Fig. 6 ❶) also had significant reduction in oil presumably because pyruvate generation was limiting for fatty acid biosynthesis. The decrease in oil in *wri1* was attributed to reductions in flux from sucrose that is consistent with transcriptionally regulated changes in glycolysis [235] and fatty acid metabolism [236]. As a result flux through cytosolic pyruvate kinase and malic enzyme was altered in part to compensate for the reduced supply of carbon to fatty acid metabolism by plastid-localized steps [59]. The role of these two enzymes have also been considered by environmental changes to temperature [226] and supplied nitrogen [47,57,61]. When inorganic nitrogen (i.e., instead of amino acids) was supplied to rapeseed the flux through malic enzyme in the mitochondria decreased 50% as a consequence of increased use of TCA intermediates for amino acid biosynthesis [61]. Rapeseed growth was depressed by 50% possibly due in part to the form of nitrogen supplied for metabolism. In soybeans, changes in the amount of organic nitrogen supplied did-not significantly impact growth but did lead to changes in final composition that included more protein [47,57]. Thus the response of metabolism to changes in nutrient or environment is variable and provides further rationale for flux analysis in many species [63].

4.2.2. Altered flux phenotypes in lipid mutants

Genetic mutants have also played a role in elucidating the most active pathways that contribute to fatty acid and lipid production. In the examples of *act1(ats1)* and *tgd1* lines [145,146] each mutant was identified from a forward genetic screen by an easily detected lipid phenotype (e.g., fatty acid or lipid class composition changes), therefore changes in acyl fluxes were expected and confirmed by labeling analysis. However reverse genetics-derived mutants that do not display significant growth or lipid composition phenotypes have also benefited from tracer studies. In particular the acyl editing mechanism has been supported by lyso-phosphatidylcholine acyltransferase (LPCAT) mutants. Since acyl editing can result in the removal of PUFA from PC for TAG synthesis in seed tissue, defects in the LPCAT enzymes required for acyl editing cycle would be expected to reduce the PUFA composition of TAG. Of the four putative Arabidopsis gene products with *in vitro* lyso-phosphatidylcholine activity [119,237] a double knockout comprised of the two enzymes with highest LPCAT activity gave very little change to Arabidopsis seed FA composition [120,238]. The TAG composition suggested the two LPCATs may not have been greatly involved in acyl editing, or that other enzymes with lysophospholipid acyltransferase activity can compensate for their loss in seed tissue. However, [^{14}C]acetate labeling of the double mutant revealed a different conclusion. Wild-type seeds incorporated nascent fatty acids into *sn*-2 PC prior to *sn*-1/*sn*-2 DAG, characteristic of the seed acyl editing mechanism [172]. However, the *lpcat1/lpcat2* double mutant had a DAG → PC precursor–product relationship more similar to the flux of *de novo* DAG synthesis by the Kennedy pathway, and the stereochemistry of fatty acid incorporation was essentially the same in both lipids. The results suggested that acyl editing was impaired within the *lpcat1/lpcat2* mutant [5,120,174,238]. It appears that a compensatory increase in the PC-derived DAG pathway of TAG synthesis provided a way for nascent fatty acids to flux through PC for desaturation prior to TAG synthesis. Thus, the true phenotype of a mutant was not revealed by static pool measurements of lipid composition. Analysis of metabolic fluxes with isotopic labeling identified the "hidden" mutant phenotype and also underlined the compensatory metabolism needed to maintain a seed composition similar to wild type.

4.2.3. Oil with modified FA composition

The majority of fatty acids found in plants are 16 or 18 carbons long with 0–3 methylene interrupted double bonds. These acyl groups are valuable sources of nutrition, but have a select range of uses as biofuels and industrial feed stocks. However, within the Plant Kingdom there are greater than 300 different types of "unusual fatty acids" that have desirable physical properties and functional groups (e.g., short chain, medium chain, hydroxy, epoxy, cyclopropane, and conjugated double bonds). Capitalizing on natural diversity is an attractive proposition for biotechnology and could result in value-added fuels, lubricants, polymers, coatings, adhesives, surfactants, resins, and other products that reduce dependency on petroleum [19,239,240]. However, many plants that produce these unusual fatty acids have agronomic features which make them less suitable as major crops. Over the past two decades attempts to genetically engineer unusual fatty acids into common oilseed crops or model species with few exceptions have produced modest proportions of desirable fatty acids [18,19,240–243]. To understand why, isotopic labeling has been used to identify limiting steps. California bay (*Umbellularia californica*) plants contain a medium-chain acyl-acyl carrier protein thioesterase (MCTE) that terminates FA synthesis at 12 carbons [244] (Fig. 6 ❷). When the MCTE was expressed in *B. napus*, lauric acid accumulation was positively correlated with MCTE activity up to approximately 30% 12:0 in TAG. A further 30-fold increase in MCTE enzyme activity increased the concentration of 12:0 to near 60% and indicated that this enzyme was no longer limiting lauric acid accumulation in TAG [118,245–247]. Though [^{14}C]acetate was predominantly converted to lipid in wild-type *B. napus* seeds, in the high lauric transgenic B. napus line only one-half of the [^{14}C]acetate went to lipids with the rest contributing to water soluble products including sucrose and malate [246]. Along with high enzyme activities for 12:0-CoA oxidase, isocitrate lyase, and malate synthesis the results suggested a high rate of beta-oxidation and gluconeogenesis that likely breaks

down newly synthesized 12:0 and limits its accumulation in TAG. Even though FA beta-oxidation was increased in these seeds total oil accumulation was unaffected because of a concomitant increase in fatty acid synthesis (also elucidated by metabolic labeling) indicating plants can respond to futile cycles of FA synthesis and degradation to maintain seed lipid levels.

Significant production of unusual oils does occur in a few crops including castor. Castor (*Ricinus communis*) produces a significant amount of the hydroxylated fatty acid ricinoleic acid (12-hydroxy-9-cis-octadecenoic acid) through a FA hydroxylase that is a variant of the FAD2 enzyme and utilizes 18:1 esterified to PC as a substrate [166,239,248]. The seed endosperm contains ~90% ricinoleic acid in castor TAG; though only 5% of the PC fatty acid profile is hydroxylated [249]. Thus at least 90% of seed 18:1 fluxed through the *sn*-2 position of PC. An *in vitro* analysis of TAG synthesis within castor endosperm microsomes suggested that supplied [^{14}C]18:1 was hydroxylated on PC, released from PC through an acyl editing mechanism, and the hydroxy-FA-CoA utilized to synthesize *de novo* DAG and TAG through a classical Kennedy pathway (Fig. 7B; [250]).

Attempts to engineer other plants to produce castor type oils have resulted in significant, but far less, hydroxylated fatty acid content. Heterologous expression of the castor fatty acid hydroxylase produced only ~17% modified fatty acids in Arabidopsis, suggesting other factors may limit their accumulation in TAG [251–255]. Based on short time point [^{14}C]glycerol pulse labeling approximately 50% of newly synthesized *de novo* DAG produced by the Kennedy pathway contained a hydroxylated fatty acid, but this *de novo* DAG was turned over and not utilized for synthesis of PC or TAG [174]. Since Arabidopsis utilizes the PC-derived DAG pathway of TAG synthesis, the inefficient utilization of unusual-fatty-acid-containing *de novo* DAG for PC synthesis represents a limitation for synthesis of TAG containing unusual fatty acids at the *sn*-1 position. Additionally, hydroxy-fatty acid production within transgenic *Arabidopsis* seeds reduced total seed oil levels by 30–50% [251,256] and revealed a concomitant decrease in the rate of fatty acid synthesis based on [^{14}C]acetate and [^{3}H]$_2$O labeling studies [257]. Comparative [^{14}C]acetate and [^{14}C]malonate labeling suggested acetyl-CoA carboxylase activity was reduced by one half in seeds producing hydroxy-fatty acids relative to the wild-type. Together these results indicated that the lower oil content of the hydroxy-fatty acid producing lines was a consequence of reduced fatty acid biosynthesis rather than fatty acid beta-oxidation [257]. Subsequent engineering attempts to increase the proportion of hydroxy-fatty acids in seed oils through co-expression of the castor fatty acid hydroxylase and selective TAG synthesis enzymes from castor [251,254] have resulted in significant increases in the proportion of modified fatty acids in TAG (i.e., to over 25%), relieved the acetyl-CoA carboxylase inhibition, and restored near wild-type rates of fatty acid synthesis and oil production [257]. One implication from this work is that the inefficient utilization of unusual fatty acids within ER glycerolipid synthesis can inhibit *de novo* fatty acid synthesis in the plastid, potentially indicating an uncharacterized endogenous mechanism that coordinates ER lipid assembly with plastid FA synthesis [257]. Similar reductions in TAG in other transgenic oilseeds [258,259] may suggest a common mechanism that coordinates fatty acid biosynthesis with TAG containing unusual fatty acids.

4.2.4. Assessing regulation with metabolic control analysis

Though perturbation experiments can provide insights into carbon partitioning, attempts to unify this data with other "omics" technologies have not led to straightforward interpretations. For example Junker and colleagues measured the protein activities of 22 enzymes of central metabolism at saturating substrate concentrations in Brassica [61]. Their data, obtained from cultured embryos, was reasonably consistent with tissues measured directly after harvest [260] but activities did not agree closely with metabolic fluxes. This cautionary tale supports the shared control of flux through multiple pathway enzymes [261] and demonstrated that metabolism can be poised to adjust to environmental perturbation without significant regulatory reprogramming at the proteome level. Therefore catalytic activities may not provide meaningful estimates of *in vivo* flux. Analogously, changes in metabolite levels from perturbed oxygen supply [62] or through *in silico* explorations [262] do not require obvious changes in fluxes either; indicating our understanding of the control of oil biosynthesis and regulation of its accumulation remain primitive [263].

As a complementary approach the relationship between activities and fluxes has been considered by metabolic control analysis [264–266]. MCA first gained prominence because early analyses described the shared control of pathway flux that was inconsistent with notions of enzymatic bottlenecking that still pervades the literature and common thought today. Assessment of control can entail either an enzyme level exploration that is a "bottom up" approach or alternatively divide metabolism into groups of pathway steps referred to as "blocks" with top down control analysis (TDCA) [267]. Through a series of investigations Harwood and colleagues have applied the latter approach to assess the control structure of oil metabolism in crops. Fatty acid biosynthesis and lipid assembly were considered as separate blocks in soybean, palm, olive and brassica species [268–274]. The flux through both blocks was measured using ^{14}C labeling with acetate and glycerol and then again after perturbing metabolism. By adding exogenous fatty acids (i.e., oleate) or enzyme inhibitors, altering temperature, or through changes in gene expression the studies monitored the responses of fatty acid biosynthesis and lipid assembly that were the metabolic blocks. In this case, the blocks were separated by the cytosolic acyl-CoA pool which was manipulated using oleate. This approach is referred to as single manipulation TDCA. Then inhibitors can be used to further probe control, known as double manipulation TDCA. In soybean and palm, fatty acid biosynthesis contributed approximately two-thirds of the flux control, slightly more than in olive [268,270,274]. In oilseed rape, the flux control coefficient for lipid assembly was predominant [273]. Double manipulation studies with combinations of inhibitors including 2-bromooctanoate or diazepam that inhibit lipid assembly [272–274] and diflufenican and triclosan that inhibit fatty acid synthesis [271,273] provided further confirmation of the findings. One unique observation came from the specific comparison of Kennedy pathways between olive and palm callus cultures by Ramli and coworkers. They observed that radioactive DAG in addition to TAG accumulated at increased levels in olive relative to palm [270]. They attributed the change in labeling to enhanced flux control specific to DGAT in olive and used the DGAT specific inhibitor 2-bromooctanoate to compare olive and palm. Results from the studies indicated the flux control coefficient for DGAT was 74% in olive but only 12% in palm and therefore DGAT played a more significant role in controlling olive oil production. Subsequent studies overexpressing DGAT indicated a shift in the distribution of flux control, consistent with TDCA theory, but also demonstrated a related increase in seed oil content [e.g., 14% increased oil in Brassica [272]]. These studies have since been further validated with results from field trials [16]. Together they describe promising enzymatic targets for enhanced oil biosynthesis on the basis of their contribution of flux control through lipid biosynthetic pathways. Control analysis is equally well-suited to other tissues such as leaves [275] and may provide insights to overcome current challenges that limit oil production in vegetative tissues. Altogether these investigations establish that even with shared flux control, the modification of steps exerting greatest control can result in altered overall flux, however with the additional

consequence that control of flux is likely shifted to other steps in the process.

4.3. Future techniques and technology to address longstanding challenges

4.3.1. Developments in MS technology to enable labeling and flux studies

Deciphering unknown plant function and the operation of lipid metabolic networks in the future will greatly benefit from technological advances. Historically separation techniques such as thin layer and gas chromatography that are highly reproducible have been invaluable in segregating lipid species; however transformative progress in lipid analysis was more recently aided with the development of electrospray ionization (ESI) technology [276]. The ionization of intact molecular species of lipids through "soft" techniques that minimize fragmentation has helped avoid artifacts from preparation and allowed direct verification of acyl composition in different lipids [277–279]. ESI tandem MS can reliably quantify picomole levels of known compounds and identify hundreds of lipids species [280] leading to estimates that approximately 90% of all lipid pools can be measured [281,282]. There is hope that fragmentation patterns may even be capable of discerning the regiospecific attachment at *sn*-1 and *sn*-2 positions [277,279,283], though acyl or double bond migration artifacts remain a concern [284]. As ESI-related technology is several orders of magnitude more sensitive than other technologies [283], it has found application in quantifying isotopic labeling [67,285] leading to assessments of metabolic flux in central metabolism [e.g., [66,70,286]] as well as recent mammalian studies examining flux of cellular lipids [278]. By tracking deuterium or phospholipid head group labeling, newly synthesized PC and mechanisms of PLA and PLB deacylation-reacylation mechanism have been described [277] and the status of progress in this area was recently reviewed by Ecker and Liebisch within this journal [287]. Presumably similar approaches could be applied to plant tissues if commensurate amounts of labeled substrate were metabolized by plants. Given the number of different labeling experiments described for plants in the literature that range from *in planta*, to leaf disks, embryos, cell or root tip cultures, tissue slices, or homogenates and organelle preparations, such approaches seem promising.

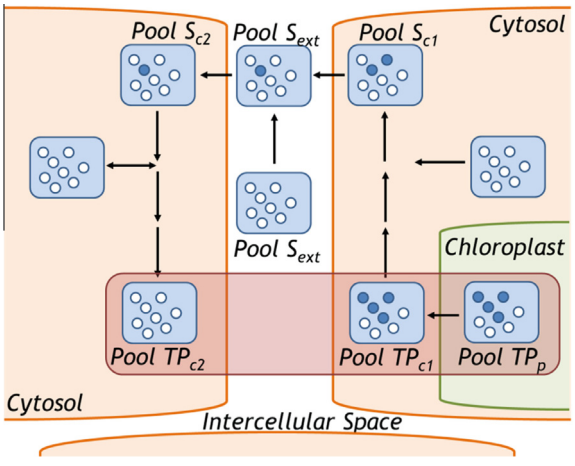

Fig. 11. Cellular heterogeneity contributes to spatial labeling differences in metabolites. The complexity of plant cells includes subcellular pools of metabolites separated at cellular and subcellular levels, however most experimental methods are incapable of examining pools specific to a compartment. Portions of three individual cells are shown in orange along with the intercellular space between them. Individual metabolite pools shown in blue contain both unlabeled and labeled atoms indicated by white and dark blue filled circles. In this example, triose phosphate that is labeled autotrophically in the chloroplast (TP_p) can be exported to the cytosol (TP_{c1}) and converted to sucrose by combining with other metabolites that are unlabeled. The resulting sucrose (S_{c1}) is less labeled and may be further diluted when exported to the exterior of the cell (S_{ext}) and into other cells (S_{c2}). Conversion of sucrose to triose (TP_{c2}) results in at least three triose pools that are mixed when metabolites are extracted from biomass. The heterogeneity in cells and the distinct metabolism at the subcellular level can limit the conclusions that can be drawn.

4.3.2. Addressing the challenges of multicellular eukaryotic metabolism

One of the practical limitations to interpretation of labeling experiments in eukaryotes is the ability to resolve the spatial distribution of compounds at cellular and subcellular levels. Plant cells come in dozens of different types that perform different

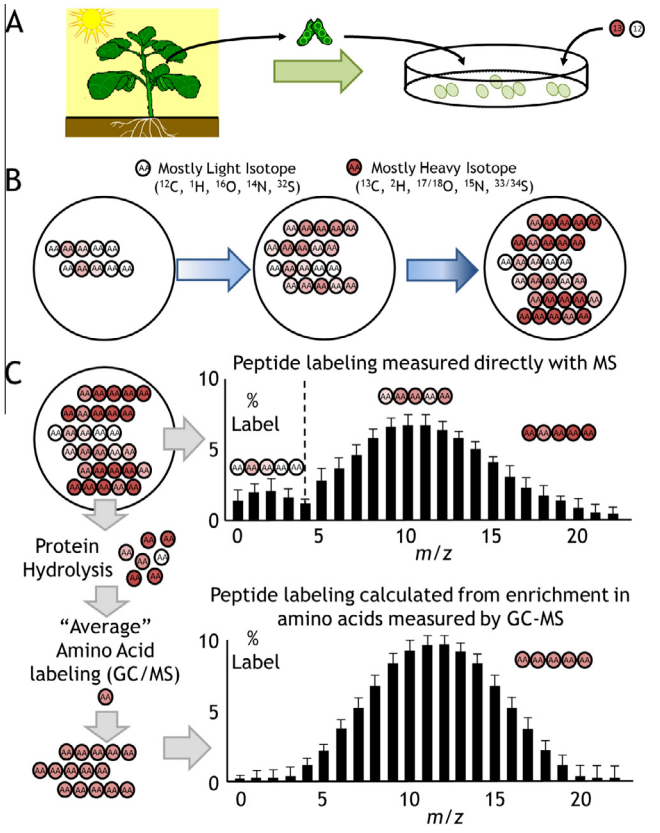

Fig. 12. Analysis of ^{13}C peptide labeling in developing soybeans for spatial and temporal metabolic information. (A) Developing soybeans were taken from pods and cultured with ^{13}C resulting in partially labeled storage proteins (inspired by [319]). (B) Initially seed metabolism on the vine resulted in isotopic concentrations consistent with natural abundance. As the labeling experiment progressed new proteins were made using amino acids that contained ^{13}C. (C) Examination of the peptides indicated the presence of a small fraction of storage protein that had not been turned over by culturing and had isotopic concentrations reflecting natural abundance. In addition a second fraction that was more isotopically enriched reflected the labeling process. Thus the peptide mass isotopomers formed a bimodal distribution that reflected the temporal labeling process. Some proteins were the result of unlabeled amino acids present in planta whereas others were the result of amino acids made by labeled amino acids generated during culturing. For comparison purposes the same protein harvested after culturing was hydrolyzed and amino acids mass isotopomers were measured. The amino acid descriptions were mathematically convolved to generate a labeling description of the same peptides for comparison. However when hydrolyzed amino acids were convolved, the connectivity of labeled or unlabeled amino acids specific to a time on the vine or in culture was lost. Therefore GC–MS analysis of amino acids and mathematical convolution could not account for the specific attachment of labeled amino acids next to each other which was a result of the temporal labeling process in seed development and culturing. Thus the direct measurement of peptides provided enhanced and more accurate labeling information.

functions (e.g., leaf mesophyll and epidermal cells) and that are themselves comprised of heterogeneous populations. The metabolite heterogeneity including lipid compositions at the cellular level examined through MRI [288,289] and imaging mass spec [290–293] is visually striking [see figures and further description in [294]] and reminds us that plants operate at a systems level (Fig. 1) with individual cells predisposed to differing metabolic objectives. Lessons learned from a specific tissue such as oil rich cells within seeds could be used to characterize mechanisms that limit oil production elsewhere, including leaves and other vegetative tissue that are less than 5% lipid [295] but that could be engineered for increased energy content [8–14,296–299].

Eukaryotic and prokaryotic pathways and the transport between them in lipid metabolism emphasize the complicated subcellular compartmentation that convolutes the interpretation of experiments. Relative to other species, plants have an increased number of duplicated metabolic pathways within different organelles [300] including both lipid and central metabolism. Glycolysis has been reported in at least three locations of a plant cell [301] including cytosolic, plastidic, and a possible role on the surface of mitochondria [302]. Likewise both cytosolic and plastidic forms of enzymes functioning with pentose phosphate metabolism exist [210] (Fig. 10). These intricate network descriptions are a well-recognized challenge [6,303–308] (Fig. 11) and the implications for modeling in multiple locations have been described [222] including labeling experiments to maximize information content from the labeling experiment [75].

Experimental techniques that provide information specific to cellular or subcellular locations represent one approach to more accurately assess metabolism. Organelle fractionation using non-aqueous buffers at reduced temperatures to rapidly quench metabolism produces minimal artefacts [309–311] and has allowed metabolite profiling at the subcellular level [312]. Extraction of single cells [313] or subcellular treatments [314] have also been developed to assess the metabolome and the status of profiling methods was recently reviewed [315]. Thus the methods to combine very specific spatial profiling approaches with isotopic labeling are becoming more refined. A different strategy is to make use of the known biosynthetic locations of metabolite biosynthesis and analyze the differences in enrichment from a labeling experiment. Experiments have capitalized on the distinct biosynthetic locations of fatty acids [52,59], carbohydrates [51,52], or protein-derived amino acids [316] to inform isotopic labeling on subcellular metabolism. The production of fatty acids includes a fatty acid synthase complex located in the chloroplast stroma that synthesizes acyl chains to lengths of 16 or 18 depending on the species. Further elongation or assembly to make lipids occurs after export to the ER. Thus the distinct locations of fatty acid biosynthesis and elongation result in the incorporation of acetate groups from distinct plastidic or extra-plastidial sources. The inspection of the terminal acetate on labeled fatty acids of different lengths provides a means of comparing the spatially distinct acetyl-CoA pools [52,317]. Similarly, methods for carbohydrates [51,52] and amino acids [316] produced in distinct biosynthetic locations of plants can provide analogous information about sugars and amino acids from different locations and have been recently extended with high resolution MS to indirectly assess amino acids through peptide labeling descriptions [318,319] assisting flux analysis [320].

Developing soybean embryos were cultured with ^{13}C to generate significant amounts of biomass labeled through metabolism (Fig. 12A). The isotopic labeling was measured in peptides obtained from proteolysis of storage proteins. A fraction of the storage protein was nearly unlabeled because it was produced "on the vine" prior to embryo culturing (Fig. 12B). This resulted in a subset of mass isotopomers with little ^{13}C incorporation (Fig. 12C inspired by [319]; $m/z < 5$ has low histogram values). The subsequent metabolic labeling and growth of the embryos in culture resulted in a second distribution of mass isotopomers in newly synthesized storage proteins that correspond to significant ^{13}C in the amino acids used in protein biosynthesis (i.e., $m/z > 5$ in Fig. 12C). Thus, the MS measurements produced a bimodal distribution within the peptides that reflected two distinct metabolic events for the embryos: growth in planta initially, without isotopic labeling, followed by growth in culture with ^{13}C substrates. Thus the final labeling in protein observed through the mass isotopomer description, served as a record of the growth over time with and without provision of isotope. The labeling distributions in the individual amino acids that were necessary to generate the mass spectral distribution can be determined through a computational fitting process where many peptides of different amino acid compositions and labeling descriptions are considered. Additionally the unlabeled fraction can be accurately established and accounted for based upon the composition and spectral distribution. The study indicated that protein made with isotopes during metabolism can be used to track differences in metabolism that occur temporally [319]. For comparison purposes, the same protein was hydrolyzed and labeling in individual amino acids measured using GC–MS. The amino acid mass isotopomer descriptions were mathematically convolved to regenerate a labeling description in peptides; however as presented in Fig. 12C, the temporally averaged amino acid labeling resulted in a mass isotopomer profile that did not exhibit a bimodal labeling pattern. The information about [^{13}C]-amino acids connected to each other in peptides that occurred when made in culture and the connectivity information of [^{12}C]-amino acids in peptides that were produced on the vine prior to culturing was lost when all peptides were hydrolyzed and measured by GC–MS. The GC–MS-based measurement of hydrolyzed peptides resulted in a labeling description for each amino acid that was the combination of all growth including the initial growth in planta without isotopes, as well as in culture labeling. Thus peptide measurements present a strategy for temporal metabolism (and analogously for spatial metabolism, see [316]) and compartmentalized flux analyses [320].

4.3.3. From CO_2 to lipid: temporal labeling-based MFA approaches

Steady state MFA descriptions are limited to tissues that exhibit unchanging metabolism for long durations and to networks containing branch points with enzymatic bond-breaking and reforming reactions. The steady state enrichments of different atoms within a metabolite or end product can indicate the relative use

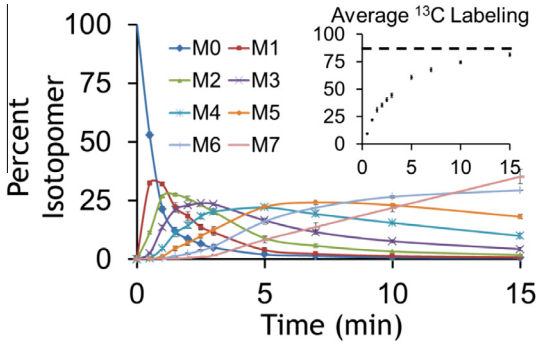

Fig. 13. S7P isotopic labeling during autotrophic metabolism in leaves. Isotopic labeling of sedoheptulose-7-phosphate (S7P), an intermediate of the Calvin cycle. The mass isotopomers indicate the labeling trajectories that reflect the incorporation of [^{13}C]O_2 sequentially into S7P. Initially the metabolite is unlabeled as indicated by 100% M0 composition at time zero, then as the time exposed to [^{13}C]O_2 increases the M1 pool increases but at some point is replaced by M2. The pattern continues until S7P becomes highly labeled. The inset graph indicates the average labeling per carbon. The average labeling is approaching a value of ~80% with time, indicating the presence of pools that were inactive within the labeling duration (~20% of the total S7P pool size).

of different pathways; however many tissues exhibit only brief periods of constant metabolism (e.g., leaf metabolism is diurnal) or in some cases all metabolites become fully labeled with time due to an exclusive source of carbon (e.g., autotrophic metabolism with $[^{13}C]O_2$ provision). Still other paths lack branch points and do not exhibit enzymatic isotope rearrangements within a molecule. The latter is often a feature of secondary metabolic pathways that are linear or fatty acid biosynthesis where the repeated addition of labeled acetyl groups results in nearly completely labeled intermediates at isotopic steady state. Thus additional information such as the enrichment over time is needed. For this reason continuous pulse or pulse-chase metabolic labeling of lipid assembly are preferred.

The temporal measurement of stable isotopic labeling is analogous to the dynamic radiolabeling approaches (but with stable isotopes) and is an appealing development because each time point contributes information that complements steady state labeling analysis. Therefore the additional measurements of labeling over time in a temporal analysis (Fig. 3) provide a richer set of data for modeling purposes [321] over steady state analysis alone. Additionally, because the earliest time points are the most sensitive to label provision, they provide a significant amount of information and the experimental duration can be shortened [322,323]. Recent studies have described the incorporation of ^{13}C into whole plants [324–326] and specific photosynthetic tissues, cells or unicellular organisms [66,67,70,327,328]. When $[^{13}C]O_2$ is provided to plants, the experiment is non-invasive and metabolic data reflects *in vivo* operation. Fluxes in Arabidopsis leaves were recently assessed by kinetic flux profiling that models unlabeled mass isotopomers levels in metabolites [66]. Estimates for active and inactive pools were used to overcome the spatial challenges of a plant system and describe photosynthetic metabolism. Autotrophic metabolism has also been described mathematically using non-stationary state MFA applied to unicellular systems [65,70] and the same approach has now been leveraged to investigate higher plants [69]. The application of non-stationary MFA utilizes all isotopomer data, which is fitted to accommodate the labeling trajectories between time points (Fig. 13). Thus multiple evaluations in time provide an enriched data set relative to steady state investigations (Fig. 3A) and can be used to distinguish pathways. The modeling of isotopomer pools allows estimation of the inactive pools through pool dilution fluxes and therefore presents an alternative way to obtain information on spatial complexity, possibly without some of the pitfalls associated with direct experimental measurements. For example, the flux through photorespiration, a pathway with up to 16 spatially resolved pools was examined through this strategy and led to quantification without assumptions about the ratio of carboxylation to oxygenation [69]. A number of other reports, mostly in non-plant systems are starting to surface on related use and applications [199,329–333].

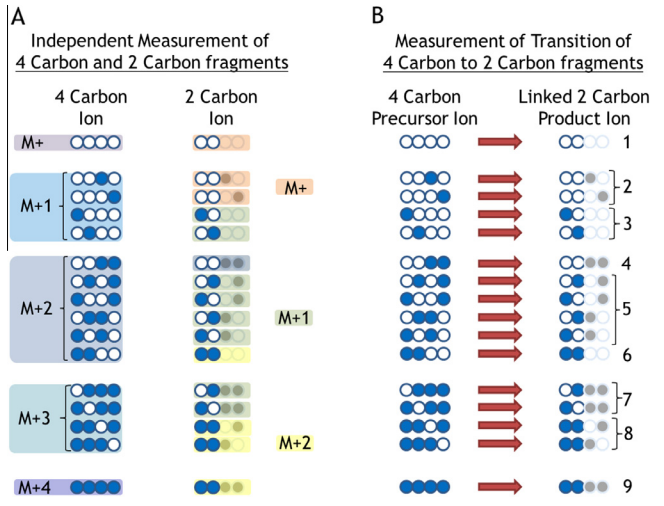

Fig. 14. Comparison of mass isotopomer measurements for isotopically labeled fragments using single and tandem mass spectrometers. Assessment of mass isotopomers with mass spectrometry can benefit from linking fragments (product ions) to their precursor molecules. In the example a four carbon compound is fragmented in the mass spectrometer resulting in a 2 carbon product that can be measured. (A) In a single quadrupole instrument the fragment may be detectable along with some of the remaining intact four carbon molecule resulting in 8 mass spectral measurements, 5 from the four carbon product and 3 from the two carbon fragment. In both cases the mass isotopomers must account for 100% of the fractional labeling, thus the number of independent measurements are 4 and 2, respectively. (B) When the measurements for the same four and two carbon compound can be directly linked through the use of a tandem mass spectrometer, the number of independent measurements increases to 8 (i.e., 9 measurements, 8 that are independent).

The power of computational nonstationary MFA and other methods lies in the abundance of measurements that contribute to establish an overdetermined set of network differential mass balance equations that describe metabolism. Though the networks are generally simplifications, future analyses can be expected to become more complicated and benefit from advances in labeling and mass spectrometry. In particular mass spectrometry collision cell technologies are available but largely untapped such as electron transfer dissociation (ETD), and higher energy collision dissociation (HCD). Together with CID methods, fragmentation can be optimized for lipids or other macromolecules depending on the biological question of interest. Regiospecific information, higher resolution techniques to distinguish similar compounds, positive and negative ionization for different lipid classes, increased MS^n afforded in linear and orbital trap MS with small quantities, and separation technologies such as ion mobility that capitalize on other physical properties all represent technological opportunities that remain largely unexplored. As these capacities are further developed, compounds will be identified less ambiguously and positional (not just mass) isotopomer descriptions will become available. Fig. 14 illustrates the additional measurement information that can be obtained from tandem MS that is a step in this direction. Whereas a single quadrupole has the capacity to measure intact as well as fragments of a metabolite, tandem MS can link fragmented products back to labeling in their precursors. Thus the connection between precursor and product ions results in additional information relative to independent monitoring of each. As indicated in the figure a four carbon compound (i.e., 4 circles) results in five mass isotopomers of which four are independent (i.e., one is redundant if they must sum to 100% to account for total labeling description), and the measurement of a second, two carbon fragment adds an additional three measurements of which two are independent. Together the MS of these two fragments provides six independent mass isotopomer measurements; however, by monitoring the transition from precursor to product ions, nine mass isotopomers groups can be measured of which eight are independent from one another. Thus the tandem MS of the same two fragments results in 25% (i.e., 6 *vs.* 8 measurements) more information. Further details on the number of independent measurements from tandem MS is presented elsewhere [334]. It is reasonable to expect that with higher power MS^n techniques or different fragment evaluations a complete isotopomer description may be achieved; reducing further the guesswork in model descriptions and biological interpretation.

5. Conclusions and perspective

The engineering of primary metabolism including the accumulation of lipids in plant tissues remains a challenging endeavor despite intensive research efforts. Fundamental to our

understanding of lipid pathways, isotopic labeling methods provide a dynamic description of metabolic operation including network fluxes. The most basic aspects of lipid metabolism in plants including the eukaryotic and prokaryotic pathways of membrane and storage lipid assembly were largely defined by isotopic labeling studies. Future investigations can be anticipated to be equally transformative for our understanding of lipid metabolism and have a significant impact on modern day problems in food and energy, in part because of the availability of high purity commercial isotopes and technologies such as MS that are sensitive with high resolution. Coupled with MS imaging, or more specific metabolic readouts, isotopic labels can account for heterogeneity, tissue-level and subcellular differences in metabolism. Other dynamic lipidomic studies involving isotopic labeling with or without high resolution MS, and nonstationary MFA will define fluxes quantitatively and establish emergent biological properties. Finally, the use of isotopic labeling methods with environmental or genetically altered plants will be essential in the further elucidation of unknown reactions and/or pathways within lipid metabolism, and is starting to allow metabolic assessment of regulation and control mechanisms that remain the current frontier of metabolic research in many species. Elucidation of lipid metabolism is no longer technologically limited, but possibly our imagination and ability to leverage available tools in new clever experiments present the greatest hurdle to Progress in Lipid Research.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We gratefully acknowledge conversations with Drs. John Ohlrogge and Jan Jaworski on topics related to the review and its revision. Work in the authors' labs was supported by a Department of Energy grant (DE-AR0000202; D.K.A.) and the Great Lakes Bioenergy Research Center Cooperative Agreement (DE-FC02-07ER64494; H.T.), the National Science Foundation (EF-1105249; D.K.A.), and the USDA-ARS. Any product or trademark mentioned here does not imply a warranty, guarantee, or endorsement by the authors or their affiliations over other suitable products.

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Glossary of abbreviations

AA: amino acid
ACP: acyl carrier protein
ADPG: adenosine diphosphoglucose
AKG: alpha-ketoglutarate
ATP: adenosine triphosphate
ACL: ATP-citrate lyase
CoA: coenzyme A
CPT: CDP-choline:diacylglycerol cholinephosphotransferase
DAG: diacylglycerol
DGAT: acyl-CoA:diacylglycerol acyltransferase
DGDG: digalactosyldiacylglycerol
DHAP: dihydroxyacetonephosphate
ER: endoplasmic reticulum
ESI: electrospray ionization
E4P: erythrose-4-phosphate

FA: fatty acid
FAT A/B: fatty acid thioesterase A and B
F6P: fructose-6-phosphate
G6P: glucose-6-phosphate
G3P: glycerol-3-phosphate
GC: gas chromatography
HP: hexose phosphate
ICITDH: isocitrate dehydrogenase
LACS: long chain acyl-CoA synthetase
LC: liquid chromatography
LPA: lyso-phosphatidic acid
LPAAT: acyl-CoA:lyso-phosphatidic acid acyltransferase
LPCAT: lysophosphatidylcholine acyltransferase
ME: malic enzyme
MFA: metabolic flux analysis
MGDG: monogalactosyldiacylglycerol
MRI: magnetic resonance imaging
MS: mass spectrometry
NADH: nicotinamide adenine dinucleotide
NADPH: nicotinamide adenine dinucleotide phosphate
NEFA: non-esterified fatty acid
NMR: nuclear magnetic resonance
OAA: oxaloacetate
OPPP: oxidative pentose phosphate pathway
PA: phosphatidic acid
PAP: phosphatidic acid phosphatase
PC: phosphatidylcholine
PDAT: phospholipid:diacylglycerol acyltransferase
PDCT: phosphatydlcholine:diacylglycerol cholinephosphotransferase
PG: phosphatidylglycerol
PUFA: polyunsaturated fatty acid
PYR: pyruvate
R5P: ribose-5-phosphate
RuBP: ribulose 1,5-bisphosphate
RuBisCO: ribulose bis-phosphate carboxylase/oxygenase
S7P: sedoheptulose-7-phosphate
TAG: triacylglycerol
TCA: tricarboxylic acid cycle
TGD: trigalactosyldiacylglycerol
TP: triose phosphate
UDPG: uridine diphosphoglucose
3-PGA: 3-phosphoglyceric acid