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Metabolic flux analysis: Recent advances in carbon metabolism in plants

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Abstract

Isotopic tracers are used to both trace metabolic pathways and quantify fluxes through these pathways. The use of different labeling methods recently led to profound changes in our views of plant metabolism. Examples are taken from primary metabolism, with sugar interconversions, carbon partitioning between glycolysis and the pentose phosphate pathway, or metabolite inputs into the tricarboxylic acid (TCA) cycle, as well as from secondary metabolism with the relative contribution of the plastidial and cytosolic pathways to the biosynthesis of terpenoids. While labeling methods are often distinguished according to the instruments used for label detection, emphasis is put here on labeling duration. Short time labeling is adequate to study limited areas of the metabolic network. Long-term labeling, when designed to obtain metabolic and isotopic steady-state, allows to calculate various fluxes in large areas of central metabolism. After longer labeling periods, large amounts of label accumulate in structural or storage compounds: their detailed study through the retrobiosynthetic method gives access to the biosynthetic pathways of otherwise undetectable precursors. This chapter presents the power and limits of the different methods, and illustrates how they can be associated with each other and with other methods of cell biology, to provide the information needed for a rational approach of metabolic engineering.

Introduction

Curiosity about metabolic pathways arises from the need to understand the biological mechanisms of plant life or from intents to improve the yield or quality of a plant product like wood, fruits or flowers, or the production of particular compounds. The first answers can be obtained from the analysis of metabolites, either by specific assays or by comprehensive methods of metabolite profiling. More specific questions that may require the use of tracers arise after the observa-

tions of changes in the levels of a metabolite of interest in relation to the genotype, development stages or the environment, or from unexpected results of carbon balance calculations. In recent years, labeling experiments have been used to unravel the function of regulatory or structural proteins in genetic engineering experiments.

Isotopic tracers are used to study metabolic pathways both qualitatively, to identify fluxes, and quantitatively, to quantify the fluxes in the pathways. The tracers may be either radioactive (^{14}C) or stable (^{13}C) isotopes. A wide range of enrichments is used for [^{13}C] labeled precursors, from about 100%, as in most of the works reviewed here, to around 1% with natural substrates when small variations around the natural abundance of ^{13}C are studied [1, 2]. Analyses are performed either by nuclear magnetic resonance (NMR) [3], or by mass spectrometry [4]. The combination of tracers, tracer concentrations and detection methods constitute a large number of methods. In addition, it must be noted that time is an essential parameter in labeling experiments because the duration of labeling determines how the labeling results can be handled and, more specifically, which type of model is adequate for the quantitative interpretation of enrichments in terms of flux values.

The experimental setup for a labeling experiment may be 'hypothesis free', but the interpretation of labeling data benefits from computational modeling of the metabolic pathways, which is necessarily based on hypotheses on the occurrence of certain metabolic pathways. The basic principles of modeling were established many years ago [5–8]. Establishing the set of metabolic pathways is the first step of setting up a model: the preliminary metabolic scheme is derived from published data on enzyme activities and compartmentation obtained from the literature. It should be noted that as long as the model fits the experimental data, the proposed pathways are validated, but the model itself does not lead to pathway discovery. The systematic search for pathways by methods such as elementary flux mode analysis [9] will provide more certainty in including all the pathways that may account for the observed label distribution. In addition, as underlined in [10], various sets of reactions may lead to similar label distribution from one given substrate. Therefore, fitting the model with experimental data is no proof that the metabolic scheme is valid. Redundancy is required in tracer experiments, i.e., a conclusion must be obtained through various means: by complementary labeling experiments with precursors labeled on different positions or with different labeling times, or by different methods like enzyme assays, enzyme inhibition, gene disruption or overexpression, etc.

Properties of labeling methods according to the length of labeling

Short-term labeling

In a typical short-term experiment (Fig. 1), the flow of tracer can be followed along the pathway: the amount of label in the pools, expressed as a percentage of the total incorporated label, decreases along the sequence. Similarly, the enrichment, or spe-

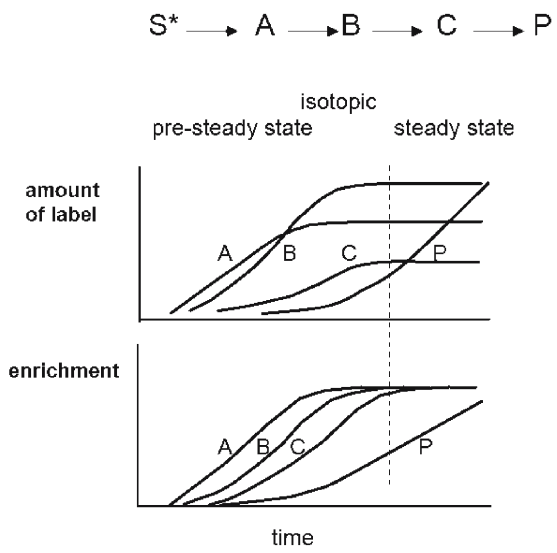

Figure 1. Labeling of pools in a pathway as a function of time. In labeling experiments, a pool may be a group of metabolites (proteins), a metabolite from a given cell compartment, or a particular moiety, or atom, of a metabolite. A purified metabolite may be a mixture of different pools of this compound from different cellular compartments, or from different cells of a tissue, each with different metabolic fates.
The results of tracer experiments are expressed as the amount of tracer in a given pool of metabolite (A) or as enrichment of the pools (B). ^{13}C enrichment is expressed as % and varies between 1.1%, the natural enrichment of carbon, and 100%, the enrichment of commercial tracers. For ^{14}C and other radioactive isotopes, enrichment is expressed as specific radioactivity which is an amount of radioactivity per mol (dpm (or Bq)/mol). In early pre-steady-state, both the amount of label per pool or the enrichment decrease along the pathway: both can be used as indicators of the position of the metabolites in the pathways (pool compartmentation, or branching pathways are possible complications). Unidirectional fluxes are calculated as the ratio (amount of label accumulated)/(enrichment of the precursor); underestimation may happen where labeling time is so long that label is lost from the product of interest. At isotopic and metabolic steady-state, the labeling and concentration of the intermediates remain constant: in a linear pathway, as illustrated here, the amount of label per pool is proportional to pool size, which brings no information on the pathway itself.

cific radioactivity, of the different pools decreases along the pathway. Short-term experiments are useful to solve three types of problems:

1. to establish the sequence of metabolites in a pathway; for example, the C3 and C4 photosynthesis types were named from the first metabolite found to be labeled after a few seconds of labeling with $^{14}CO_2$.
2. to quantify the absolute flux in the pathway: the number of moles of a metabolite, or group of metabolites, produced is calculated by dividing the amount of label accumulated by the enrichment of the precursor in the pathway (Fig. 1).

3. to deduce kinetic parameters of enzymes in the pathway from the kinetics of label distribution, by using models that include kinetic parameters of the enzymes. However, many kinetic parameters that are typically calculated from *in vitro* experiments with isolated enzymes may fail to meet the actual values under *in vivo* conditions of a compartmentalized plant cell or whole plant. Therefore, on the basis of the current technologies, modeling short-term labeling data in plant cells is intended with only limited areas of the metabolic network. As an example, this method was used for the identification of constraints in the accumulation of glycine betaine in plants [11, 12].

Steady-state labeling

As labeling time increases, isotopic steady-state is established in the pathway. In plants labeled with glucose, this was found to take a few hours. At this stage, the enrichments of different pools in the pathway are found to be constant, but the whole cells are not yet uniformly labeled. This was called 'relative steady-state' [13]. When a uniformly labeled substrate is provided, the steady-state enrichment in a linear pathway is uniform. This provides no information on fluxes in the pathway. However, where entering fluxes of unlabeled endogenous substrates lead to a dilution of label, the relative values of the labeled and unlabeled fluxes can be quantified from the decreased enrichment induced at the entry step (see Fig. 2). With non-uniformly labeled substrates, such as [1-^{13}C]glucose, the redistribution of the labeled atom(s) provides additional qualitative and quantitative information on substrate cycles in the pathway. This steady-state labeling method has been applied to the relatively large network formed by central metabolism (see below).

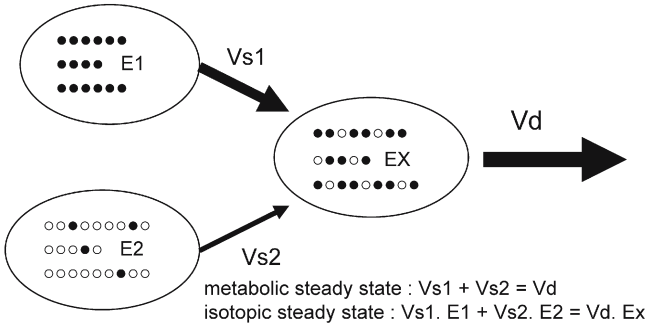

Figure 2. Modeling label distribution at metabolic and isotopic steady-state. Labeling to metabolic and isotopic steady-state enrichments provides information on joining pathways. For each pool (metabolite, or part of a metabolite) formed from two or more precursors, enrichment depends on both the enrichment of and the relative flux from each of the precursors. Two sets of equations can be written for each pool of metabolite or metabolite moiety: the metabolic steady-state equations state that C input = C output; the isotopic steady-state equations state that label input = label output. These equations link fluxes to enrichments. Relative values of Vs fluxes are calculated from measured enrichments of the precursors (E1 and E2) and product (EX).

Modeling of the isotopic and metabolic steady-state uses relatively simple linear equations, which link enrichment ratios with relative rates (Fig. 2). The amount of experimental data required to feed the model is lower after steady-state than after short-term labeling because after the long labeling times used, rapidly exchanging pools of a metabolite that are present in two or more compartments can be considered to have the same labeling. The review by Roscher et al. [14] discusses the effects of compartmentation and of transient conditions in long-term labeling experiments. In most experimental conditions, near steady-state rather than true steady-state conditions is obtained: applying steady-state models creates a problem when transient situations are studied, because the metabolic steady-state condition is not verified [3]. When the changes occur slowly, the turnover of the metabolites may be sufficient to ensure that changes in labeling in one step will be transmitted to the whole system. When changes in the level of a metabolite cannot be neglected, the metabolic steady-state equation must be modified to take this particular flux into account.

Long-term labeling for retrobiosynthetic analysis

After longer labeling time, final metabolites like protein amino acids become strongly labeled. Information is obtained from the relative abundances of different isotopologs in the sink metabolites (e.g., amino acids from proteins, starch, lipids) in these experiments. The isotopolog profiles of their respective precursors can be reconstructed by retrobiosynthetic analysis. The wealth of the method is that, on this basis, otherwise inaccessible metabolic intermediates can be analyzed that also constitute the central nodes of a metabolic network.

This chapter shows how labeling methods of metabolic flux analysis have recently led to a renewal of our views of the pathways of central metabolism, from sugars and hexose-P to the TCA cycle, and of isoprenoid biosynthesis. Clearly, many fields where sound approaches were developed are not treated here. The aims of this limited presentation are to illustrate the basic principles as well as the power and limits of the different methods, and to show how the qualitative and quantitative information provided by labeling experiments may contribute to the global approaches of systems biology.

Sucrose, glucose and hexose-P interconversions in heterotrophic cells

Heterotrophic cells import sugars, usually sucrose, from photosynthetic tissues. Sucrose enters the cell as sucrose or as glucose and fructose after hydrolysis by cell wall invertase. In the cell, sucrose can be hydrolyzed to glucose and fructose by invertase or cleaved to UDP-Glc and fructose by sucrose synthase. Intracellular glucose is also formed by substrate cycles similar to the turnover of sucrose, starch or cell wall polysaccharides. The operation of sucrose cycling was deduced after pulse/chase labeling experiments with labeled Glc where the decrease of the radioactivity measured in sucrose was more rapid than the decrease in the amount of sucrose [15]. It was deduced that sucrose was simultaneously synthesized (incorpo-

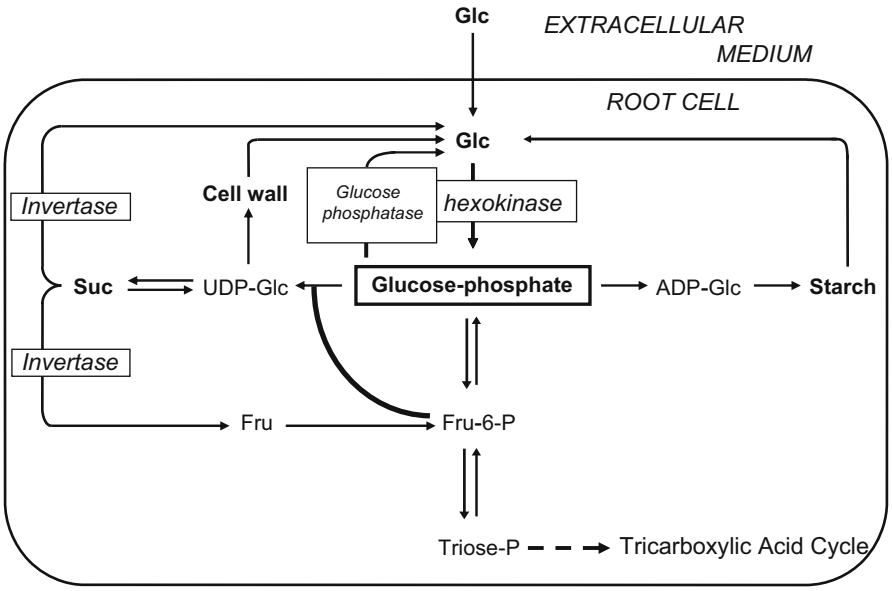

Figure 3. The sources of intracellular Glc in non photosynthetic plant cells. Glc is imported from the apoplast (extracellular medium). It is also a product of the turnover of intracellular oligo- and polysaccharides. This global flux was calculated after steady-state labeling experiments. The flux of Glc import and the fluxes of Glc formation from cell walls, starch and sucrose were measured by short-time labeling experiments. The occurrence of a Glc-phosphatase reaction results from the comparison of the global and individual fluxes towards intracellular Glc [22].

ration of label during the pulse) and degraded (decrease of labeling during the chase). In contrast, starch was found to be stable. The turnover of sucrose and starch was then quantified in other tissues: *Chenopodium* cells [16], ripening banana [17], potato tubers [18], and tomato fruit [19]. Using an approach of steady-state labeling in maize root tips [20], and in tomato cells [21], a high rate of cycling between hexose-P and glucose was observed and, based on enzyme activity data, it was suggested that this cycle was the result of sucrose turnover. More recently [22], a combination of short time and steady-state labeling approaches led to an evaluation of the respective role of the different pathways that may be involved in the Glc-P to Glc conversion (see Fig. 3). This work is presented in more detail here as an illustration of the properties of these two methods of labeling.

Short-term labeling estimations of free Glc formation in plant cells

Short-term labeling experiments were used, together with metabolite measurements, to evaluate the flux of external Glc uptake and the fluxes of Glc formation from the turnover of sucrose, starch and cell wall polysaccharides (Fig. 3). The approach was similar to that used in [16], and consists of:

1. Measuring the unidirectional flux of synthesis (Vs) using short-term labeling experiments.
2. Calculating the net flux of sugar (i.e., sucrose or starch) accumulation (Va), as the variation in sugar content, measured by a method of quantitative analysis of metabolites, over a time period: Va = Δ sugar content/Δt.
3. Deducing the unidirectional flux of degradation (Vd) as: Vd = Va-Vs.

The unidirectional flux of synthesis of a compound is calculated as the rate of incorporation of radioactivity (V_{RA}) divided by the specific radioactivity of its precursor. The precursors of sucrose and starch are UDP-Glc and ADP-Glc, respectively. Because measuring their specific radioactivity is difficult, glucose [16], or hexose-P [15, 23] were used as indicators because they give more certainty and they were expected to be in rapid exchange with UDPGlc and ADPGlc. In maize root tips, it was verified that Glc-6P and UDP-Glc were identically labeled, even after a very short time of labeling [22]. On the other hand, intracellular Glc was not identically labeled to UDPGlc, which may be explained by the slow labeling of the Glc vacuolar pool [20].

In growing maize root tips, short-term labeling experiments showed that the turnover of cell walls and starch were low compared to sucrose turnover and could therefore be neglected as sources of intracellular glucose. Steady-state labeling was used to examine whether sucrose turnover accounts for Glc-6P turnover.

Steady-state labeling measurements of Glc-P cycling

At isotopic steady-state the labeling of intracellular Glc results from the relative values of the flux of external Glc uptake (external Glc is labeled on C1 only) and the sum of the intracellular fluxes of Glc production from cellular oligo- and polysaccharides. The Glc molecules formed from these reactions derive from the hexose-P pool: they are less labeled on C1 than external Glc, and more labeled on C6. The enrichment of C1 and C6 of intracellular Glc and of the sucrose glucosyl was measured by ^{1}H and ^{13}C NMR. Resolution of the equations for either C1 or C6 leads to estimations of the flux ratio of total intracellular flux of Glc production (called Vrem) to the flux of Glc uptake. The absolute value of Vrem was then calculated using this ratio and the absolute value of Glc uptake measured in the short-term experiment. Vrem was found to be very much higher than the flux of Glc production from sucrose turnover determined by short time labeling. This result pointed to the operation of another substrate cycle in maize root tips, possibly the direct hydrolysis of Glc-P to Glc by a Glc-phosphatase [22].

This work illustrates how short- and steady-state labeling are complementary approaches to a better insight into central metabolism.

Partitioning of Glc-P through the pentose phosphate pathway and glycolysis

Glucose 6P can be catabolized through glycolysis or the oxidative pentose phosphate pathway (OPPP) which plays an important role in cell biosyntheses and de-

fence through the production of NADPH. Measuring the partition of hexose-P between OPPP and glycolysis is important to establish the function of the pathways. This is difficult in all organisms because the two pathways are interconnected through the exchange of fructose-6-P and triose-P. In addition, in plants, both pathways are present in two compartments, the cytosol and the plastids.

Classic assays with [1-^{14}C]- and [6-^{14}C]glucose

The approaches used to compare the fluxes in glycolysis and the OPPP have been elaborated by Katz and collaborators [6]. A model was set up to calculate the contribution of each pathway by using [^{14}C]glucose labeled on C1 or C6, through the specific yields of evolved $^{14}CO_2$ (the C1/C6 ratio) or the enrichments ratios of the triose-P and their derivatives (alanine, malate, etc.). Glucose labeled on C2 or C3 was also used to obtain complementary information through the redistribution of label in the Glc molecule. The specific yield of CO_2 is higher, and the enrichment of triose-P is usually found to be lower with [1-^{14}C]glucose than with [6-^{14}C]glucose. This is explained by the different fates of the Glc-C1 and -C6 through the OPPP. For Glc-6-P that enters the OPPP, C1 is lost as CO_2 at the second step of this pathway, whereas the C6 is incorporated into fructose-P or glyceraldehyde-3-P via the non-oxidative part of the pentose phosphate pathway. It may either be lost as CO_2 much further along the metabolic pathway, after two turns in the TCA cycle, or be retained in biosynthetic products, the most important, quantitatively, being the proteinogenic amino acids. Conversely, the fate of Glc-6-P C1 and C6 through glycolysis, is the same. Therefore, the differences observed in the labeling of CO_2 or triose-P derivatives are attributed to the OPPP. In fact, two distinct mechanisms affect the production of $^{14}CO_2$ from [1-^{14}C]glucose or [6-^{14}C]glucose: with [1-^{14}C]glucose, $^{14}CO_2$ evolves earlier as can be seen in short-term experiments, and in higher amounts when in an isotopic steady-state. Very often the two effects are confused. For example, the fact that the C1 of Glc-1-P is lost earlier in the OPPP does not explain that the specific yield of CO_2 is higher with [1-^{14}C]glucose than with [6-^{14}C]glucose, because the specific yields, (which give the C1/C6 ratio) are measured in near steady-state conditions. Indeed, if glucose was fully oxidized to CO_2, the C1/C6 ratio (at steady-state) would be 1, whatever the flux through the OPPP. The difference in specific CO_2 yields essentially depends on the incomplete oxidation of the triose-P derivatives [6, 8].

The problem is then to derive flux quantification from the observed differences in specific yields or enrichments. The method most often used because of its apparent simplicity was to incubate the tissues with either [1-^{14}C]glucose or [6-^{14}C]glucose and measure the specific yields of CO_2 and calculate the C1/C6 ratio: the C1/C6 ratio higher than 1 was used as an indicator of the operation of the OPPP [6]. The application to plants has been critically analyzed by ap Rees [8]. It was noted that, in plants, the pathway of pentosan synthesis which releases the Glc carbon 6 as CO_2 would be a cause of error. The results obtained on maize root tips show that this method is effectively unreliable with plant tissues: the same production of $^{14}CO_2$ was measured from [1-^{14}C]glucose and [6-^{14}C]glucose, which confirmed previous

data that had been interpreted as an indication that the OPPP was not active in this material [20]. However, the decreased enrichment of triose-P derivatives compared to that of hexose-P after steady-state labeling experiment [20] (see below) strongly suggested that the OPPP was highly active. In addition, this is consistent with the high biosynthetic activity of the growing root tips, which requires a source of NADPH. It was suggested that the C1/C6 ratio was disturbed by the pathway of pentosan synthesis. This example demonstrates that the method based on $^{14}CO_2$ yields is not reliable with plant tissues, as previously indicated [8]. It may be noted that, in the same labeling conditions, the observation of triose derivatives, instead of CO_2, would be less prone to errors.

As an improvement to this method, Garlick et al. [24] replaced [1-^{14}C]glucose with [1-^{14}C]gluconate. They showed that plant cells can take up [1-^{14}C]gluconate and metabolize it essentially by direct phosphorylation into [1-^{14}C]6-phosphogluconate which is then decarboxylated. Therefore, the release of $^{14}CO_2$ from [1-^{14}C]gluconate is a reliable indicator of the occurrence of a flux through the OPPP. The C1*/C6 ratio, with [1-^{14}C]gluconate and [6-^{14}C]glucose, respectively, was used. The method was found to be broadly applicable to plants, and showed that the OPPP was active in a number of plant materials, including maize root tips. However, it would be difficult to make this method quantitative. The C1*/C6 ratio depends on both the flux through the OPPP relative to that of glycolysis, and on the fraction of triose-P oxidized to CO_2. Therefore, a variation in the C1*/C6 ratio would not be reliably interpreted as a change in the flux through the OPPP relative to glycolysis, since it may also reflect a change in the fraction of triose-P retained in stored products. A quantification of the absolute flux through the OPPP could be made in short-term labeling experiments from the rate of $^{14}CO_2$ evolution if the specific radioactivity of the pool of 6-phosphogluconate could be measured; however, as discussed in [24], the cellular location of the reaction, cytosolic or plastidial, is not known.

Assays through NMR measurements of carbon enrichments

Steady-state labeling of plant tissues with stable isotopes ([1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, or [U-$^{13}C_6$]-Glc) associated with NMR or MS label measurements of metabolites provides a great deal of information about the reactions of intermediary metabolism. Estimations of the partitioning of hexose-P between glycolysis and the OPPP can be obtained after steady-state labeling with [1-^{13}C]glucose, through the analysis of sucrose, starch and alanine. The labeling of sucrose and starch reflects that of the cytosolic and plastidial hexose-phosphates, respectively, and the labeling of alanine reflects that of pyruvate, which derives from the triose-P. The information that was obtained by the comparison of specific CO_2 yields with [1-^{14}C]- or [6-^{14}C]Glc can be obtained with [1-^{13}C]glucose alone because, in the latter case, the carbon enrichments of hexose-P and triose-P can be compared. However, redundancy through the use of other tracers is still useful.

This approach was used to study the intermediary metabolism of maize root tips [20] and in tomato cells [21]. After incubation with [1-^{13}C]Glc up to isotopic steady-

state, the enrichments of carbon atoms in glucose, sucrose, starch and alanine were determined. Initially, the qualitative analysis of data were used to determine which metabolic pathways had to be included in the model, an important step before writing the equations that relate fluxes (the unknowns) to enrichments (experimental data). As an example, the OPPP was included in the model after the observation that alanine C3 was less labeled than the average of Glc C1 and C6. In a second step, fluxes were calculated to fit experimental enrichments. The carbon flux entering the OPPP was found to be higher than the flux of glycolysis measured at the PEP formation step [20, 21].

It is characteristic of steady-state labeling studies that fluxes can be quantified but the pathway involved cannot be identified with certainty. Since, in maize root tips, the ratio of enrichments of C6 to C1 was higher in starch than in sucrose, the plastidial OPPP was considered as a possibility to explain the loss of label from the Glc-P C1 position. In a complementary experiment with [2-^{14}C]Glc, the transfer of label to Glc C1, which characterizes the operation of the OPPP, was sought in the glucosyl units of sucrose and starch: it was found essentially in starch, thus confirming the plastidial location of the OPPP. In maize root tips, it was possible to fit the model with a null flux through the cytosolic OPPP [20]. In tomato cells the situation was found to be different: sucrose and starch were identically labeled, which was interpreted as a rapid exchange between the cytosolic and plastidial hexose-P; consequently, it was not possible to estimate the flux of the OPPP in each of these subcellular compartments [21]. It must be observed that in these two studies [20, 21] not all the possible reactions in the non-oxidative branch of the PPP were considered: the ribose-5P isomerase and ribulose-5P isomerase reaction were assumed to function close to equilibrium.

A more complete description of the pentose phosphate pathway was obtained by the complete analysis of the intramolecular labeling of sucrose and starch in *Brassica napus* embryos incubated to isotopic steady-state with [U-$^{13}C_6$]glucose, [1-^{13}C]glucose, [6-^{13}C]glucose, [U-$^{13}C_{12}$]sucrose, and [1,2-$^{13}C_2$]glucose [25]. Labeling with [2-^{13}C]Glc was used to evaluate the reversibility of the transketalose and transaldolase reactions. The labeling in amino acids, lipids, sucrose and starch was measured by GC-MS and NMR. The similar labeling of cytosolic and plastidial metabolites was interpreted as a rapid exchange of metabolites between these compartments. The measured fluxes were used to evaluate the split of hexose-P towards glycolysis and the OPPP: the latter was found to have a contribution to the supply of reductant for fatty acid biosynthesis lower than usually estimated. In a further study [26], the balance of carbohydrate to oil conversion was found to be much higher than would be expected from established pathways. Metabolic and isotopic steady-state experiments and modeling, using [1-^{13}C]alanine and [U-^{13}C]alanine as substrates, showed that a significant fraction of the CO_2 lost in the pyruvate dehydrogenase reaction, which forms the acetyl-CoA used for fatty acid biosynthesis, is recycled by Rubisco in a light dependent manner, but without Calvin cycle.

Using steady-state labeling, metabolic pathways and fluxes were also analyzed in developing maize kernels [27–29]. The *in vitro* culture of maize kernels represents a system to study the metabolism in intact kernels at different developmental

stages under defined conditions. Typically, the kernels were supplied with culture media containing a mixture of [U-$^{13}C_6$]glucose and unlabeled glucose. After growth on the labeled medium for several days, glucose was isolated from the starch hydrolysate and analyzed by NMR spectroscopy.

Due to the use of totally ^{13}C-labeled glucose as a tracer, highly complex signal patterns were detected in the ^{13}C-NMR spectra that reflect couplings between ^{13}C-atoms in a given molecule. Due to the inherently restricted coupling information in complex molecules (typically, ^{13}C-^{13}C couplings can only be observed via 1–3 bonds) and due to limited spectral resolution, isotopolog groups (so-called X-groups) [30] give sets of individual glucose isotopologs. Numerical deconvolution can then be used to determine the abundances of individual carbon isotopologs from the abundances of the X-groups.

As a major finding, the relative abundances of the [U-$^{13}C_6$]-isotopolog were low showing that the carbon skeleton of the vast majority of the applied labeled glucose had been broken and reassembled at least once. The observed [1,2,3-$^{13}C_3$]- and [4,5,6-$^{13}C_3$]-isotopologs reflected glycolytic cycling via triose phosphates. The [1,2-$^{13}C_2$]-isotopologs showed cycling via the transketolase reaction of the pentose phosphate pathway, and the [2,3-$^{13}C_2$]- and [4,5-$^{13}C_2$]-isotopologs have been explained by cycling involving the tricarboxylic acid cycle.

As outlined in more detail below, the isotopolog compositions can then be balanced by numerical or computational methods affording relative metabolic fluxes in the biosynthesis of the metabolites under study. In the kernel experiments, a computational approach [29, 31] was used that assessed the contributions and interconnections of glycolysis, glucogenesis, the pentose phosphate pathway, and the citrate pathway in considerable detail. Interestingly, minor modulations of the flux pattern were found during different phases of kernel development probably as an answer to the specific demands for metabolic precursors during kernel development [29].

Carbon inputs into the TCA cycle

The tricarboxylic acid cycle (TCA cycle) is the major pathway of respiration in all eukaryotic cells. It is well known for its energetic and biosynthetic roles. Acetyl-CoA, usually produced in the mitochondrion by the PDH reaction, is condensed with OAA to form citrate. In one ‘turn’ of the cycle, two carbons are lost as CO_2 and a new OAA molecule is formed: this is equivalent to the complete oxidation of the acetyl unit, but the entering acetyl carbons remain present in the OAA molecule. The intermediates of the TCA cycle are also used as building blocks for biosyntheses, particularly, in quantitative terms, the biosynthesis of amino acids of the glutamate and aspartate families. For each molecule taken out of the TCA cycle, so-called ‘anaplerotic’ reactions provide the OAA required as acetyl-unit acceptor. In plants, the PEP carboxylase reaction, which produces OAA in the cytosol, plays this role (Fig. 4). Equivalent anaplerotic substrates are four carbon compounds derived from the catabolism of amino acids of the aspartate family, or succinate produced by the glyoxylic acid cycle; the five C compound alpha-ketoglurate, which is de-

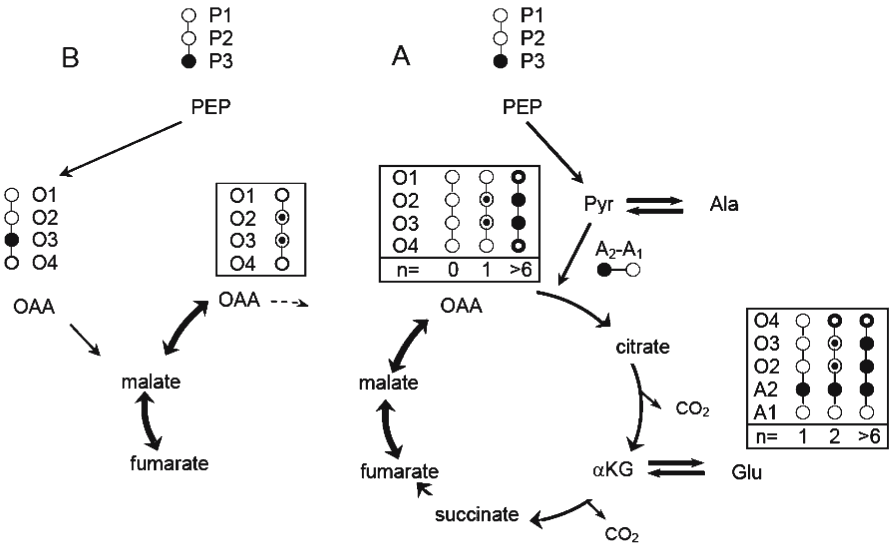

Figure 4. Glycolytic carbon input into the TCA cycle. Glc labeled on C1 or C6 produces PEP, pyruvate and alanine labeled on their C3 (●), with the other two carbons unlabeled (○). A: pyruvate dehydrogenase produces acetyl units labeled on their C2 (A2). A2 then forms the C4 of glutamate carbons. During the first turn of the TCA cycle (n=1), A2 and O3 are incorporated into the methylene carbons of succinate; because succinate is symmetrical, A2 goes to either of the central carbons of OAA. As the number of 'turns' increases, the enrichments of the OAA carbons O2 and O3 increases up that of A2 (shown here for n>6). B: The PEP carboxylase reaction forms OAA labeled on its C3 (O3), and the near equilibrium reactions between malate, fumarate and OAA randomize this label between O2 and O3 of OAA; O4 is also labeled, according to the enrichment of cytosolic CO_2. The OAA metabolized in the TCA cycle, as observed in the Glu molecule, is a mixture of the OAA formed in the TCA cycle (A) and that formed by the PEPC reaction (B).

rived from the catabolism of amino acids of the glutamate family also plays this role. The full oxidation of OAA is possible after its conversion to pyruvate through the malic enzyme reaction.

Major questions about the TCA cycle are the following:

- Among sugars, proteins and lipids, what is the substrate of respiration?
- In sugar-fed cells, where glycolysis provides both pyruvate and OAA to the TCA cycle:
  - how is the glycolytic flux partitioned between these two branches?
  - is OAA used as anaplerotic substrate only, or is it converted to pyruvate, via the malic enzyme (ME) reaction, to feed respiration?

Short-term labeling has been used for pathway identification, and steady-state labeling experiments have provided quantitative information about fluxes. The origin and fate of some carbon atoms in intermediates of the TCA cycle will be described first, because this knowledge helps to deduce qualitative information from labeling

patterns and to design experiments that can produce the information needed, even if the final, quantitative, interpretation of the data needs comprehensive modeling of the pathways.

Glutamate as the indicator molecule in studies of the TCA cycle

In steady-state labeling studies of the tricarboxylic acid cycle, the essential molecule to examine is glutamate, the indicator molecule for alpha-ketoglurate. Glutamate is a stable compound, it is usually abundant and its enrichments can be easily measured by ^{1}H and ^{13}C NMR spectroscopy (for example, see [20]). The glutamate carbons 4 and 5 are made of the acetyl units incorporated into citrate by the citrate synthase reaction, whereas the other three carbons are derived from oxaloacetate (OAA, Fig. 4A). During the first turn of the TCA cycle, the C4 and C5 glutamate carbons are incorporated into the methylene and carboxylic carbons, respectively, of succinate. Because succinate is symmetrical, the labeled methylene carbon goes to either of the central carbons of OAA; the carboxylic carbons go to either of the corresponding positions in OAA. A simple model of this sequence of reactions (input of one acetyl unit and loss of two CO_2 per turn) shows that, at steady-state, the acetyl-C2 forms the C2-C3-C4 moiety of glutamate. Therefore, after labeling with [1-^{13}C]glucose or [2-^{13}C]acetate, each of these central glutamate carbons would have the same enrichment as the acetyl-C2.

In plants, however, the NMR analysis of glutamate most often shows that the glutamate C2 and C3 are less enriched than C4. This accounts for the anaplerotic input of OAA, which is usually attributed to the PEPC reaction (see discussion below). In labeling experiments with [2-^{13}C]acetate, the OAA produced by the PEPC reaction is not labeled. In labeling experiments with [1-^{13}C]glucose, the PEPC reaction labels the OAA C3, but this label is randomized between C3 and C2 in the OAA-fumarate-succinate exchange that occurs in the TCA cycle (Fig. 4B). The average enrichment of C2 and C3 in the OAA molecules from the PEPC reaction is about half of that found in glutamate C4. Small differences observed between the C2 and C3 of glutamate have been attributed to incomplete randomization of the OAA produced by the PEC flux [20, 32]. The alternative mechanism is partial channeling of the TCA cycle flux, but there is no evidence for channeling at this step in plants [20].

In labeling experiments where labeled Glc or acetate are used as substrate, the dilution of the glutamate C2-C3 relative to C4 at isotopic steady-state can be used to calculate the anaplerotic flux, but the dilution itself does not indicate which of the different potential anaplerotic pathways is responsible for this flux. The choice of the PEPC reaction as that being responsible for the anaplerotic flux in sugar-fed tissues does not result from the observed labeling but from indications that PEPC activity is related to N assimilation [33] and protein synthesis, or to malate overproduction (see references below). On the other hand, the alternative anaplerotic pathways, proteolysis or the glyoxylic acid cycle, are found in special cases such as decaying or sugar-starved tissues [34].

Partitioning of the glycolytic flux at the PEP branch point

In plants, cytosolic glycolysis produces pyruvate or OAA, through the pyruvate kinase (PK) or the phosphoenolpyruvate carboxylase (PEPC) reactions, respectively. The partitioning of glycolysis at this branch point was studied by both short time or steady-state labeling experiments.

Changes in the PEPC/PK flux with development measured by short-term labeling

In the developing seeds of barley, at the stage of maximum fresh weight, the endosperm acidifies rapidly as it receives malic acid formed in the aleurone layer. This was found to be accompanied by a five-fold rise in the PEPC activity in the aleurone, which suggested that the increase in malic acid production was linked to an increased flux through the PEPC reaction. Alternative hypotheses included either a change of the fate of OAA produced by PEPC from amino acid synthesis to malic acid formation, or an increase in the glyoxylic acid cycle. The hypothesis of an increased PEPC/PK flux was tested by a short-term labeling experiment where uniformly labeled glucose was used as substrate, and the incorporation of radioactivity was monitored for up to 10 min in the major products of the two branches of glycolysis: alanine for the PK branch and malate + aspartate for the PEPC branch, as well as in the common products of the pathways, the TCA cycle intermediates citrate and glutamate [35]. Among the carboxylic acids and amino acids, the greater amounts of label were found in the compounds analyzed, with comparatively little label in citrate and glutamate. This showed that malate was not significantly labeled through the TCA cycle. Since, in the time period studied, most of the label was still present in the products of interest, the quantitative comparison of the PEPC and PK fluxes could be made by comparing the amounts of label incorporated in malate, aspartate and alanine. The PEPC/PK flux ratio was found to increase from 1.6 in aleurone of young seeds, to 7.5 in older, acidifying seeds. The kinetics of labeling also showed that the pattern of labeling changes in old compared to young aleurone. Alanine, aspartate and malate are labeled to similar extents in young seeds, whereas malate is the major product of glycolysis in old seeds. It should be noted that only ratios of tracer amounts were compared between materials. Amounts of incorporated label were not compared as they also depend on a number of factors that may differ according to development stages, such as the rate of tracer (Glc) input into the tissues, the size of the intracellular Glc pool, etc.

Changes of the PEPC/PK ratio according to growth conditions studied by steady-state labeling

The PEPC flux was also measured after steady-state labeling, based on its effect on the differential enrichments of the glutamate carbons. In maize root tips [20, 36] and in tomato cells [21] labeled at isotopic steady-state, the enrichments of Ala-C3 was the same as that of Glu-C4. This indicates that Pyr-C3 is the only source of Glu-C4,

in agreement with the generally accepted view that sugars are the major respiratory substrate in plant cells. The lower labeling of glutamate carbons C2 and C3 compared to C4 was related to the PEPC flux. As illustrated in details [36], the effect of the PEPC flux on the labeling of TCA cycle intermediates depends on where the carbon drain for biosyntheses occurs in the TCA cycle. In [20] the fluxes towards amino acids of the glutamate and aspartate families were assumed to be equal; this was confirmed in tomato cells in culture by analyzing the amino acid composition of the proteins [21]. From the steady-state models, the PEPC/PK flux ratio was calculated to be 0.5 in maize roots and 0.4 in tomato cells during the exponential growth phase. This means that of three PEP molecules formed by glycolysis, one goes through PEPC and two through the PK branch of glycolysis.

Changes induced in the metabolism of maize root tips submitted to sugar starvation were studied [34] by providing [1-^{13}C]glucose for 4 h, then incubating them in the absence of glucose (i.e., sugar starvation was induced in pre-labeled tissue). Modeling of these data was not intended because the system was clearly far from both isotopic and metabolic steady-state. However, the labeling data could be interpreted in qualitative terms. At the end of the 4 h labeling period, the carbons of alanine and glutamate were less enriched than at steady-state (16 h labeling) but, as expected in glucose-fed tissue, the alanine C3 and glutamate C4 enrichments were similar, and the glutamate C2-C3 were clearly less enriched than the C4, reflecting the PEPC activity. After 5 h of glucose starvation, the C2, C3 and C4 had become equal and remained so, although at a lower value at 16 h. This was interpreted as an indication that the PEPC flux had stopped as a consequence of glucose starvation.

Similarly, during the culture cycle of tomato cells, the C2-C3 *versus* C4 difference was found to decrease at the same time as protein accumulation rate decreased towards the end of the exponential growth phase [21]. At this stage, the PEPC/PK ratio had decreased to 0.25, indicating that only one PEP molecules out of five formed from hexose-P was used in the PEPC reaction. This is in keeping with the decreased rate of protein accumulation, compared to earlier stages of the culture. Together, these results support the view that the PEPC flux is linked with the biosynthetic activities of the cell. Moreover, as described below, the detailed study of the fate of OAA showed that the PEPC flux is essentially anaplerotic.

Quantification of the malic enzyme flux: The fate of oxaloacetate

How much of the PEPC flux is used for biosyntheses or is converted to pyruvate to feed respiration? OAA can be converted to pyruvate (Pyr) in the malic enzyme (ME) reaction. During [1-^{14}C]glucose labeling, the ME reaction produces Pyr and alanine molecules that are equally labeled on their C2 and C3, whereas glycolysis produces Pyr labeled on carbon 3 only. In most experiments with aerobic plant cells [21, 34, 36], the enrichment of alanine C2 was 2–3%, whereas that of Ala C3 was around 30%. The low labeling of Ala-C2 compared to Ala-C3 shows that little conversion of OAA to Pyr occurs *in vivo*. Using a comprehensive [20] or a simplified [36] model, the malic enzyme flux was found to provide only 3% or 8% of the Pyr

flux to the TCA cycle. This result was contrasted to previous studies of malate respiration by isolated mitochondria and of ME activity that suggested that the PEPC-ME couple might supply Pyr to the mitochondrial pyruvate dehydrogenase [36]. The labeling experiments *in vivo* established unambiguously that ME catalyses a minor flux in normal conditions; therefore, the PEPC flux is essentially anaplerotic.

The ME/PK ratio was found to increase six-fold under severe hypoxia, as calculated from the increase in the enrichment of Ala-C2 from 1.6 to 4.2 above natural abundance [30]. This increased ME activity is consistent with the decrease in malic acid content that occurs in most plant tissues transferred to anoxic or deeply hypoxic conditions and was explained by the rapid decrease in pH that occurs as oxygen is depleted [36].

The beta-oxidation of fatty acids as an alternative source of acetyl-CoA for respiration

A different configuration of the TCA cycle was observed in the particular case of germinating fatty seeds. In fatty seeds, the massive consumption of oil reserves starts about one day after radicle emergence. At this stage, the fatty acids are converted to sugars that are transported to the growing seedling through the concerted action of the beta-oxidation of fatty acids, the glyoxylic acid cycle and gluconeogenesis. What happens earlier, in the pre-emergence phase was less clear. The respiratory metabolism was thought to depend on sugars, with glycolysis and the pentose phosphate pathway playing a major role. However, fatty seeds such as lettuce or sunflower were found to have a very low fermentation rate under anoxia [37], which was not consistent with the known activation of glycolysis under anoxic conditions. This led to an examination of the pathways of respiration in germinating fatty seeds, using radioactive glucose, acetate and fatty acids. It was found that, similar to glucose and acetate, short chain or long chain fatty acids label the TCA cycle intermediates.

Three possible pathways were considered. The alpha oxidation of labeled fatty acids would produce CO_2 which would be incorporated by the PEPC reaction into OAA, and then be transferred to other TCA cycle intermediates. The other two pathways involved the beta-oxidation of fatty acids which produces acetyl units. The beta-oxidation of fatty acids associated with the glyoxylic acid cycle is active in growing seedlings might also present some activity in early germination. The third possibility was the beta-oxidation of fatty acids feeding the TCA cycle directly, as occurs in animal tissues.

The operation of the TCA cycle and of the glyoxylic acid cycle can be distinguished from each other by short time labeling with acetate or fatty acids because there is only one entry point for acetyl unit in the TCA cycle, the citrate synthase reaction, whereas there are two entry points in the glyoxylic acid cycle, the citrate synthase and the malate synthase reactions. In the classic experiments of Canvin and Beevers [38] which established the occurrence of the glyoxylic acid cycle in the endosperm of castor bean seedlings, more label had accumulated in malate than in

citrate, and more in aspartate than in glutamate, after 2 min of labeling with [^{14}C]acetate.

Evidence for a direct entry of acetyl-CoA into the TCA cycle by short-term labeling

When lettuce embryos were labeled with [^{14}C]palmitic acid or [^{14}C]hexanoic acid for 1–10 min, the amount of radioactivity measured in organic acids and amino acids was found to be the highest in citrate, followed by glutamate, succinate and malate [32]. This sequence clearly reflects the operation of the TCA cycle (Fig. 4), and is not consistent with either the glyoxylic acid cycle or alpha-oxidation. It shows that the acetyl units produced from fatty acids by beta-oxidation are incorporated into citrate through a citrate synthase reaction. This tells nothing of the quantitative importance of this pathway in the respiratory metabolism. Because of the multiplicity of acetyl-CoA pools in plant cells, the measurement of this flux through short time labeling experiments would be very difficult, as previously underlined after studies with animal systems [13].

Quantification of non-glycolytic carbon input by steady-state labeling

A quantitative estimation of the glycolytic and non-glycolytic origins of acetyl units into the TCA cycle was obtained from a steady-state labeling experiment with uniformly labeled glucose, i.e., only the glycolytic acetyl-units were labeled. Glutamate labeling was examined in two ways: its specific radioactivity was compared with that of aspartate, and the labeling of glutamate C1 was compared with glutamate C5 after selective decarboxylations of the molecule. It was found that the C4-C5 moiety of glutamate, which originates from the acetyl unit incorporated at the citrate synthase step, was only slightly labeled compared to the C1-C3 moiety derived from the OAA molecule. Modeling of the pathway, and assuming that the non-glycolytic pathway is essentially beta-oxidation, indicated that the beta-oxidation of fatty acids provides more than 90% of the acetyl-CoA entering the TCA cycle. The enrichments of the glutamate carbons, particularly the non-carboxylic carbons, are now easily measured by ^{13}C- and ^{1}H-NMR analysis. However, whereas [^{14}C]glucose can be used at tracer (micromolar) concentrations, [^{13}C]glucose must be provided at a high concentration which may lead to an artifactual increase in the activity of glycolysis.

Similar experiments showed that the beta-oxidation of fatty acids plays a similar role in sugar-starved tissues [34]. Experiments aimed at providing a confirmation of these labeling experiments showed that an isolated peroxisomal fraction from germinating sunflower seeds converts labeled palmitic acid to acetyl-CoA and, when OAA is added, to citrate [39]. It was proposed that the acetyl units produced by the peroxisomal beta-oxidation of fatty acids are exported to the mitochondria as citrate.

Given the quantitative importance of fatty acid beta-oxidation during germination, mutations that affect beta-oxidation could be expected to strongly affect the germination process. Clear phenotypes were observed on seedling growth but only in two cases on germination itself [40]. The mutation of a transporter that imports

acyl-CoAs into the peroxisome and a double mutation that suppresses the citrate synthase activity in peroxisomes produce seeds that do not germinate normally but can be made to germinate by removing the seed coat and supplying sucrose. The normal development of mutants affected on other genes in this pathway is explained by the multiplicity and overlapping functions of these genes. Of the different methods used to establish the function of beta-oxidation, the labeling experiments were the most important in establishing its quantitative importance in respiration in early germination. They could not, however, resolve its cellular localization, either peroxisomal or mitochondrial. The data obtained by the molecular genetic methods indicated that the peroxisome is the major, if not unique, site of beta-oxidation in germinating seeds [40].

Steady-state model solving

The resolution of isotopic and metabolic steady-state models, which relate fluxes and enrichments through linear equations, is relatively simple. Model solving was obtained using a matricial approach with the software Excel [20], or using the resolution of simultaneous algebraic equations using the software Mathematica [21].

As the amount of experimental data increases, specific softwares such as ^{13}C-Flux [25] or 4F [29, 31] are needed. The use of ^{13}C-Flux requires writing the forward and backward reactions of glycolysis and the OPPP, specifying the transition of carbon atoms from one metabolite to another for each reaction. ^{13}C-Flux makes it possible to simulate the steady-state distribution and to calculate the isotopomers for each intermediate of these pathways. Using an optimization algorithm, flux calculations are then fitted with the labeling measurements. In addition to the simulation and optimization tools, ^{13}C-Flux provides statistical output, including a sensitivity matrix that shows which fluxes have influence in which measurements, a covariance matrix that can be derived into confidence intervals for each flux value, and a parameter sensitivity matrix that shows the impact of the change of single measurements on the estimated fluxes [41, 42]. With the large quantity of experimental data from the different ^{13}C-substrates and the GC-MS and NMR measurements used in the study of *Brassica napus* embryos [25], an overdetermination of the flux parameters was obtained, which provides an improved reliability in flux calculations. Indeed, it was possible to accurately quantify the fluxes through glycolysis and the OPPP, including the reverse fluxes of TA and TK. The development of software packages that can automatically generate and handle the equations of complex metabolic networks and manage a large quantity of experimental data offers huge advances in flux quantification.

Retrobiosynthetic analysis: The origin of plant terpenoids

Steady-state labeling experiments have a long history in the discovery and analysis of metabolic pathways. Experiments using general ^{13}C-labeled precursors (e.g., glucose, acetate) in conjunction with the retrobiosynthetic concept provided a solid

basis to reconstruct the metabolic pathways in microorganisms [43]. As already mentioned above, the use of general tracers is also a powerful method to assign and to quantify metabolic routes in plant cell cultures, organs of plants or even whole plants grown on medium supplemented with the ^{13}C-labeled tracer. As a consequence of the general nature of the precursor used, the label is typically diverted to every metabolite through the metabolic network of the plant cell. Whereas the obtained isotopolog profiles are highly complex and typically show mixtures of several isotopologs, they nevertheless reflect the metabolic history of every metabolite under study, and provide a concise data matrix for the quantitative analysis of the pathways and fluxes between the metabolites under study. The concept will be illustrated in the following chapter in light of the discovery of a novel pathway for the biosynthesis of terpenes.

Well above 20,000 plant terpenoids have been reported [44]. A subgroup comprising sterols, carotenoids, chlorophylls, geraniol and dolichol serve essential functions in all plants. On the other hand, the vast majority of plant terpenes can be classified as secondary metabolites, serving specialized functions such as pollinator attraction or defense against predators. All plant terpenoids studied up to about 1990 had been assigned a mevalonate origin (for review, see [45]). Many of these assignments were incorrect in light of more recent evidence. It is important to understand the reasons for the earlier mis-assignments of many compounds. As described in more detail below, a major reason lies in the incomplete compartmental separation of a recently discovered mevalonate-independent pathway, a phenomenon which has been addressed as a crosstalk between the two pathways and compartments, respectively.

It is now common knowledge that plants invariably use the cytosolic mevalonate pathway as well as the plastidic mevalonate-independent pathway (non-mevalonate pathway, deoxyxylulose phosphate pathway or MEP pathway) for the biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These precursors serve as the basic building blocks for all terpenoids. The genes, proteins and intermediates of the novel non-mevalonate pathway (cf. Fig. 5) have been determined over the last 10 years by a combination of bioinformatic studies, *in vitro* approaches including cloning of the genes and expression of the enzymes, as well as isotope labeling techniques (for reviews, see [46, 47]. In line with the intracellular topology of the two pathways, the open reading frames of all non-mevalonate pathway genes from plants encode N-terminal sequences which fulfill the criteria for chloroplast targeting sequences. On the other hand, the mevalonate pathway genes of plants do not specify targeting sequences, in line with their cytoplasmic location [46, 47]. Since both biosynthetic machineries for the formation of IPP/DMAPP are present in plants, it is crucial to evaluate the biogenesis of plant terpenoids on a quantitative basis.

The origin of the biosynthetic precursors (i.e., IPP and DMAPP) of different plant terpenoids is best approached by *in vivo* studies with whole plants, plant tissue or cultured cells. A powerful strategy for elucidation of the biosynthetic origin of specific plant terpenoids uses stable isotope labeled glucose as precursor. Since glucose is a general intermediary metabolite, the isotope from the proffered carbohydrate can

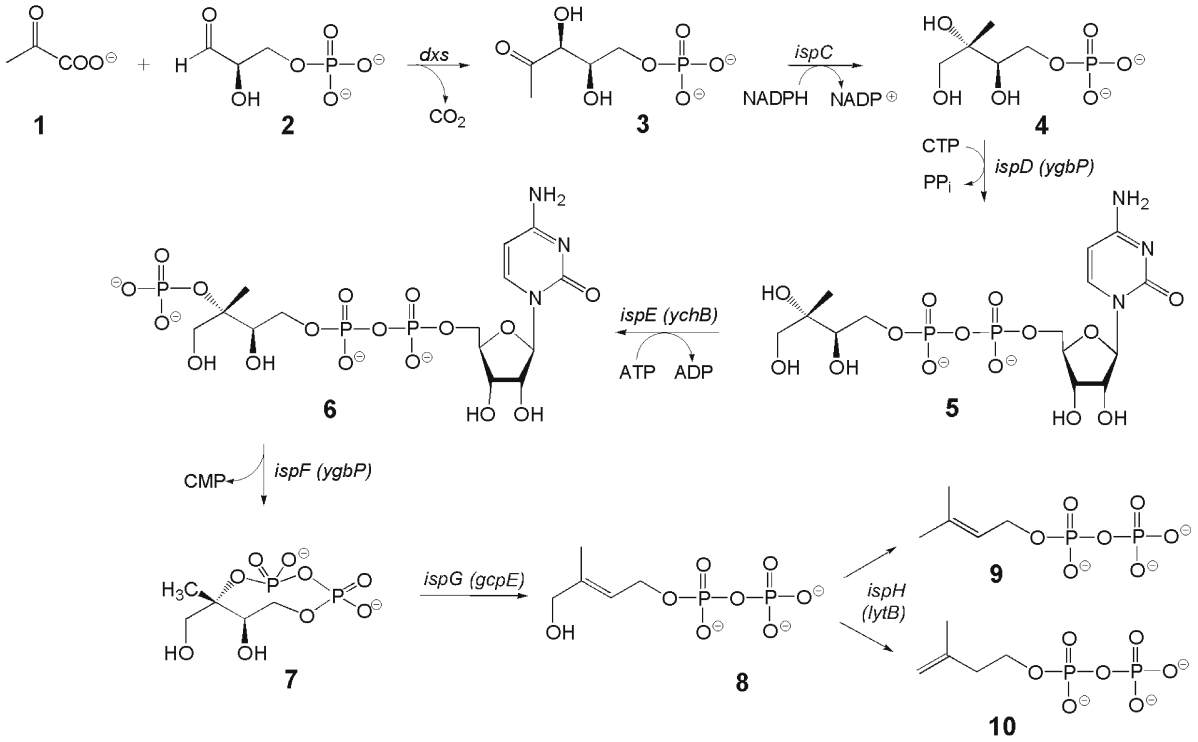

Figure 5. Biosynthetic pathways of DMAPP (9) and IPP (10), the universal precursors of terpenoids. The pathway starts with the formation of 1-deoxy-xylulose 5-phosphate (3) from pyruvate (1) (via hydroxyethyl-TPP) and glyceraldehyde 3-phosphate (2). Rearrangement and reduction yields 2-C-methy-lerythritol 4-phosphate (4), which is then converted into 4-diphosphocytidyl-2-C-ethylerythritol (5). Phosphorylation leads to the formation of the respec-tive 2-phosphate (6), which is then converted, into the cyclic diphosphate (7). Ring opening and reduction provides the hydroxymethylbutenyl diphosphate (8), which is finally reduced to IPP (10) and DMAPP (9).

be diverted to virtually all metabolic compartments of plant cells. Biosynthetic information derives from the positional aspects of the label distribution in the target molecule rather than from the net transfer of isotope. This procedure is in sharp contrast with many earlier studies where the transfer of isotope from mevalonate into a given target compound was taken as *bona fide* evidence for mevalonate origin.

Two different techniques for data interpretation will be briefly discussed below. Even on a superficial level of interpretation, it is obvious that carbon atoms 2, 4 and 5 of IPP or DMAPP, respectively, are all derived from acetate methyl groups in case of a mevalonate origin (indicated by b in Fig. 6A), and carbon atoms 1 and 3 of IPP and DMAPP are derived from the carboxylic group of acetate units (indicated by a in Fig. 6A). Irrespective of the nature of the biosynthetic precursor, carbon atoms derived from C-2, 4 and 5 of IPP/DMAPP should have the same isotope abundances in case of a mevalonate origin. Likewise, all atoms derived from C-1 and 3 of DMAPP/IPP should show identical isotope abundance. Moreover, the mevalonate pathway can at best transfer blocks of two labeled carbon atoms to the target molecule, whereas a block of three labeled carbon atoms can be transferred via the deoxyxylulose pathway, albeit under bond breakage and fragment religation brought about by 1-deoxyxylulose phosphate reductoisomerase (IspC protein) (cf. Fig. 5). Using ^{13}C NMR spectroscopy, the ^{13}C enrichment for all non-isochronous carbon atoms can be determined with high precision. Moreover, NMR can diagnose the joint transfer of ^{13}C atom groups, even in the case of an intermolecular rearrangement, by a detailed analysis of the ^{13}C coupling pattern via one- and two-dimensional experiments.

In a more rigorous approach, the entirety of all metabolic precursors in a given experimental system is treated as a network with hundreds to thousands of nodes where an isotope label can spread in every direction. If the isotope distribution in such a system is experimentally determined at a sufficient number of nodes (e.g., biosynthetic amino acids and nucleotides), then the label distribution can be assessed with high precision at a quantitative basis. As examples, the labeling patterns of the central metabolites acetyl-CoA, hydroxyethyl-TPP and glyceraldehyde phosphate can be reconstructed from the labeling patterns of leucine, valine and tyrosine on the basis of well-known pathways of amino acid biosynthesis in plants (Fig. 6). These data can then be used to construct labeling patterns of IPP/DMAPP via different hypothetical pathways, e.g., the mevalonate and non-mevalonate pathway, respectively, and the predicted patterns can be compared with the experimentally determined labeling patterns in the downstream products.

The biosynthetic origin of a considerable number of primary and secondary plant terpenoids has been reinvestigated recently using the technology described above. The experimental systems included members of the gymnosperm and angiosperm families of higher plants as well as liverworts as examples for lower plants. The data show that sterols are invariably synthesized in the cytoplasm via the mevalonate pathway [27]. Ubiquinone is biosynthesized in plant mitochondria using mevalonate-derived precursors from the cytoplasm [48].

Representative examples shown to be derived by the non-mevalonate pathway are given in Figure 7. A wide variety of monoterpenes and diterpenes is now known to be biosynthesized via the non-mevalonate pathway [49, 50]. They include com-

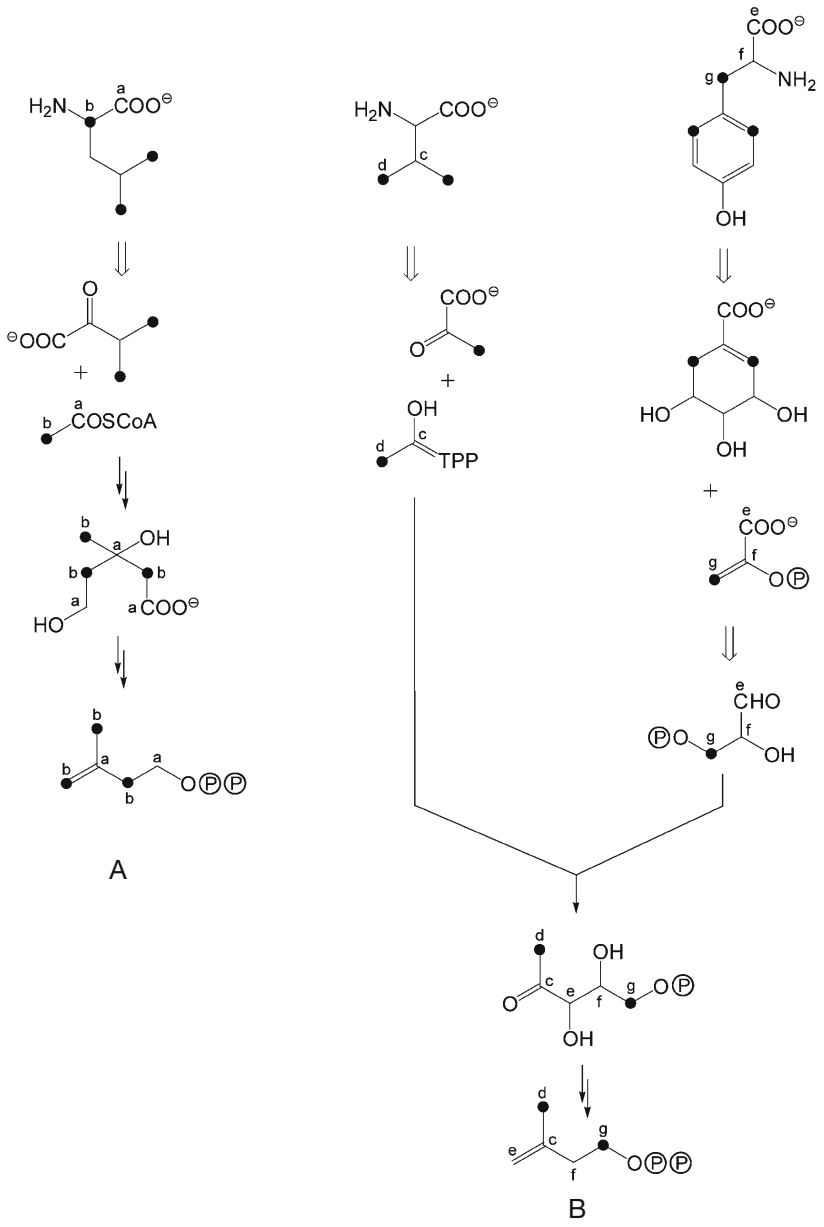

Figure 6. Retrobiosynthetic analysis of isotopolog patterns in leucine, valine and tyrosine. The isotopolog profiles of acetyl-CoA, glyceraldehyde phosphate and hydroxyethyl-TPP are reconstructed on the basis of known pathways for amino acid biosynthesis. Small characters indicate biosynthetically equivalent positions. The isotopolog compositions in the terpene building block IPP is then predicted **A**, via mevalonate or **B**, via 1-deoxyxylulose 5-phosphate, respectively. Filled dots indicate labeled positions from [1-^{13}C]glucose. It is immediately obvious that the labeling patterns differ via the two respective pathways.

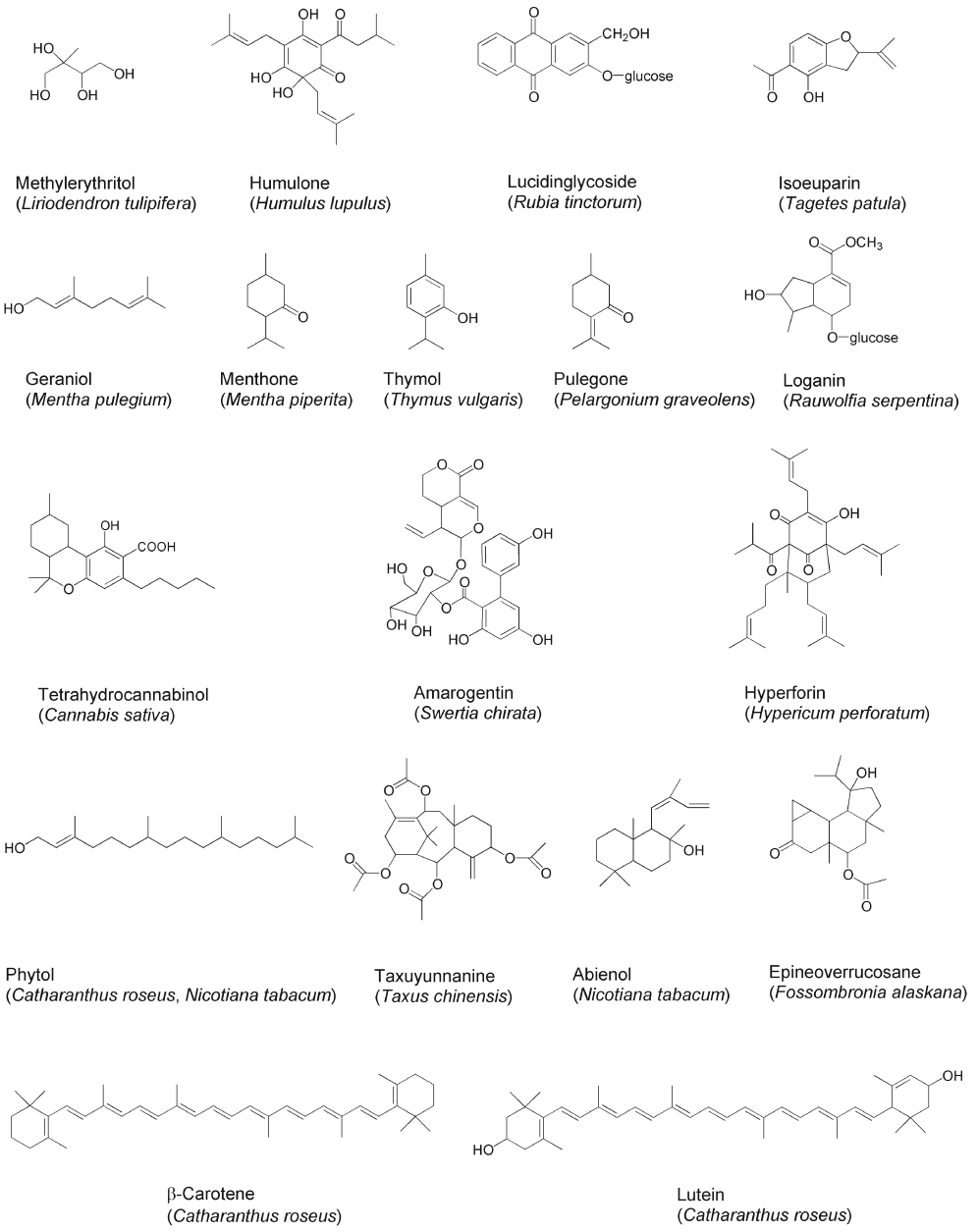

Figure 7. Examples for plant terpenoids that are predominantly or entirely derived via the non-mevalonate pathway. The biosynthetic routes of the displayed terpenoids were assigned by the retrobiosynthetic approach with the species indicated in parentheses.

pounds with central physiological significance for all plants as well as a much larger number of compounds that occur in specific taxonomic groups. Most notably, the phytol side chain which recruits chlorophyll, the most abundant organic pigments on earth, to the thylakoid membrane, is a deoxyxylulose derivative [51]. Carotenoids which play a central role in all green plants as light-protecting and light-assembling agents as well as specific roles as pigments in flowers are derived from the deoxyxylulose pathway [51].

Other examples of plant metabolites derived entirely or predominantly from the deoxyxylulose pathway include loganin, which is a basic precursor for many indole alkaloids [52], verrucosane-type compounds from liverworts [53], and taxoids from yew which play a dominant role as cytostatic agents [49]. They also comprise the isoprenoid moieties in various meroterpenoids including anthraquinone [54], benzofuran [55], tetrahydrocannabinol [56], or humulone from hops [57], the antidepressant hyperforin from St. John's wort [58], as well as the bitter-tasting amarogentin [59] (Fig. 7).

The ^{13}C incorporation studies performed with these compounds are not limited to delineating the origin of the building blocks but are also conducive to an unequivocal identification of the precursor modules. Since the biosynthesis of many terpenes involves one or more skeletal rearrangement, dissecting the isoprenoid building blocks affords important clues with regard to the downstream biosynthetic mechanism; for example, the regiochemistry in the formation of cyclic terpenes. This approach has its maximum impact for deoxyxylulose-derived compounds since universally ^{13}C-labeled 3-carbon blocks can be contributed from appropriate precursors such as [U-$^{13}C_6$]glucose and can be diagnosed in the complex metabolic products by ^{13}C homocorrelation NMR experiments. In favorable cases, very complex mechanisms of terpene formation can be extracted reliably from a small number of experiments (for a representative example, see [53]).

As mentioned above, many plant terpenoids had been incorrectly attributed in the past to the mevalonate pathway on the basis of isotope incorporation experiments with mevalonate or acetate. Whereas these experiments proceeded with minimal incorporation rates attributed to permeability barriers, the label distribution, when analyzed carefully, was in line with the mevalonate paradigm. In light of the more recent evidence described above, it is now clear that these earlier results were experimentally correct yet inappropriately interpreted. The recent studies have established that the compartmental separation between the two isoprenoid pathways is not an absolute one. Minor amounts of unidentified metabolite(s) common to both pathways can be exchanged in both directions via the chloroplast/chromoplast membranes. Thus, minor fractions of deoxyxylulose-derived isoprenoid moieties can be diverted to the cytoplasm where they can become part of sterol molecules. Likewise, a small fraction of isoprenoid moieties derived from the mevalonate pathway find their way into the chloroplast compartment where they become part of mono- and diterpenes which are predominantly obtained via the chloroplast-based deoxyxylulose pathway [60–63].

The retrobiosynthetic concept described above is a powerful tool in order to avoid pitfalls such as pathway crosstalk since it provides a quantitative dissection of

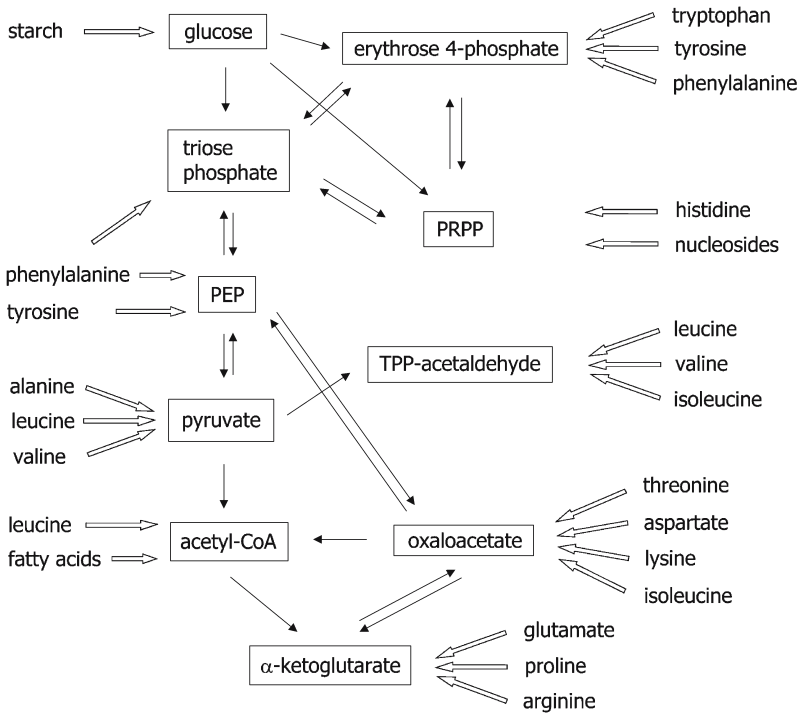

Figure 8. Scheme for the reconstruction of the labeling profiles in central metabolic intermediates ('hubs', shown in boxes) from the labeling patterns of amino acids, nucleosides, starch and fatty acids. Similar to the retrosynthesis approach for dissecting the precursors of a target compound in the organic synthesis, the retro-arrow indicates the retrobiosynthetic approach. The labeling patterns of metabolic hubs provide information about the flux through the metabolic network (schematically indicated by standard reaction arrows).

metabolite diversion as opposed to the qualitative description of net label transfer into one given metabolite that had been the source of errors in many of the earlier studies. It should be emphasized that the metabolites that can be easily used for a quantitative analysis of isotope patterns (e.g., amino acids, nucleosides, starch) provide the isotopolog profiles of approximately ten central intermediates ('hubs') in the metabolic network (Fig. 8). Since most of the basic building blocks of natural products are recruited from that cohort, the experimental approach is not limited to the question of terpene origin in plants but can be generally used to evaluate the biosynthetic history of natural products in a wide range of biological systems. However, for the complete delineation of metabolic flux in a given plant, isotopic equilibrium is one of the prerequisites. In light of the very long labeling times typically used in retrobiosynthetic studies, this assumption appears to be correct.

Conclusion

Labeling methods using isotopic tracers are in use since about 60 years and have contributed to the elucidation of most, if not all, metabolic pathways. Their power and complexity have been increased by the development of NMR methods for the analysis of enrichments and positional labeling, and of MS methods with high resolution and sensitivity for the detection of trace metabolites. In parallel, powerful softwares are being developed to handle the increasing amounts of data. In face of the considerable progress in the methods of analysis, classical limitations remain and require essential choices to be operated by the researcher. For instance, obtaining a rapid and uniform labeling of the tissues entirely depends on the structure of the plant material and is not always possible; supplying the labeled substrates by incubation in an aqueous medium requires special care to avoid disturbing the oxygenation of the tissues, which would dramatically affect their metabolism. Complementing the medium with specific nutrients or vitamins may also be necessary to reproduce physiological conditions [25]. A labeling method is defined by the substrates, the labeling time the analytical equipment and the labeling parameters analyzed, i.e., amounts of label, enrichments or positional of labeling. The present chapter emphasizes the choice of the labeling duration and its adequation with the model used for the qualitative and quantitative interpretation of the data as essential conditions for the success of labeling experiments.

While a given labeling method may appear as the most suitable for a particular material, pathway or question, more information is obtained when different methods are used in combination. The examples presented indicate that the interpretation of the labeling data depend essentially on the modeling of the pathways which is established from both labeling data and previous knowledge of either enzyme activities and their cellular localization, or genes with established or putative functions. In turn, as explained in [64] the labeling methods provide unique information on the dynamics of metabolism, which could not have been deduced from enzyme activities or gene expression data.

Short time labeling is the method of choice for the study of a particular metabolic pathway. It can also give access to the identification of rate limiting steps when coupled with models that include kinetic parameters [11]. Conversely, long-term labeling, in conditions of both metabolic and isotopic steady-state, leads to the calculation of a large number of fluxes in central metabolism. Recent studies have lead to the view of a central metabolism, from sucrose to PEP, with high rates of intermediate interconversion as compared to the fluxes towards the tricarboxylic acid cycle or the biosynthetic pathways. These results extend the concept of readily reversible reactions that was elaborated around sucrose metabolism [65] and may account for the flexibility and robustness of plant central metabolism [21, 66], at least in sugar fed sink tissues.

From the small number of detailed studies, some features, like the cycling of triose-P to hexose-P, appear to be general, while others are more variable. For example, the labeling of cytosolic and plastidial metabolites, may be similar [21, 25] or different [20] according to plant tissues, which may reflect different exchange

rates between the cytosol and plastids. The significance of these differences is not clear at the moment, since relationships between features of central metabolism and developmental conditions of the tissues have been proposed in only few particular cases. The role of Rubisco in developing green embryo was clearly related with the accumulation of triglycerides [26]. Minor differences in flux patterns during the development of maize kernels were hypothetically related with changes in the demand for certain amino acids [29]. A profound reorganization of the metabolism with increased catabolism of proteins and lipids [34, 67], and impairment of growth [68] was related with a limitation of sugar supply. A general understanding of specific patterns in the plant central metabolism could be quickly obtained through an intensive exploitation of the labeling data obtained in steady-state condition (fluxomics). Data would be provided on the enrichments and isotopolog profiles of each of the 'central' metabolites presented in Figure 8, and probably a few others. They would be made available through database, and different models could be compared in the interpretation of these data.

As illustrated here through the example of isoprenoids, the use of positional labeling in the retrobiosynthetic analysis of steady-state labeling data makes it possible to establish the contribution of distinct pathways to the formation of stored compounds where the amounts of intermediates are too low to be analyzed. The incredible diversity of plant secondary metabolites has been revealed by MS-based metabolomics [69]. This diversity is probably sensitive to growth conditions and developmental stages [70]. For metabolites of interest, the aim will be to improve their production or accumulation in plants. The task would be relatively easy if they were end-products of linear pathway supplied with non-limiting substrates. More probably, some of the precursors may be limiting; and the metabolites of interest exposed to further conversion. The way of increasing their production will therefore be not obvious. Establishing the metabolic architecture leading to these metabolites (as in [11]), through short time label transfer or retrobiosynthetic analyses may be of great help. Associating this information, obtained in selected genotypes, to gene expression and metabolomic data would make a useful contribution to systems biology.

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