Analysis of metabolic flux using dynamic labeling and metabolic modeling

A.R. FERNIE[1] AND J.A. MORGAN[2]

[1]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, 14476 Potsdam- Golm

[2]FRNY 1158, Purdue University, School of Chemical Engineering, Forney Hall of Chemical Engineering, 480 Stadium Mall Drive, West Lafayette, IN 47907-2100

Correspondance**:** Dr A.R. Fernie. Fax: +49 331 5678408; email: fernie@mpimp-golm.mpg.de and Prof. J.A. Morgan. Fax : +1 765 494-0805; email: jamorgan@purdue.edu

Summary statement: Diverse approaches have been developed in order to analyse metabolic fluxes. These can be broadly divided into stationary methods which require that the system under study is at isotopic as well as metabolic steady state and instationary methods for which only metabolic steady state is a pre-requisite. Here we review the use of instationary methods to interrogate plant metabolism and its regulation.

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ABSTRACT

Metabolic fluxes and the capacity to modulate them are a crucial component of the ability of the plant cell to react to environmental perturbations. Our ability to quantify them and to attain information concerning the regulatory mechanisms which control them is therefore essential to understand and influence metabolic networks. For all but the simplest of flux measurements labelling methods have proven to be the most informative. Both steady-state and dynamic labelling approaches having been adopted in the study of plant metabolism. Here the conceptual basis of these complementary approaches, as well as their historical application in microbial, mammalian and plant sciences are reviewed and an update on technical developments in label distribution analyses is provided. This is supported by illustrative cases studies involving the kinetic modelling of secondary metabolism. One issue that is particularly complex in the analysis of plant fluxes is the extensive compartmentation of the plant cell. This problem is discussed from both theoretical and experimental perspectives and the current approaches used to address it are assessed. Finally, current limitations and future perspectives of kinetic modelling of plant metabolism are discussed.

Key-words: steady-state modelling, kinetic flux profiling, metabolite, flux, compartmentation, isotope labelling, metabolic regulation

INTRODUCTION

The last fifteen to twenty years have seen the advent and adoption of a battery of technologies which have transformed biology by greatly accelerating data acquisition to the level where it is often no longer limiting and provides systems-wide information. The most frequently adopted approaches remain focused on levels of mRNA particularly since next generation RNAseq approaches have been widely embraced (Weber *et al*., 2007; Matas *et al*., 2011; Lohse *et al*., 2012), however, proteomics platforms are affording ever greater coverage (Baerenfaller *et al*., 2008) and providing ever finer spatial resolution of organellar and sub-organellar proteomes (Giege *et al*., 2003; Ito *et al*., 2011; Hu *et al*., 2012). In a similar vein, developments in metabolic profiling have led to increased coverage from 100s to 1000s of metabolites and have additionally begun to map the spatial abudance of these metabolites (Kruger *et al*., 2012). In parallel to these huge inventories of changes in steady-state levels, which have allowed hypothesis generation concerning many diverse aspects of biological interactions, sophisticated intergrative analyses and higher level cellular understanding of cellular dynamics have begun to be attempted (Sweetlove, Last & Fernie, 2003; Schwender, Ohrogge & Schacher-Hill, 2004; Sulpice *et al*., 2009). Here we concentrate on metabolic fluxes and especially those based on isotope labeling. We outline both steady-state and dynamic labeling approaches illustrating the latter with examples from plant specialized metabolism. We additionally discuss the problems arising from the extensive compartmentation of the plant cell before providing our own view as to the current limitations and future perspectives of kinetic modeling in plants.

Whilst the focus of this article is on modelling of fluxes the value of tracer experiments in establishing the structures underlying metabolic pathways should not be underestimated (Fernie, Geigenberger & Stitt, 2005). Similarly, studies into metabolic flux based on simply acquired experimental data such as gas exchange, end-product accumulation or spatial resolution of single metabolite concentrations provided invaluable insight into how the study of flux can inform our understanding of metabolic regulation (for recent examples see (Araujo *et al*. 2012, Geigenberger, Stitt & Fernie, 2004, Wiechert *et al.* 2007). For these reasons before describing studies in which isotope labelling is used as a means to determine metabolic fluxes

per se we will begin by describing both early and current studies reliant on less-sophisticated or, in the case of genetically-encoded metabolite sensors, more specific analytical techniques.

There are many examples of the importance of the use of tracer studies; classical examples include, but are in no means limited to, the elucidation of
And the Calvin-Benson cycle in Chlorella (Calvin, 1962) and the glyoxylate cycle in *Pseudomonas fluorescens* (Kornberg, 2000, Kornberg, 1958) and plants (Kornberg & Beevers, 1957). More recently, the approach has been highly instructive in establishing the primary plant pathway for ascorbate biosynthesis (Wheeler, Jones & Smirnoff, 1998), as well as, amongst others, clarifying pathways or lipid, volatile dimethylsulphonate and isoprenoid biosynthesis in plants and photosynthetic bacteria (Bao *et al*. 2002, Gage *et al*. 1997, Tieman *et al*. 2006), as well as lysine degradation in plants (Araujo *et al*., 2010, Dal Cin *et al*., 2011). At a more fundamental level, the tracer approach has also been employed to great extent to confirm that metabolites detected in plant extracts were truly of plant origin. An early example of this was the investigation by Graham and ap Rees of the occurrence of fructose 1-phosphate in higher plant species (Graham & ap Rees, 1965). This method has, however, been embraced on a much broader level for the evaluation of the 10s to 100s of metabolites detected by current metabolomics methodologies (Feldberg *et al*.,2009, Giavalisco *et al*., 2009, Giavalisco *et al*., 2011). The use of isotope tracers moreover often provide the prerequisite underlying structures of metabolic pathways – information that is essential for any but the most simplest type of modelling. An alternative route to this information is that described by the seminal paper of Schuster and co-workers (Schuster, Fell & Dandekar, 2000) whereby metabolic models are constructed *in silico* by a method reliant entirely on their stoichiometry. Several groups have recently adopted combined experimental and bioinformatics approaches to interrogate biochemical pathways (see for example; Schwender *et al*. 2004, Williams *et al*. 2010) and detailed discussion below) and it is widely acknowledged that such a route will ultimately be a very powerful one (Sweetlove & Ratcliffe, 2011).

Before moving onto the modelling of complex fluxes we feel it is important to note that isotope labelling has had a long and highly useful history in providing crucial

information concerning the regulation of metabolic processes in plants. Whilst the above examples illustrate the importance of the tracer approach in the qualitative determination of pathway structure it is important to acknowledge the vast literature emanating from radiolabel studies which addressed many important questions regarding the regulation of end-product synthesis. Early studies include, but are in no means limited to, seminal studies into the effect of oxygen tension on rates of plant respiration and its elevation during fruit ripening (Blackman, 1954), the relative production of organic acids by dark fixation reactions and the TCA cycle in corn roots (Lipps & Beevers, 1966), evaluation of conditional glucose dissimilation rates in germinating castor beans (Gibbs & Beevers, 1955) and other species including corn, pea, sunflower, parsley, soybean, carrot and Arum spadix (ap Rees, Fuller & Wright, 1976, ap Rees & Royston, 1971, Beevers & Gibbs, 1954, Bryant & ap Rees, 1971) relative rates of incorporation of ^{14}C derived from radiolabelled acetate into organic acids, amino acids, protein and CO_2 in rose cell suspension cultures (Fletcher & Beevers, 1970). In addition early studies were carried out employing positionally radiolabelled glucoses to ascertain of the oxidative pentose phosphate pathway flux in *Pisum sativum* and *Triticium aestivum* (Stitt and ap Rees, 1978).These studies all provided important insights into relative rates of the various routes of carbohydrate oxidation as well as mechanistic understanding of their regulation. More recent studies developed on these ground breaking studies as well as those from non-plant systems notably a series of papers by Katz and co-workers in the 1960s whereby radiolabel experiments were combined with chemical dissection in order to better characterise both forward, reverse and exchange fluxes through the major pathways of mammalian carbohydrate oxidation (Katz, Landau & Bartsch, 1966, Katz & Rognstad, 1966, Katz & Wood 1960, Katz & Wood 1963). Initially similar methods were used in plants (Stitt et al., 1983; Hajirezaei *et al*. 1994, Hill & ap Rees, 1994, Hill & ap Rees, 1995, Hill *et al*. 1995), however, such laborious methods have over time been replaced, or at least augmented, by targeted NMR studies (Fernie *et al.* 2001, Hajirezaei *et al*., 1994, Hatzfeld & Stitt, 1990, Keeling *et al.* 1988, Viola, Davies & Chudeck, 1991). Regardless of the methods by which these fluxes were assessed all of these approaches allowed a far greater comprehension of the molecular exchange occurring within and between the major plant intracellular carbon pools and also required the development of more sophisticated

mathematical descriptors – which we will return to in subsequent sections. Use of such methods have been important in the systematic analysis of the metabolic control of flux both of starch biosynthesis in leaves (Neuhaus & Stitt, 1990) and potato tubers (Geigenberger *et al*., 2004), whilst simple gas exchange measurements allowed the assessment of the control of flux through the TCA cycle (Araujo *et al*., 2012).

It should be stressed that the measurement of steady-state metabolite levels alone provides strong clues both to the metabolic activity of a tissue and to the metabolic response of an organism to environmental or genetic perturbation (Soga, 2007). Such studies have led to a considerable broadening of our understanding of for example the metabolic response to both biotic (Chen *et al.* 2009) and abiotic stress (Obata & Fernie, 2012, Urano *et al.* 2010) as well as helping to provide insight into how such responses are mechanistically invoked (Schauer & Fernie, 2006). It is, however, important to note that since the majority of metabolites determined by current profiling techniques are not end-products but intermediates of metabolic pathways, such approaches are generally incapable of resolving issues of metabolic dynamics (Fernie *et al*., 2005). Although it has previously been argued that changes in enzyme activity will provoke greater impact on steady-state metabolite levels than on fluxes (Cornish-Bowden & Cardenas, 2001, Raamsdonk *et al.* 2001), there are several examples in which fluxes through a pathway change without corresponding changes in the levels of the metabolic intermediates or even the products of a pathway. A particularly striking example of this is the fact that the rate of photosynthesis can change several-fold without detectable changes in the levels of metabolites of the Calvin-Benson cycle (Stitt, 1996). More recent examples include the increase in flux to starch without an increase in starch accumulation on the overexpression of an unregulated bacterial ADPglucose pyrophosphorylase (AGPase) in potato tubers (Sweetlove, Burrell & ap Rees, 1996) and metabolic inefficiencies in plant metabolism revealed by the metabolic flux analysis of ATP dissipation in substrate cycles (Chen & Shachar-Hill, 2012). These three examples all demonstrate that just measuring steady-state concentrations may be insufficient and as such suggest that the measurement of flux is a highly important component of modern functional genomics and systems biology (Fernie, 2012).

Although we present several examples of the modelling of simple fluxes which do not require labelling, methods that use isotopes are essential for the analysis of complex metabolic networks. Pathways often branch in a manner in which several products are formed from one substrate. Conversely, it is also often possible to generate a given product from several alternative substrates. The use of isotope labelling is further complicated by the issue of multiple pools of metabolites present at distinct sub-cellular locations within the cell. In this review we will provide an overview of the principles of steady-state and kinetic modelling and discuss illustrative examples of their application in plant systems. Following this we will review the state-of the art of experimental label distribution analysis before providing a detailed description of kinetic flux profiling with specific examples of its application in the green lineage. Kinetic modelling of plant secondary metabolic pathways will be discussed. This section will be followed by the important issue in dealing with subcellular compartmentation. Finally, we conclude with a description of future challenges and opportunities in the field of dynamic analysis and modelling of plant metabolism.

TECHNICAL DEVELOPMENTS IN LABEL DISTRIBUTION ANALYSIS

Before discussing examples of the use of flux profiling in plants it seems fitting to first provide a brief update of technical developments which have facilitated these types of isotope analyses. The labelling of metabolites and end-products is routinely analysed by nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry (MS) (Allen & Ratcliffe, 2009, O'Grady*et al*. 2012, Ratcliffe & Shachar-Hill, 2006; Kleijn *et al.* 2007, Krueger *et al*. 2012)) We will here briefly describe the current state-of-the art of both techniques individually and then also describe other developments which are needed in future attempts to more accurately estimate intracellular metabolite fluxes.

NMR which is sometime also coupled with liquid chromatography (LC) for metabolite separation (Exarchou *et al*. 2005) offers the advantage that it is facile to detect the labelling in different positions within a metabolite. This maximises the amount of information available to solve the metabolic flux map and allows information concerning transfer fluxes to be computed. The use of

multidimensional NMR has improved resolution to an extent that it is often possible to gain informative data from protein hydrolysates without the need for chromatographic separation – a fact that reduces the cost and machine time need for each analysis. It is important to note that the complexity of plant metabolic networks is so great that it is often difficult to decipher meaningful results from plant extracts without additional upstream separation (Kim, Choi & Verpoorte, 2011, O'Grady *et al.*, 2012). However, it seems reasonable to assume that these problems are not insurmountable. A larger problem for NMR is its relative insensitivity in comparison to MS based methods (O'Grady *et al.*, 2012). However, recent advances in NMR spectroscopy, such as cryo- and small sample volume probes and multinuclear and multidimensional techniques, have attempted to ameliorate this drawback (Fan & Lane, 2008). By contrast to most MS techniques, it is also possible to carry out monitoring of metabolism within living plant cells via NMR, which has proven useful in analysing unidirectional reaction rates in plant cells in response to changes in temperature or oxygen concentration (Roscher *et al.*, 1998).

MS can either be paired with gas or liquid chromatography or capillary electrophoresis for effective separation and detection of metabolites. Moreover it can be used in tandem to increase resolution. Gas chromatography (GC) is attractive given is stability and resolution capacity (Fernie *et al.* 2004) although it is important to note that it is not suitable for all metabolite types with for example phosphorylated intermediates are much more reliably measured by LC based methods (Arrivault *et al.* 2009). The requirement that the metabolite is volatile or can be rendered as such by derivatization also constrains the size of the metabolites which can be measured rendering it ineffective for the analysis of most secondary metabolites. For this reason LC methods are often preferable whilst it does not resolve peaks as sharply as GC it is capable of resolving many key metabolites. Furthermore, recent years have seen the development of suggestions for the standardized reporting of MS based metabolomics data (Fernie *et al*. 2011, Fiehn *et al.*, 2008, Tohge *et al.*, 2011), these will likely be equally useful for those concerned with flux profiling. Also of interest in this context are the recent papers highlighting the expansion of metabolomics methods by the incorporation of heavy isotope into plants (Feldberg *et al.*, 2009, Giavalisco *et al.*, 2009, Giavalisco *et al.*,

2011), whilst not designed for the determination of flux themselves these experiments clearly highlight the breadth of applicability that this approach has the potential to achieve. A further capacity of the MS approach is the use of tandem MS or of pre-separation by tandem chromatography frequent examples of both of these have appeared in the literature in recent years (see for example Tohge *et al.*, 2011, Vogel *et al.*, 2010). The other separation technique commonly coupled with MS is capillary electrophoresis this, like LC is a gentler method of separation than GC and is thus able both to maintain the more labile metabolites in their native form and exhibits a high degree of resolution (Monton & Soga, 2007). That said this approach is currently less commonly used following isotope labelling than GC or LC-MS based methods.

Whilst the sensitivity of the MS is the main advantage of this approach the fact that it does not readily yield positional labelling information is its major drawback. This effectively means that it can only group isotopomers by mass and not by positional information. Whilst even in this manner they can provide enough information for the fitting of flux values there are possibilities of using the ion fragmentation pattern to recover a portion of the positional data. This works because metabolites are known and indeed well documented to display consistent fragmentation patterns (Christensen and Nielsen, 1999; Dauner and Sauer, 2000; Schwender, Ohlrogge & Shachar-Hill Y., 2003; 2006; Huege et al., 2007). The mass distribution of key fragments in comparison to that of the molecular ion can thus be used to decipher labelling of individual metabolite fragments (Christensen and Nielsen, 1999; Dauner and Sauer, 2000; Schwender, Ohlrogge & Shachar-Hill Y., 2003; 2006; Huege et al., 2007; Allen, Shachar-Hill & Ohlrogge, 2007). The absence of derivatization and less harsh ionization methods render the availability of comparable information in LC-MS experiments considerably less problematic (Szecowka et al.,2012). However, analysis of tandem MS can on comparison of the labelling between daughter and parent ions facilitate the collection of positional information (Choi & Antoniewicz, 2011). Thus whilst in principle the combination of NMR and MS methods is complimentary and they have often been used synergistically in metabolic flux analysis of plant systems (see for example (Alonso, Goffman, Ohlrogge & Shachar-Hill, 2007, Hay & Schwender, 2011, Lonien & Schwender, 2009, Williams *et al.*, 2010), method developments may render the

practice of one using MS methods more common in the future. Such an approach is already taken particularly in cases where intermediates are analysed but also in studies of prokaryotes or seed plants in which simple networks or models are being used (Colon *et al.*, 2010, Szecowka *et al.*, 2012, Young *et al.*, 2011).

Another area in which considerable technical advances are currently underway is at the level of spatial resolution of metabolism (Aharoni & Brandizzi, 2012, Krueger *et al.*, 2012). Whilst ultimately it may be possible to adapt mass spectral and NMR imaging approaches for use in flux profiling this currently seems some way off.

STEADY-STATE VERSUS DYNAMIC LABELLING

Whilst a growing number of attempts to use computational approaches such as flux balancing in plants and photosynthetic bacteria (Boyle & Morgan, 2011, Dal'Molin *et al.*, 2011, Grafahrend-Belau *et al.*, 2009, Hay & Schwender, 2011, Knopp *et al.*, 2010, Mintz-Oron *et al.*, 2012, Pilalis *et al.*, 2011, Poolman *et al.*, 2009) have been made, utilizing similar methods to those which have proven highly successful for microbes (Bonarius, Schmid & Tramper, 1997, Borodina & Nielsen, 2005, Converti & Perego, 2002, Reed & Palsson, 2003), the majority of flux studies in plants depend on labelling. This fact notwithstanding such methods have clear utility and are convincingly argued as being complementary to one another (Schwender, 2011, Sweetlove & Ratcliffe, 2011), a postulate that is supported by the fact that experimental labelling data from models of the cellular metabolism of Arabidopsis closely fit fluxes previously determined by the flux balance approach (Williams *et al.*, 2010). It is not our intention in this article to review such methods in detail here since they have recently been comprehensively covered (Converti & Perego, 2002, Sweetlove & Ratcliffe, 2011) but rather to focus on isotope based methodologies for flux determination. Such approaches all follow one of two styles namely steady-state or dynamic labelling (see Table 1 for a comparison between the two). The latter relies on the kinetically-resolved measurement of the flow of label through a network whilst the former aims rather to characterize the steady-state reached upon continuous supply of a labelled precursor.

Steady-state labelling

The principles of steady-state labelling have been established for many years (Borowitz, Stein & Blum, 1977, Katz & Rognstad, 1966) and this approach has been used extensively in microbial (Fischer & Sauer, 2003, Moxley *et al.*, 2009, Schmidt, Nielsen & Villadsen, 1999, Vallino & Stephanopoulos, 1993, van Rijsewijk *et al.*, 2011), mammalian (Brodie et al., 1966, Brunengraber, Kelleher & DesRosiers, 1997, Malloy, Sherry & Jeffrey, 1988, Matthews *et al.*, 1980) and lately plant systems (Alonso *et al.*, 2007, Dieuaide-Noubhani *et al.*, 1995, Iyer *et al.*, 2008, Rontein *et al.*, 2002, Schwender *et al.*, 2004, Spielbauer *et al.*, 2006, Sriram *et al.*, 2004, Williams *et al.*, 2008). The more widespread adoption of this method in plants has been facilitated by the considerable advances in analytical methodology (discussed in detail below) as well as in approaches in computation which render flux estimates more accessible (Wiechert *et al.*,2001, Zamboni, Fischer & Sauer, 2005). It is important to note that steady-state labelling is crucially only applicable if the system under study can be demonstrated to reach *both* isotopic and metabolic steady states during the experiment (Roscher *et al.*, 2000, Wiechert & Nöh, 2005). One cannot merely assume that this is the case however; the attainment of steady-states must be empirically determined. In plants whilst central metabolites generally reach steady state in the range of minutes to hours (Dieuaide-Noubhani *et al.*, 1995, Ettenhuber *et al.*, 2005, Roessner-Tunali *et al.*, 2004, Schwender *et al.*, 2004, Sriram *et al.*, 2004, Williams *et al.*, 2010), polymeric storage molecules, structural components and many secondary metabolites often take considerably longer to achieve this (Kruger, Ratcliffe & Roscher, 2003, Matsuda *et al.*, 2005, Rontein *et al.*, 2002, Roscher *et al.*, 2000). Problems can also arise with specific experimental tissues when using the steady-state approach. Although the majority of central metabolites apparently reach isotopic steady state after 5h in excised potato tuber discs (Roessner-Tunali *et al.*, 2004) the onset of the wounding response in these discs already occurs after 2h (Kahl, 1974). This represents a good example of a physiological condition rendering the steady-state labelling approach inappropriate. Such features of metabolism can prove restrictive for steady-state modeling, however, it is nevertheless the best suited approach for the analysis of complex networks (Ratcliffe & Shachar-Hill, 2006). The reason for this is the fact that it depends on the assignment of the observed labelling of a metabolite to competing fluxes from sources with different positional enrichments. When applicable, steady-state analysis is better equipped to cope with reversible

steps, cyclic fluxes and the additional complexities provided by the extensive sub-compartmentation of the plant cell (Krueger *et al*., 2011) and discussed in detail below). However, this is only true for heterotrophic systems since the assimilation of labelled carbon dioxide leads to an uninformative steady state in which all metabolites are uniformly labelled (Ratcliffe & Shachar-Hill, 2006, Roscher *et al*., 2000). Despite this drawback the steady-state approach has proven highly useful in plants; particularly in studies with seeds (Allen, Ohlrogge & Shachar-Hill, 2009, Alonso, Val & Shachar-Hill, 2011, Schwender *et al*., 2004, Schwender, Ohlrogge & Shachar-Hill, 2003, Sriram *et al*., 2004), cell suspension cultures (Kruger *et al*., 2007, Rontein *et al*., 2002, Williams *et al*., 2010) and also in excised maize root tips (Dieuaide-Noubhani *et al*., 1995). Given that we have chosen to focus this review largely on dynamic labelling approaches we will not extensively review the outcomes of these studies here but rather highlight a few illustrative examples. Early applications of this approach in plant systems determined exact solutions for a set of steady-state equations using experimentally determined positional fractional enrichment data as inputs (Dieuaide-Noubhani *et al*., 1995, Edwards *et al*., 1988, Fernie *et al*., 2001, Rontein *et al*., 2002, Roscher *et al*., 2000). Whilst relatively simple to carry out, such approaches suffer from the drawback that they have no inbuilt manner of testing the validity of the resultant flux maps. One way around this is to test the map by measuring the positional enrichments obtained from differentially labelled precursors for example [1-^{13}C]- and [2-^{13}C]-glucoses (Ratcliffe & Shachar-Hill, 2006). Another approach drawing on extensive work carried out in microbial flux analysis (Ruehl *et al*., 2012, Schmidt *et al*., 1999, Wiechert, 2001, Wiechert *et al*., 2001) is to adopt full isotopomeric (cumomer) analysis by which the system is over-determined thus facilitating statistical assessment of the quality of fit and determination of a confidence range for each fitted parameter. Whilst discussed earlier by plant scientists (see for example Kruger et al., 2003) the cunomer approach in plants was first adopted by Schwender *et al.* (2006),) and subsequently by several other plant research groups (Alonso *et al*., 2007, Williams *et al*., 2010; Masakapalli *et al.* 2010). This approach has proven instrumental in recent evaluations of, amongst other things, light and energy efficiencies in plant cells (for a review see Chen & Shachar-Hill, 2012) and in the comparison of flux estimates from metabolic flux analysis and flux balance analysis (Williams *et al*., 2010). Of particular note here is the work of Schwender *et*

al. (2006) which, at least to our knowledge, was the first approach to employ Wiecherts approach and the cunomer framework. It additionally applies a test of goodness of fit and discusses the robustness of the modelling outcome to the removal of measurement data, to differently ^{13}C labelled substrates and to changes in the topology of the reaction network. A further important recent advance in computational efficiency is the Elementary Metabolite Unit (EMU) approach which writes balances only on the carbon fragments for which experimental information is obtained, rather than the full set of isotopomers. This is results in a significant computational reduction in calculating fluxes, which is especially important in computations used to estimate fluxes in large networks (Antoniewicz, Kelleher & Stephanopoulos, 2007).

Dynamic labelling

The alternate approach to steady-state labelling is the dynamic labelling approach wherein the concentration and labelling status of various metabolite pools are monitored at multiple time points either following a pulse of label or during a time-course of continuous labelling (Ratcliffe & Shachar-Hill, 2006, Roscher *et al.*, 2000). An advantage of the pulse labelling approach is that since the specific activities of the partially labelled isotopomers initially increase before declining further constraints are provided for fitting procedures. McNeil *et al*. (2000) took advantage of this feature in the quantitative analysis of the pathways of choline biosynthesis in tobacco using ^{33}P-labelled precursors and were able to reveal that phospho-base methylation is the main route of choline biosynthesis in this species. Whilst not suffering the restrictions steady state analysis does, with respect to tissue types and the requirement that pool sizes do not vary during the experiment, dynamic labelling is best suited for small-scale networks with analysis being progressively more difficult as the label moves further away from its point of entry into the metabolic network (Ratcliffe & Shachar-Hill, 2006). Thus dynamic labelling is highly suited to analysis of peripheral areas of metabolism such as secondary metabolism (Boatright *et al.*,2004, Colon *et al.*, 2010, Dal Cin *et al.*, 2011, Heinzle *et al.*, 2010, Matsuda *et al.*, 2003) but perhaps less so to central metabolic pathways for which it can prove very difficult to analyse absolute fluxes (Baxter *et al.*,2007). In this section we will merely outline the use of dynamic labelling in non-

plant systems with its use in plants being detailed later. The dynamic profiling approach has its roots in non-photosynthetic microbial research where it has been used extensively to characterise fluxes within primary metabolism (Hiller, Metallo & Stephanopolous, 2011; Zamboni, 2011; Rühl, Zamboni & Sauer, 2010; Schaub, Mauch & Reuss, 2008; Selivandro *et al.*, 2006). Whilst traditionally this was used for the analysis of relatively simple systems recent years have witnessed its extension to more complex systems by the development of non-stationary (alternatively named instationary) metabolic flux analysis and the kinetic flux profiling approach (Noeh, *et al.*, 2007, Noeh & Wiechert, 2006, Yuan, Bennett & Rabinowitz, 2008).

This approach has additionally recently been used to provide a comprehensive quantitative analysis of carbohydrate oxidation in *Penecillium chrysogenum* (Zhao, et al.,, 2008). Whilst such approaches are more demanding in computational time they are more informative than the stationary approach. An extension of the EMU approach was recently formulation for the instationary metabolic flux analysis (Young, et al., 2008). It was successfully applied to the analysis of primary photosynthetic metabolism in cyanobacteria (Young *et al.*, 2011; described in the next section).

EARLY APPLICATIONS OF DYNAMIC LABELLING IN THE GREEN LINEAGE

As seen above for microbial flux analysis, applications of dynamic labelling in the green lineage can also be split into simple pathway based analysis and more complex network based analysis. We will review the former in passing only and concentrate on the latter here. The use of dynamic labelling in pathway based analysis has already been alluded to above. A plethora of examples exist by which dynamic labelling has been used in order to gain quantitative information concerning flux distributions through various plant pathways including C1 metabolism, monoterpenoids, monlignols and phenylpropanoid derivatives (Boatright *et al.*, 2004, Colon *et al.*, 2010, Hatzfeld & Stitt, 1990 , Matsuda *et al.*, 2003, McNeil *et al.*, 2000, Rios-Estepa & Lange, 2007, Rios-Estepa *et al.*, 2010, Rios-Estepa *et al.*, 2008(Dal Cin *et al.*, 2011, Lee & Voit, 2010, Maeda *et al.*, 2010))as well as to computationally model photosynthesis (Zhu, de Sturler & Long, 2007). Importantly, not only is dynamic metabolic flux analysis necessary for plant tissues, in which physiologically relevant steady-states are not easy to achieve, but it also offers the advantage of being able to yield models which can predict the effects of genetic or environmental perturbation (Allen *et al.*, 2009, Morgan & Rhodes, 2002, Poolman, Assmus & Fell, 2004, Rohwer & Botha, 2001). As such it is likely to develop into a highly important tool for the metabolic engineering of useful natural products. That said early studies demonstrated that it is by no means trivial to apply such methods in order to define absolute fluxes in central metabolism (Baxter *et al.*, 2007, Roessner-Tunali *et al.*, 2004). It is perhaps telling to note that in both of these studies heterotrophic systems were analysed - heterotrophic Arabidopsis cell suspension cultures and excised potato tuber discs, respectively.

Two studies in autotrophic systems have recently demonstrated that this approach can indeed have considerable utility in the analysis of primary carbon assimilation and the subsequent central metabolism of the photoassimilate. The first of these reconstructed comprehensive flux maps of photoautotrophic metabolism by computational analysis of dynamic isotope labeling measurements and applied these to determine metabolic pathway fluxes in the cyanobacterium Synechocystis

sp. PCC6803. The approach taken was to rapidly sample the cells in response to a step change in the ratio of ^{13}C to ^{12}C CO_2.The isotopic distribution of label within central metabolic precursors was analyzed by a combination of LC/MS/MS and GC/MS techniques (Shastri & Morgan, 2007). Intriguingly, comparison to a theoretically predicted flux map revealed inefficiencies in photosynthesis due to considerable flux through the oxidative pentose phosphate pathway and malic enzyme, despite this species being characterized by negligible photorespiration (Young *et al.*, 2011). The authors additionally noted that the distribution of labeling displayed patterns consistent with metabolite channeling – the process by which metabolites are transferred directly between enzymes without transfer through the bulk phase. Similar patterns have previously been described in the seed plant tobacco (Hasunuma *et al.,* 2009). Whilst this is reasonable circumstantial evidence for channeling, more convincing experiments are provided by so called dilution experiments in which the specific activities of precursor and product of a pathway are contrasted in the presence and absence of added unlabeled intermediates of the pathway (Moller and Cohn, 1980; Graham *et al.*, 2007) That said compelling arguments have recently also been put forward suggesting data from steady-state metabolic flux analysis can reveal metabolite channeling (Williams *et al.*, 2010). The work of Young and co-workers therefore clearly illustrates the clear potential of the approach to fill gaps in our understanding of metabolic carbon and energy flows in cyanobacteria and likely amenable to unicellular algae, if compartmentation of the key metabolic intermediates can be resolved.

The second study focused on resolving intracellular fluxes in intact Arabidopsis rosettes, based on time-dependent labeling patterns in the metabolome. Plants photosynthesizing under limiting irradiance and ambient CO_2 in a custom built chamber were transferred into a $^{13}CO_2$ enriched environment. The isotope labeling patterns of 40 metabolites were subsequently obtained using liquid- or gas chromatography coupled to mass spectrometry. Labeling kinetics revealed striking differences between metabolites. Whilst these generally matched qualitative expectations from pathway topology and stoichiometry, they revealed some unexpected features that point to complexity of subcellular and cellular compartmentation. To achieve quantitative insights, the dataset was used for *in silico* modeling in the framework of kinetic flux profiling (Szecowka *et al.*, 2013).

The obtained flux estimates were benchmarked to four classically determined fluxes signatures of photosynthesis and the model was able to assess the robustness of the estimates with respect to different features of the underlying metabolic model and the time-resolved dataset. As for the studies mentioned above some unusual labeling patterns were observed that hinted at multiple spatially resolved pools of certain metabolites. When taken together these results present a convincing argument for the key importance of enhancing subcellular resolution in flux analysis (Sweetlove and Fernie, 2013).

KINETIC MODELING OF PLANT SECONDARY METABOLISM

Kinetic (also called mechanistic or dynamic) models are able to quantitatively represent metabolism based on formulation that includes information regarding the stoichiometry, kinetics, and regulation of sets of metabolic reactions. They are different from the previously described metabolic flux analysis in that the fluxes and pool sizes are no longer constrained to be at a steady-state. Most critically, they differ from stoichiometric models in their ability to be predictive of dynamic events. Wiechert and Novak (2011) have written a useful recent review of the steps of formulating and validating a kinetic model. An excellent illustration of the approach is a detailed kinetic model that described the hierarchical regulation of central metabolism in *E. coli* (Kotte, Zaugg & Heinemann, 2010). Secondary metabolism in plants whilst being amazingly diverse can still be organized into families of structurally related compounds (D´Auria & Gershenzon, 2005). Such classification facilitates the grouping of pathways and even their in order to render their modeling more tractable. This core structure forms the logical basis of organization of models of fluxes as well as kinetic models (Morgan & Shanks, 2002). Next we present selected examples from the recent literature focused on kinetic models of secondary metabolism.

In kinetic model formulation, there are two basic approaches, which can be classified as top-down or bottom-up approaches. In a bottom-up approach one builds the model on as many individual reactions with *in vitro* determined parameters as feasible and utilizes the correct enzyme mechanism if possible. This can represent an enormous amount of work for a pathway of more than a few

enzymes, especially poorly studied pathways. However, in some cases, the remaining unknown parameters can be obtained by optimization (Knoke *et al.*, 2009). In a series of two papers, a seminal work used such a bottom-up approach to model monoterpene formation in peppermint leaves (Rios-Estepa *et al.*, 2010, Rios-Estepa *et al.*, 2008). The bottom-up approach was able to take advantage of significant enzymatic and regulatory knowledge concerning this well-studied pathway (Croteau *et al.*, 2005). In the original model, the presence of inhibition kinetics was found to be a key aspect to fit the experimental data. This newly discovered inhibition was verified subsequently by further *in vitro* and *in vivo* measurements. Importantly, the model also included developmental profiles of enzyme activity as well as trichome gland expansion. The biochemical profile of the monoterpenes was found to be highly correlated with the gene expression of key enzymes. However, the expression of these enzymes had low correlation to the overall yield, which was found to be governed by the density of trichomes on leaves.

In contrast to the bottom-up approach, a simpler method to model is to use a top down approach. The advantage of such an approach is one is not reliant on the tenuous assumption that *in vitro* kinetic parameters apply to *in vivo* conditions. A particularly illustrative example is the kinetic model developed for benzenoid metabolism in Petunia (Colon *et al.*, 2010). Previous to this work little was known about the *in vitro* kinetics of many of the constituent enzymes of this pathway. Therefore, several reactions in the pathway were lumped. The pathway was studied by feeding several different concentrations of isotopically labeled Phe, the precursor, to the pathway. This perturbation was followed by measuring the transient changes in intracellular concentrations of pathway intermediates and extracellular volatiles and the specific isotopic labeling percentage enabled the kinetic parameters for the pathway to be determined. The model was then validated by successfully making a prediction of the metabolic kinetics in a flower exhibiting lower expression of benzyl CoA:benzyl alcohol/phenylethanol benzoyltransferase.

In both the bottom-up or top-down kinetic models, the distribution of flux control can be determined for the pathway (Morgan & Rhodes, 2002). This analysis is

actually more significant than simply having a model that fits the data as control coefficients tell one where the control within the pathway resides and how much increase or decrease in the expression of certain enzymes is required to significantly alter fluxes. A vital consideration to keep in mind is that the flux control coefficients are local properties of the pathway and hence are specific to a given set of environmental conditions. Under different conditions the distribution of control coefficients can shift. In other words, generalizing that a given enzyme is rate limiting should not be done.

APPROACHES TO TACKLE THE COMPLEXITY OF COMPARTMENTATION

Plants display extensive compartmentation at the tissue and subcellular levels (Figure 1). Even cells within a single tissue are differentiated with some 40 odd cell types present in a typical plant (Fernie, 2007). Thus techniques to selectively sample metabolites and pinpoint their concentrations *in planta* are extremely valuable. Knowledge of the subcellular metabolic compartmentation is one key challenge facing the development of predictive models of plant metabolism. Techniques have been developed that are based upon the knowledge of biosynthetic specificity of metabolic pathways. These have been successfully applied to quantify metabolic fluxes (Allen *et al.*, 2007, Schwender et al., 2006; Sriram *et al.*, 2004). In each of these studies the authors fed ^{13}C labeled precursors and waited until an isotopic steady-state was achieved. In Allen et al. (2007), as in many previous studies carried out on a narrower range of compounds (Schwender et al., 2006), the labeling patterns of fatty acids, cell wall sugars, protein hydrolysates and starch were analyzed to ”retrobiosynthetically” estimate the flux contributions of plastid and cytosol specific pathways. In a recent paper on analysis of labeling patterns, the differences in labeling of amino acids obtained from digestion of the small and large subunits of RuBisCO were analyzed (Allen *et al.*, 2012). Significant differences in labeling of several amino acids pointed to non-equilibrium pools in plastids compared to the cytosol. It should be noted, that these methods can be extended to other proteins or molecules derived from precursors specific to a subcellular compartment. Moreover, protein digests are more interconnected with many parts of central metabolism than sugars due to separate amino acid groups coming from distinct compartmental pathways. However, the analysis of biomass derived molecules for flux analysis is restricted to reaching labeling at isotopic steady-state.

A second widely employed technique that preserves intracellular metabolite concentration is non-aqueous fractionation (Benkeblia, Shinano & Osaki, 2007, Farré *et al.*, 2001, Gerhardt & Heldt, 1984, Stitt *et al.*, 1989). This technique ruptures cell material after quenching which arrests metabolism, and the removal of water by lyophilization prevents polar metabolites from being able to diffuse – instead they remain assocaited with the insoluble macromolecules of the

subcellular compartments. The advantage is that both the enzymes and metabolites are retained compartmentally but the enzymes are inactive. The cellular material is then stratified by centrifugation in a non-aqueous density gradient. Fractions are collected and the separation is quantified by extracting the enzymes and measuring the relative activity of the marker enzymes in each fraction and correlating them to the metabolite concentrations. This technique produces a continuous distribution of fractions rather than isolated organelles. From this information, the metabolic concentration profiles of each compartment can be deconvoluted and the resulting data employed for further analysis. A recent paper has demonstrated the power of the technique in analysis of Arabidopsis leaf metabolome (Krueger *et al.*, 2011). This study was able to identify more than 2,000 analytes by a combination of LC/MS and GC/MS. The assignment of compartment localization was both correlated to known marker enzyme activity distributions as well as by a marker free approach. Furthermore, after deconvolution the localization of a significant number of metabolites was still difficult to unambiguously assign. The importance of the computational deconvolution procedure has recently been published together with a method demonstrating the utility of principal components analysis to visualize the data (Klie *et al.*, 2011). While non-aqueous fractionation is labor intensive, it is currently the most comprehensive technique of metabolite profiling that is also capable of capturing accurate quantitative metabolite distributions.

CURRENT LIMITATIONS IN KINETIC MODELING OF PLANT METABOLISM

It is our contention, that the key limitations for building kinetic models are in obtaining reliable quantitative metabolomics date at high spatial and temporal resolution. In other words, the mathematical modeling framework is in place and capable to support significant amounts of data. However, precise data amenable for quantitative modeling are far more difficult to obtain in plants for several reasons. One of the current hurdles is the ability to spatially resolve intracellular plant metabolism (see previous section) as well as at the whole plant level. Obviously many cell types exists in whole plants and they have differentiated into physically and likely metabolically distinct roles. The ability to routinely examine the entire metabolome of plant cells by a single analytical technique is far beyond our reach due to the enormous chemical diversity of molecules found in (Krueger *et al.*,

2012). Even targeted analysis of specific pathways often requires multiple analytical techniques. While substantial progress on the analytical chemistry of metabolomics for single cells is being made, the ability to get reliable samples from single cells is a significant challenge (Heinemann & Zenobi, 2011, Svatoš, 2011). With application to plants, mass spectrometry imaging has been reported to be successful at the single cell level for several secondary metabolites of relatively high abundance (Hölscher *et al.*, 2009).

We should not overlook that we still do not know all the metabolites or pathways present in plants. An example of a technology that will be of prime use is the combined isotopic feeding studies with non-targeted MS studies. In a recent paper, Creek et al. use non-targeted LC-MS and ^{13}C-glucose feeding to identify metabolites not previously known in *Trypanosoma brucei* (Creek *et al.*, 2012).

Another key challenge is the variance of enzyme expression and metabolite concentrations over time. There is a large variance in time scales in which metabolic processes change; from the very rapid sub second time scale in response to light and to biotic or abiotic stress which occur on the minutes to hours time scale, to metabolic changes governed by circadian rhythms which are on multiple hours time scales (Figure 2). These disparate time scales require experimental sampling at numerous discrete time points and further require distinct mathematical models that capture the relevant information. Studies are beginning to address this issue at least at the experimental level. One early study analyzed the dynamics of transcripts, proteins (using enzyme activities as a surrogate) and metabolites in Arabidopsis diurnal cycles revealed that transcript levels undergo marked and rapid changes during diurnal cycles and after transfer to darkness, whereas changes in activities are smaller and delayed (Gibon et al 2006). However, it is worth noting that the difficulties in integrating such datasets are well acknowledged (Fernie and Stitt, 2012). That said advances in non-invasive imaging techniques such as NMR (Kim *et al.*, 2011) or fluorescence resonance energy transfer based metabolite sensors (Lalonde, Ehrhardt & Frommer, 2005) could play an important role to complement destructive, but highly informative, sampling techniques.

FUTURE PESPECTIVES FOR KINETIC MODELING OF PLANT METABOLISM

From the previously mentioned key challenges, the ability of mathematical models to integrate disparate –omics data sets is key to systems biology. A promising approach that combines proteomic and metabolomic data with stoichiometric network models was recently published with human erythrocytes as a model metabolic system (Yizhak *et al.*, 2010). In this approach, both proteomic and metabolic data is utilized to constrain a stoichiometric metabolic model and produces better fits of fluxes than other flux balance techniques.

Secondly, as highlighted earlier, non-aqueous fractionation is an important analytical technique. The combination of the technique with isotopically labeled metabolites offers great promise for determining metabolic fluxes as illustrated in the work described above aimed at characterizing photosynthetic fluxes in the Arabidopsis rosette (Szecowka *et al.,* 2012). However, further standardization of the technique by characterizing the particle sizes of the homogenized tissue as well as examining how different plant tissues separate will be required to fully optimize this method. Particularly encouraged will be detailed protocols, e.g. how gradients are formed, how fractions are collected, influence of temperature and humidity, to allow robust results from within a laboratory as well as validation by other laboratories. This is similar in vein to the technology and standards developed for electrophoresis of proteins.

Several key challenges and avenues for future research are modeling diurnal rhythms and developmental modeling. As compared to studies in cell or protoplast cultures, whole plants go through a lengthy complicated developmental process. The application of both kinetic models as well as dynamic metabolic flux analysis (Leighty & Antoniewicz, 2011) are promising areas for exploration to capture such interesting and relevant physiological phenomena. The combination of mathematical models iteratively with increasing –omic and specific biochemical characterization will progress rapidly in the coming years. Certainly there is the continued need for interdisciplinary efforts between experimentalists, statisticians and mathematical modelers to make the most significant progress. A key outcome of having detailed kinetic models is the predictive ability to engineer plants to accumulate desired compounds or lower the flux towards undesired compounds.

We hope this review goes some way to convincing the reader that this goal should be achievable in the near future.

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REFERENCES

Aharoni A. & Brandizzi F. (2012) High-Resolution Measurements in Plant Biology. *Plant Journal*, **70**, 1-4.

Allen D.K., Laclair R.W., Ohlrogge J.B. & Shachar-Hill Y. (2012) Isotope labelling of Rubisco subunits provides in vivo information on subcellular biosynthesis and exchange of amino acids between compartments. *Plant Cell and Environment*, **35**, 1232-1244.

Allen D.K., Ohlrogge J.B. & Shachar-Hill Y. (2009) The role of light in soybean seed filling metabolism. *Plant Journal*, **58**, 220-234.

Allen D.K. & Ratcliffe R.G. (2009) Quantification of isotope label. In: *Plant Metabolic Networks* (ed J. Schwender), pp. 105-149. Springer, New York.

Allen D.K., Shachar-Hill Y. & Ohlrogge J.B. (2007) Compartment-specific labeling information in C-13 metabolic flux analysis of plants. *Phytochemistry*, **68**, 2197-2210.

Alonso A.P., Goffman F.D., Ohlrogge J.B. & Shachar-Hill Y. (2007) Carbon conversion efficiency and central metabolic fluxes in developing sunflower (Helianthus annuus L.) embryos. *Plant Journal*, **52**, 296-308.

Alonso A.P., Val D.L. & Shachar-Hill Y. (2011) Central metabolic fluxes in the endosperm of developing maize seeds and their implication for metabolic engineering. *Metabolic Engineering*, **13**, 96-107.

Antoniewicz M.R., Kelleher J.K. & Stephanopoulos G. (2007) Elementary metabolite units (EMU): A novel framework for modeling isotopic distributions. *Metabolic Engineering*, **9**, 68-86.

Ap Rees T., Fuller W.A. & Wright B.W. (1976) Pathways of carbohydrate oxidation during thermogenesis by the spadix of Arum maculatum. *Biochimica et Biophysica Acta*, **437**, 22-35.

Ap Rees T. & Royston B.J. (1971) Control of respiration in disks of carrot storage tissue. *Phytochemistry*, **10**, 1199-1206.

Araujo W.L., Ishizaki K., Nunes-Nesi A., Larson T.R., Tohge T., Krahnert I., Witt S., Obata T., Schauer N., Graham I.A., Leaver C.J. & Fernie A.R. (2010) Identification of the 2-Hydroxyglutarate and Isovaleryl-CoA Dehydrogenases as Alternative Electron Donors Linking Lysine Catabolism to the Electron Transport Chain of Arabidopsis Mitochondria. *Plant Cell*, **22**, 1549-1563.

Araujo W.L., Nunes-Nesi A., Nikoloski Z., Sweetlove L.J. & Fernie A.R. (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. *Plant Cell and Environment*, **35**, 1-21.

Arrivault S., Guenther M., Ivakov A., Feil R., Vosloh D., van Dongen J.T., Sulpice R. & Stitt M. (2009) Use of reverse-phase liquid chromatography, linked to tandem mass spectrometry, to profile the Calvin cycle and other metabolic intermediates in Arabidopsis rosettes at different carbon dioxide concentrations. *Plant Journal*, **59**, 824-839.

Bao X.M., Katz S., Pollard M. & Ohlrogge J. (2002) Carbocyclic fatty acids in plants: Biochemical and molecular genetic characterization of cyclopropane fatty acid synthesis of Sterculia foetida. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 7172-7177.

Baerenfaller K., Grossmann J., Grobei M.A., Hull R., Hirsch-Hoffmann M., Yalovsky S., Zimmermann P., Grossniklaus U., Gruissem W., Baginsky S. (2008). Genome-scale proteomics reveals Arabidopsis thalaina gene models and proteome dynamics. *Science*, **320**, 938-941.

Baxter C.J., Liu J.L., Fernie A.R. & Sweetlove L.J. (2007) Determination of metabolic fluxes in a non-steady-state system. *Phytochemistry*, **68**, 2313-2319.
Beevers H. & Gibbs M. (1954) The direct oxidation pathway in plant respiration. *Plant Physiology*, **29**, 322-324.
Benkeblia N., Shinano T. & Osaki M. (2007) Metabolite profiling and assessment of metabolome compartmentation of soybean leaves using non-aqueous fractionation and GC-MS analysis. *Metabolomics*, **3**, 297-305.
Blackman F.F. (1954) Analytic studies in plant respiration. Cambridge University Press, New York.
Boatright J., Negre F., Chen X.L., Kish C.M., Wood B., Peel G., Orlova I., Gang D., Rhodes D. & Dudareva N. (2004) Understanding in vivo benzenoid metabolism in petunia petal tissue. *Plant Physiology*, **135**, 1993-2011.
Bonarius H.P.J., Schmid G. & Tramper J. (1997) Flux analysis of undetermined metabolic networks: the quest for the missing constraints. *Trends in Biotechnology*, **15**, 308-314.
Borodina I. & Nielsen L.K. (2005) From genomes to in silico cells via metabolic networks. *Curr Opin Biotech*, **16**, 350-355.
Borowitz M.J., Stein R.B. & Blum J.J. (1977) Quantitative analysis of the change of metabolic fluxes along the pentose phosphate and glycolytic pathways in Tetrahymena in response to carbohydrates. *Journal of Biological Chemistry*, **252**, 1589-1605.
Boyle N.R. & Morgan J.A. (2011) Computation of metabolic fluxes and efficiencies for biological carbon dioxide fixation. *Metabolic Engineering*, **13**, 150-158.
Brodie B.B., Costa E., Dlabac A., Neff N.H. & Smookler H.H. (1966) Application of steady state kinetics to estimation of synthesis rate and turnover time of tissue catecholamines. *Journal of Pharmacology and Experimental Therapeutics*, **154**, 493-&.
Brunengraber H., Kelleher J.K. & DesRosiers C. (1997) Applications of mass isotopomer analysis to nutrition research. *Annual Review of Nutrition*, **17**, 559-596.
Bryant J.A. & Ap Rees T. (1971) Nucleic acid synthesis and induced respiration by disks of carrot storage tissue. *Phytochemistry*, **10**, 1191-1197.
Calvin M. (1962) Path of carbon in photosynthesis. *Science*, **135**, 879-&.
Chen F., Liu C.J., Tschaplinski T.J. & Zhao N. (2009) Genomics of Secondary Metabolism in Populus: Interactions with Biotic and Abiotic Environments. *Critical Reviews in Plant Sciences*, **28**, 375-392.
Chen X. & Shachar-Hill Y. (2012) Insights into metabolic efficiency from flux analysis. *Journal of Experimental Botany*, **63**, 2343-2351.
Choi J. & Antoniewicz M.R. (2011) Tandem mass spectrometry: A novel approach for metabolic flux analysis. *Metabolic Engineering*, **13**, 225-233
Christensen B., Nielsen, J. (1999). Isotopomer analysis using GC-MS. *Metabolic Engineering* 1, 282-290.
Colon A.M., Sengupta N., Rhodes D., Dudareva N. & Morgan J. (2010) A kinetic model describes metabolic response to perturbations and distribution of flux control in the benzenoid network of Petunia hybrida. *Plant Journal*, **62**, 64-76.
Converti P. & Perego P. (2002) Use of carbon and energy balances in the study of the anaerobic metabolism of Enterobacter aerogenes at variable starting glucose concentrations. *Applied Microbiology and Biotechnology*, **59**, 303-309.

Cornish-Bowden A. & Cardenas M.L. (2001) Functional genomics - Silent genes given voice. *Nature*, **409**, 571-572.
Creek D.J., Chokkathukalam A., Jankevics A., Burgess K.E.V., Breitling R. & Barrett M.P. (2012) Stable Isotope-Assisted Metabolomics for Network-Wide Metabolic Pathway Elucidation. *Analytical Chemistry*, **84**, 8442-8447.
Croteau R., Davis E., Ringer K. & Wildung M. (2005) (-)-Menthol biosynthesis and molecular genetics. *Naturwissenschaften*, **92**, 562-577.
D'Auria J.C.,Gershenzon J. (2005). The secondary metabolism of Arabidopsis thaliana: growing like a weed. Curr Opin Plant Biol., 8, 308-316.
Dal'Molin C.G.O., Quek L.E., Palfreyman R.W., Brumbley S., M., & Nielsen L.K. (2011) AraGEM, a Genome-Scale Reconstruction of the Primary Metabolic Network in Arabidopsis. *Plant Physiology*, **152**, 579-589.
Dal Cin V., Tieman D.M., Tohge T., McQuinn R., de Vos R.C.H., Osorio S., Schmelz E.A., Taylor M.G., Smits-Kroon M.T., Schuurink R.C., Haring M.A., Giovannoni J., Fernie A.R. & Klee H.J. (2011) Identification of Genes in the Phenylalanine Metabolic Pathway by Ectopic Expression of a MYB Transcription Factor in Tomato Fruit. *Plant Cell*, **23**, 2738-2753.
Dauner M., Sauer, U. (2000). GC-MS analysis of amino acids rapidly provides rich information for isotopomer balancing. *Biotechnology Progress* 16, 642-649.
Dieuaide-Noubhani M., Raffard G., Canioni P., Pradet A. & Raymond P. (1995) Quantification of compartmented metabolic fluxes in maize root-tips using isotope distribution from C-13 labeled or C-14 labeled glucose. *Journal of Biological Chemistry*, **270**, 13147-13159.
Edwards S., Nguyen B.T., Do B. & Roberts J.K.M. (1988) Contributions of malic enzyme, pyruvate kinase, phosphoenolpyruvate carboxylase, and the Krebs cycle to respiration and biosynthesis and to intracellular pH regulation during hypoxia in maize root tips observed by nuclear magnetics resonance and gas chromatography mass spectrometry. *Plant Physiology*, **116**, 1073-1081.
Ettenhuber C., Spielbauer G., Margl L., Hannah L.C., Gierl A., Bacher A., Genschel U. & Eisenreich W. (2005) Changes in flux pattern of the central carbohydrate metabolism during kernel development in maize. *Phytochemistry*, **66**, 2632-2642.
Exarchou V., Krucker M., van Beek T.A., Vervoort J., Gerothanassis I.P. & Albert K. (2005) LC-NMR coupling technology: recent advancements and applications in natural products analysis. *Magnetic Resonance in Chemistry*, **43**, 681-687.
Fan T.W.M. & Lane A.N. (2008) Structure-based profiling of metabolites and isotopomers by NMR. *Progress in Nuclear Magnetic Resonance Spectroscopy*, **52**, 69-117.
Farré E.M., Tiessen A., Roessner U., Geigenberger P., Trethewey R.N. & Willmitzer L. (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. *Plant Physiology*, **127**, 685-700.
Feldberg L., Venger I., Malitsky S., Rogachev I. & Aharoni A. (2009) Dual Labeling of Metabolites for Metabolome Analysis (DLEMMA): A New Approach for the Identification and Relative Quantification of Metabolites by Means of Dual Isotope Labeling and Liquid Chromatography-Mass Spectrometry. *Analytical Chemistry*, **81**, 9257-9266.

Fernie A.R. (2007) The future of metabolic phytochemistry: larger numbers of metabolites, higher resolution, greater understanding. *Phytochem*, 69, 2861-2880.
Fernie A.R. (2012) Grand challenges in plant systems biology: closing the circle(s). *Frontiers in Plant Science*, **3**, 35.
Fernie A.R., Stitt M. (2012). On the discordance of metabolomics with proteomics and transcriptomics: coping with increasing complexity in logic, chemistry and network interactions. *Plant Physiol.* 158, 1139-1145.Fernie A.R., Aharoni A., Willmitzer L., Stitt M., Tohge T., Kopka J., Carroll A.J., Saito K., Fraser P.D. & DeLuca V. (2011) Recommendations for Reporting Metabolite Data. *Plant Cell*, **23**, 2477-2482.
Fernie A.R., Geigenberger P. & Stitt M. (2005) Flux an important, but neglected, component of functional genomics. *Current Opinion in Plant Biology*, **8**, 174-182.
Fernie A.R., Roscher A., Ratcliffe R.G. & Kruger N.J. (2001) Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. *Planta*, **212**, 250-263.
Fernie A.R., Trethewey R.N., Krotzky A.J. & Willmitzer L. (2004) Metabolite profiling: from diagnostics to systems biology. *Nat Rev Mol Cell Biol*, **5**, 763-769.
Fiehn O., Wohlgemuth G., Scholz M., Kind T., Lee D.Y., Lu Y., Moon S. & Nikolau B. (2008) Quality control for plant metabolomics: reporting MSI-compliant studies. *Plant Journal*, **53**, 691-704.
Fischer E. & Sauer U. (2003) Metabolic flux profiling of Escherichia coli mutants in central carbon metabolism using GC-MS. *European Journal of Biochemistry*, **270**, 880-891.
Fletcher J.S. & Beevers H. (1970) Acetate metabolism in cell suspension cultures. *Plant Physiology*, **45**.
Gage D.A., Rhodes D., Nolte K.D., Hicks W.A., Leustek T., Cooper A.J.L. & Hanson A.D. (1997) A new route for synthesis of dimethylsulphoniopropionate in marine algae. *Nature*, **387**, 891-894.
Geigenberger P., Stitt M. & Fernie A.R. (2004) Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers. *Plant Cell and Environment*, **27**, 655-673.
Gerhardt R. & Heldt H.W. (1984) Measurement of Subcellular Metabolite Levels in Leaves by Fractionation of Freeze-Stopped Material in Nonaqueous Media. *Plant Physiology*, **75**, 542-547.
Giavalisco P., Koehl K., Hummel J., Seiwert B. & Willmitzer L. (2009) C-13 Isotope-Labeled Metabolomes Allowing for Improved Compound Annotation and Relative Quantification in Liquid Chromatography-Mass Spectrometry-based Metabolomic Research. *Analytical Chemistry*, **81**, 6546-6551.
Giavalisco P., Li Y., Matthes A., Eckhardt A., Hubberten H.-M., Hesse H., Segu S., Hummel J., Koehl K. & Willmitzer L. (2011) Elemental formula annotation of polar and lipophilic metabolites using C-13, N-15 and S-34 isotope labelling, in combination with high- resolution mass spectrometry. *Plant Journal*, **68**, 364-376.
Gibon Y., Usadel B., Blaesing O.E., Kamlage, B., Hoehne M., Trethewey, R., Stitt M. (2006). Integration of metabolite with transcript and enzyme activity profiling during diurnal cycles in Arabidopsis rosettes.

Giege P., Heazlewood J.L., Roessner-Tunali U., Millar A.H., Fernie A.R., Leaver C.J., Sweetlove L.J. (2003). Enzymes of glycolysis are functionally associated with the mitochondrion in Arabidopsis cells. *Plant Cell*, **15**, 2140-2151.

Gibbs M. & Beevers H. (1955) Glucose dissimilation in the higher plant. Effect of age of tissue. *Plant Physiology*, **30**, 343-347.

Grafahrend-Belau E., Schreiber F., Koschuetzki D. & Junker B.H. (2009) Flux Balance Analysis of Barley Seeds: A Computational Approach to Study Systemic Properties of Central Metabolism. *Plant Physiology*, **149**, 585-598.

Graham D. & ap Rees T. (1965) Occurence of fructose 1-phosphate in tissues of higher plants O. *Nature*, **208**, 88-&.

Graham J.W.A., Williams T.C.R., Morgan M., Fernie A.R., Ratcliffe R.G. & Sweetlove L.J. (2007) Glycolytic enzymes associate dynamically with mitochondria in response to respiratory demand and support substrate channeling. *Plant Cell*, **19**, 3723-3738.

Hajirezaei M., Sonnewald U., Viola R., Carlisle S., Dennis D. & Stitt M. (1994) Transgenic potato plants with strongly decreased expression of pyrophosphate fructose-6-phosphate phosphotransferase show no visibile phenotype and only minor changes in metabolic fluxes in their tubers. *Planta*, **192**, 16-30.

Hasunuma T., Harada K., Miyazawa S.-I., Kondo A., Fukusaki E. & Miyake C. (2009) Metabolic turnover analysis by a combination of in vivo C-13-labelling from (CO2)-C-13 and metabolic profiling with CE-MS/MS reveals rate-limiting steps of the C-3 photosynthetic pathway in Nicotiana tabacum leaves. *Journal of Experimental Botany*, **61**, 1041-1051.

Hatzfeld W.D. & Stitt M. (1990) A study of the rate of recycling of triose phosphates in heterotrophic Chenopodium rubrum cells, potato tubers, and maize endosperm. *Planta* **180**, 198–204.

Hay J. & Schwender J. (2011) Metabolic network reconstruction and flux variability analysis of storage synthesis in developing oilseed rape (Brassica napus L.) embryos. *Plant Journal*, **67**, 526-541.

Heinemann M. & Zenobi R. (2011) Single cell metabolomics. *Current Opinion in Biotechnology*, **22**, 26-31.

Heinzle E., Matsuda F., Miyagawa H., Wakasa K. & Nishioka T. (2007) Estimation of metabolic fluxes, expression levels and metabolite dynamics of a secondary metabolic pathway in potato using label pulse-feeding experiments combined with kinetic network modelling and simulation. *Plant Journal*, **50**, 176-187.

Hill S.A. & Ap Rees T. (1994) Fluxes of carbohydrate metabolism in ripening bananas. *Planta*, **192**, 52-60.

Hill S.A. & Ap Rees T. (1995) The effect of hypoxia on the control of carbohydrate metabolism in ripening bananas. *Planta*, **197**, 313-323.

Hill S.A., Waterhouse J.S., Field E.M., Switsur V.R. & Aprees T. (1995) Rapid recycling if triose phosphates in oak stem tissue. *Plant Cell and Environment*, **18**, 931-936.

Hiller K., Metallo C. & Stephanopoulos G. (2011). Elucidation of cellular metabolism via metabolomics and stable-isotope assisted metabolomics. *Curr Pharm Biotechnol*, **12** ,1075-86.

Hölscher D., Shroff R., Knop K., Gottschaldt M., Crecelius A., Schneider B., Heckel D.G., Schubert U.S. & Svatoš A. (2009) Matrix-free UV-laser desorption/ionization (LDI) mass spectrometric imaging at the single-cell level: distribution of

secondary metabolites of Arabidopsis thaliana and Hypericum species. *The Plant Journal*, **60**, 907-918.Hu J., Baker A., Bartel B, Linka N, Mullen RT, Reumann S, Zolman BK. (2012). Plant peroxisomes: biogenesis and function. *Plant Cell,* **24**, 2279-303

Huege J., Sulpice R., Gibon Y., Lisec J., Koehl K. & Kopka J. (2007) GC-EI-TOF-MS analysis of in vivo carbon-partitioning into soluble metabolite pools of higher plants by monitoring isotope dilution after (CO2)-C-13 labelling. *Phytochemistry*, **68**, 2258-2272.

Ito J., Taylor N.L., Castleden I., Weckwerth W., Millar A.H., Heazlewood J.L. (2009). A survey of the Arabidopsis thaliana mitochondrial phosphoproteome. *Proteomics*, **9**, 4229-4440.

Iyer V.V., Sriram G., Fulton D.B., Zhou R., Westgate M.E. & Shanks J.V. (2008) Metabolic flux maps comparing the effect of temperature on protein and oil biosynthesis in developing soybean cotyledons. *Plant Cell and Environment*, **31**, 506-517.

Kahl G. (1974) Metabolism in plant storage tissue slices. *Botanical Review*, **40**, 263-314.

Katz J., Landau B.R. & Bartsch G.E. (1966) The pentose cycle, triose phosphate isomerization and lipogenesis in rat adipose tissue. *Journal of Biological Chemistry*, **241**, 727-740.

Katz J. & Rognstad R. (1966) The labelling of pentose phosphate from glucose ^{14}C and the estimation of the rates of trtransaldolase transketolase and contribution of the pentose cycle and ribulode phosphate synthesis. *Biochemistry*, **6,**, 2227-2247.

Katz J. & Wood H.G. (1960) The use of Glucose C^{14} for the evaluation of the pathways of glucose metabolism. *Journal of Biological Chemistry*, **235,**, 2166-2177.

Katz J. & Wood H.G. (1963) The use of $C^{14}O_2$ yields from glucose-1 and -6 C^{14} for the evaluation of pathways of glucose metabolism. *Journal of Biological Chemistry*, **238**, 517-523.

Keeling P.L., Wood J.R., Tyson R.H. & Bridges I.G. (1988) Starch biosynthesis in developing wheat grain - evidence against the direct involvement of triose phosphates in the metabolic pathway. *Plant Physiology*, **87**, 311-319.

Kim H.K., Choi Y.H. & Verpoorte R. (2011) NMR-based plant metabolomics: where do we stand, where do we go? *Trends in Biotechnology*, **29**, 267-275.

Kleijn R.J., Geertman J.M.A., Nfor B.K., Ras C., Schipper D., Pronk J.T., Heijnen J.J., van Maris A.J.A. & van Winden W.A. (2007) Metabolic flux analysis of a glycerol-overproducing Saccharomyces cerevisiae strain based on GC-MS, LC-MS and NMR 13C-labelling data. *FEMS Yeast Reasearch*, **7**, 216-231.

Klie S., Krueger S., Krall L., Giavalisco P., Flügge U.-I., Willmitzer L. & Steinhauser D. (2011) Analysis of the compartmentalized metabolome – a validation of the non-aqueous fractionation technique. *Frontiers in Plant Science*, **2**.

Knoke B., Textor S., Gershenzon J. & Schuster S. (2009) Mathematical modelling of aliphatic glucosinolate chain length distribution in Arabidopsis thaliana leaves. *Phytochemistry Reviews*, **8**, 39-51.

Knopp H., Zilliges Y., Lockau W. & Steuer R. (2010) The metabolic network of Synechocystis sp PCC 6803: systemic properties of autotrophic growth. *Plant Physiology*, **154**, 410-422.

Kornberg H. (2000) Krebs and his trinity of cycles. *Nature Reviews Molecular Cell Biology*, **1**, 225-228.

Kornberg H. & Beevers H. (1957) A mechanism of conversion of fat to carbohydrate in castor beans. *Nature*, **180**, 35-36.
Kornberg H.L. (1958) Metabolism of C2 compounds in microorganisms.1. Incorporation of [2-C-14]acetate by Psedomonas fluorescens, and by a corynebacterium, grown on ammonium acetate. . *Biochemical Journal*, **68**, 535-542.
Kotte O., Zaugg J.B. & Heinemann M. (2010) Bacterial adaptation through distributed sensing of metabolic fluxes. *Molecular Systems Biology*, **6**.
Krueger S., Giavalisco P., Krall L., Steinhauser M.C., Büssis D., Usadel B., Flügge U.I., Fernie A.R., Willmitzer L. & Steinhauser D. (2011) A topological map of the compartmentalized Arabidopsis thaliana leaf metabolome. *PLoS One*, **15**, e17806.
Krueger S., Steinhauser D., Willmitzer L. & Giavalisco P. (2012) High-resolution plant metabolomics: from mass spectral features to metabolites and from whole-cell analysis to subcellular metabolite distributions. *Plant Journal*, **70**, 39-50.
Kruger N.J., Huddlestone J.E., Le Lay P., Brown N.D. & Ratcliffe R.G. (2007) Network flux analysis: impact of C-13 substrates on metabolism in Arabidopsis thaliana cell suspension cultures. *Phytochemistry*, **68**, 2176-2188.
Kruger N.J., Ratcliffe R.G. & Roscher A. (2003) Quantitative approaches for analysing fluxes through plant metabolic networks using NMR and stable isotope labelling. *Phytochemistry Reviews*, **2**, 17-30.
Lalonde S., Ehrhardt D.W. & Frommer W.B. (2005) Shining light on signaling and metabolic networks by genetically encoded biosensors. *Current Opinion in Plant Biology*, **8**, 574-581.
Lee Y. & Voit E.O. (2010) Mathematical modeling of monolignol biosynthesis in Populus xylem. *Mathematical Biosciences*, **228**, 78-89.
Leighty R.W. & Antoniewicz M.R. (2011) Dynamic metabolic flux analysis (DMFA): A framework for determining fluxes at metabolic non-steady state. *Metabolic Engineering*, **13**, 745-755.
Lipps S.H. & Beevers H. (1966) Compartmentation of organic acids in corn roots I. Differential labeling of 2 malate pools. *Plant Physiology*, 709-712.Lohse M., Bolger A.M., Nagel A., Fernie A.R., Lunn J.E., Stitt M., & Usadel B. (2012). RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. *Nucleic Acids Res* **40(Web Server issue)**:W622-7
Lonien J. & Schwender J. (2009) Analysis of Metabolic Flux Phenotypes for Two Arabidopsis Mutants with Severe Impairment in Seed Storage Lipid Synthesis. *Plant Physiology*, **151**, 1617-1634.
Maeda H., Shasany A.K., Schnepp J., Orlova I., Taguchi G., Cooper B.R., Rhodes D., Pichersky E. & Dudareva N. (2010) RNAi Suppression of Arogenate Dehydratase1 Reveals That Phenylalanine Is Synthesized Predominantly via the Arogenate Pathway in Petunia Petals. *Plant Cell*, **22**, 832-849.
Malloy C.R., Sherry A.D. & Jeffrey F.M.H. (1988) Evaluation of carbon flux and substrate selection through alternate pathways involving the citric-acid cycle of the heart by c-13 nmr-spectroscopy. *Journal of Biological Chemistry*, **263**, 6964-6971.
Masakapalli S.K., Le Lay P., Huddleston J.E., Pollock N.L., Kruger N.J. & Ratcliffe R.G. (2010) Subcellular Flux Analysis of Central Metabolism in a Heterotrophic Arabidopsis Cell Suspension Using Steady-State Stable Isotope Labeling. *Plant Physiology*, **152**, 602-619.

Matas A.J., Yeats T.H., Buda G.J., Zheng Y., Chatterjee S., Tohge T., Ponnala L., Adato A., Aharoni A., Stark R., Fernie A.R., Fei Z., Giovannoni J.J., Rose J.K.C. (2011). Tissue- and cell-type specific transcriptome profiling of expanding tomato fruit provides insights into metabolic and regulatory specialization and cuticle formation. *Plant Cell*, **23**, 3893-910

Matsuda F., Morino K., Ano R., Kuzawa M., Wakasa K. & Miyagawa H. (2005) Metabolic flux analysis of the phenylpropanoid pathway in elicitor-treated potato tuber tissue. *Plant and Cell Physiology*, **46**, 454-466.

Matsuda F., Morino K., Miyashita M. & Miyagawa H. (2003) Metabolic flux analysis of the phenylpropanoid pathway in wound-healing potato tuber tissue using stable isotope-labeled tracer and LC-MS spectroscopy. *Plant and Cell Physiology*, **44**, 510-517.

Matthews D.E., Motil K.J., Rohrbaugh D.K., Burke J.F., Young V.R. & Bier D.M. (1980) Measurement of leucine metabolism in man from a primed continuous infusion of L-[1-C-13]leucine. *American Journal of Physiology*, **238**, E473-E479.

McNeil S.D., Nuccio M.L., Rhodes D., Shachar-Hill Y. & Hanson A.D. (2000) Radiotracer and computer modeling evidence that phospho-base methylation is the main route of choline synthesis in tobacco. *Plant Physiology*, **123**, 371-380.

Mintz-Oron S., Meir S., Malitsky S., Ruppin E., Aharoni A. & Shlomi T. (2012) Reconstruction of Arabidopsis metabolic network models accounting for subcellular compartmentalization and tissue-specificity. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 339-344.

Møller B.L., Conn E.E. (1980). The biosynthesis of cyanogenic glucosides in higher plants. Channeling of intermediates in dhurrin biosynthesis by a microsomal system from Sorghum bicolor (linn) Moench. *J Biol Chem* **255**, 3049-3056.

Monton M.R.N. & Soga T. (2007) Metabolome analysis by capillary electrophoresis-mass spectrometry. *Journal of Chromatography A*, **1168**, 237-246.

Morgan J.A. & Rhodes D. (2002) Mathematical Modeling of plant metabolic pathways. *Metabolic Engineering*, **4**, 80-89.

Morgan J.A. & Shanks J.V. (2002) Quantification of metabolic flux in plant secondary metabolism by a biogenetic organizational approach. *Metabolic Engineering*, **4**, 257-262.

Moxley J.F., Jewett M.C., Antoniewicz M.R., Villas-Boas S.G., Alper H., Wheeler R.T., Tong L., Hinnebusch A.G., Ideker T., Nielsen J. & Stephanopoulos G. (2009) Linking high-resolution metabolic flux phenotypes and transcriptional regulation in yeast modulated by the global regulator Gcn4p. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 6477-6482.

Neuhaus H.E. & Stitt M. (1990) Control analysis of photosynthate partitioning - impact of reduced activity of ADP-glucose pyrophosphorylase or plastid phosphoglucomutase on the fluxes to starch and sucrose in Arabidopsis thaliana (L) Heynh. *Planta*, **182**, 445-454.

Noeh K., Groenke K., Luo B., Takors R., Oldiges M. & Wiechert W. (2007) Metabolic flux analysis at ultra short time scale: Isotopically non-stationary C-13 labeling experiments. *Journal of Biotechnology*, **129**, 249-267.

Noeh K. & Wiechert W. (2006) Experimental design principles for isotopically instationary (13)C labeling experiments. *Biotechnology and Bioengineering*, **94**, 234-251.
O'Grady J., Schwender J., Shachar-Hill Y. & Morgan J.A. (2012) Metabolic cartography: experimental quantification of metabolic fluxes from isotopic labelling studies. *Journal of Experimental Botany*, **63**, 2293-2308.
Obata T. & Fernie A.R. (2012) The use of metabolomics to dissect plant responses to abiotic stresses. *Cellular and Molecular Life Sciences*, **in press**.
Pilalis E., Chatziioannou A., Thomasset B. & Kolisis F. (2011) An in silico compartmentalized metabolic model of Brassica napus enables the systemic study of regulatory aspects of plant central metabolism. *Biotechnology Bioengineering*, **108**, 1673-1682.
Poolman M.G., Assmus H.E. & Fell D.A. (2004) Applications of metabolic modelling to plant metabolism. *Journal of Experimental Botany*, **55**, 1177-1186.
Poolman M.G., Miguet L., Sweetlove L.J. & Fell D.A. (2009) A Genome-Scale Metabolic Model of Arabidopsis and Some of Its Properties. *Plant Physiology*, **151**, 1570-1581.
Raamsdonk L.M., Teusink B., Broadhurst D., Zhang N.S., Hayes A., Walsh M.C., Berden J.A., Brindle K.M., Kell D.B., Rowland J.J., Westerhoff H.V., van Dam K. & Oliver S.G. (2001) A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *Nature Biotechnology*, **19**, 45-50.
Ratcliffe R.G. & Shachar-Hill Y. (2006) Measuring multiple fluxes through plant metabolic networks. *Plant Journal*, **45**, 490-511.
Reed J.L. & Palsson B.O. (2003) Thirteen years of building constraint-based in silico models of Escherichia colis. *Journalof Bacteriology*, **185**, 2692-2699.
Rios-Estepa R. & Lange B.M. (2007) Experimental and mathematical approaches to modeling plant metabolic networks. *Phytochemistry*, **68**, 2351-2374.
Rios-Estepa R., Lange I., Lee J.M. & Lange B.M. (2010) Mathematical Modeling-Guided Evaluation of Biochemical, Developmental, Environmental, and Genotypic Determinants of Essential Oil Composition and Yield in Peppermint Leaves. *Plant Physiology*, **152**, 2105-2119.
Rios-Estepa R., Turner G.W., Lee J.M., Croteau R.B. & Lange B.M. (2008) A systems biology approach identifies the biochemical mechanisms regulating monoterpenoid essential oil composition in peppermint. *Proceedings of the National Academy of Sciences*, **105**, 2818-2823.
Roessner-Tunali U., Liu J.L., Leisse A., Balbo I., Perez-Melis A., Willmitzer L. & Fernie A.R. (2004) Kinetics of labelling of organic and amino acids in potato tubers by gas chromatography-mass spectrometry following incubation in C-13 labelled isotopes. *Plant Journal*, **39**, 668-679.
Rohwer J.M. & Botha F.C. (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of in vitro kinetic data. *Biochemical Journal*, **358**, 437-445.
Rontein D., Dieuaide-Noubhani M., Dufourc E.J., Raymond P. & Rolin D. (2002) The metabolic architecture of plant cells - Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. *Journal of Biological Chemistry*, **277**, 43948-43960.
Roscher A., Emsley L., Raymond P. & Roby C. (1998) Unidirectional steady state rates of central metabolism enzymes measured simultaneously in a living plant tissue. *Journal of Biological Chemistry*, **273**, 25053-25061.

Roscher A., Kruger N.J. & Ratcliffe R.G. (2000) Strategies for metabolic flux analysis in plants using isotope labelling. *Journal of Biotechnology*, **77**, 81-102.
Rühl M., Zamboni N., Sauer U. (2010). Dynamic flux responses in riboflavin overproducing Bacillus subtilis to increasing glucose limitation in fed-batch culture. *Biotechnol Bioeng* **105**: 795-804.
Ruehl M., Rupp B., Noeh K., Wiechert W., Sauer U. & Zamboni N. (2012) Collisional fragmentation of central carbon metabolites in LC-MS/MS increases precision of 13C metabolic flux analysis. *Biotechnology and Bioengineering*, **109**, 763-771.
Schaub J., Mauch K., Reuss M. (2008). Metabolic flux analysis in Escherichia coli by intergrating isotopic dynamic and isotopic stationary 13C labeling data. *Biotech Bioeng*, **99**, 1170-1185.
Schauer N. & Fernie A.R. (2006) Plant metabolomics: towards biological function and mechanism. *Trends in Plant Science*, **11**, 508-516.
Schmidt K., Nielsen J. & Villadsen J. (1999) Quantitative analysis of metabolic fluxes in Escherichia coli, using two-dimensional NMR spectroscopy and complete isotopomer models. *Journal of Biotechnology*, **71**, 175-189.
Schuster S., Fell D.A. & Dandekar T. (2000) A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. *Nature Biotechnology*, **18**, 326-332.
Schwender J. (2011) Experimental flux measurements on a network scale. *Frontiers in Plant Science*, **2**, 63.
Schwender J., Goffman F., Ohlrogge J.B. & Shachar-Hill Y. (2004) Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. *Nature*, **432**, 779-782.
Schwender J., Ohlrogge J.B. & Shachar-Hill Y. (2003) A flux model of glycolysis and the oxidative pentosephosphate pathway in developing Brassica napus embryos. *Journal of Biological Chemistry*, **278**, 29442-29453.
Schwender J., Ohlrogge J.B. & Shachar-Hill Y. (2004). Understanding flux in plant metabolic networks. *Curr Opin Plant Biol*, **7**, 309-317
Schwender J., Ohlrogge J.B. & Shachar-Hill Y. (2006). Mitochondrial metabolism in developing embryos of Brassica napus. J Biol Chem, 281, 34040-34047.
Selivanov V.A., Marin S., Lee P.W., Cascante M. (2006). Software for dynamic analysis of tracer-based metabolomic data: estimation of metabolic fluxes and their statistical analysis. Bioinformatics 22: 2806-2812.
Shastri A.A. & Morgan J.A. (2007) A transient isotopic labeling methodology for C-13 metabolic flux analysis of photo auto trophic microorganisms. *Phytochemistry*, **68**, 2302-2312.
Soga T. (2007) Biomarker discovery by metabolome analysis. *Drug Metabolism Reviews*, **39**, 27-28.
Spielbauer G., Margl L., Hannah L.C., Roemisch W., Ettenhuber C., Bacher A., Gierl A., Eisenreich W. & Genschel U. (2006) Robustness of central carbohydrate metabolism in developing maize kernels. *Phytochemistry*, **67**, 1460-1475.
Sriram G., Fulton D.B., Iyer V.V., Peterson J.M., Zhou R.L., Westgate M.E., Spalding M.H. & Shanks J.V. (2004) Quantification of compartmented metabolic fluxes in developing soybean embryos by employing Biosynthetic ally directed fractional C-13 labeling, [C-13, H-1] two-dimensional nuclear magnetic resonance, and comprehensive isotopomer balancing. *Plant Physiology*, **136**, 3043-3057.

Stitt M. (1996) Metabolic regulation of photosynthesis. In: *Advances in Photosynthesis* (ed N. Baker). Academic Press.
Stitt M., ap Rees T. (1978). Pathways of carbohydrate oxidation in leaves of *Pisum sativum* and *Triticum aestivum. Phytochem*, 17, 1251-1256.
Stitt M., Wirtz W., Heldt H.W. (1983). Regulation of sucrose synthesis by cytoplasmic fructosebisphosphatase and sucrose phosphate synthase during photosynthesis in varying light and carbon dioxide. *Plant Physiol,* 72, 767-774.
Stitt M., Lilley R.M., Gerhardt R., Heldt H.W. & Sidney Fleischer B.F. (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. In: *Methods in Enzymology*, pp. 518-552. Academic Press.
Sulpice R., Pyl E.T., Ishihara H., Trenkamp S., Steinfath M., Witucka-Wall H., Gibon Y., Usadel B., Poree F., Piques M.C., Von Korff M., Steinhauser M.C., Keurentjes J.J., Guenther M., Hoehne M., Selbig J., Fernie A.R., Altmann T., Stitt M. (2009). Starch as a major integrator in the regulation of plant growth. *Proc. Natl. Acad. Sci. USA*, **106**, 10348-10353.
Svatoš A. (2011) Single-cell metabolomics comes of age: new developments in mass spectrometry profiling and imaging. *Analytical Chemistry*, **83**, 5037-5044.
Sweetlove L.J., Burrell M.M. & apRees T. (1996) Starch metabolism in tubers of transgenic potato (Solanum tuberosum) with increased ADPglucose pyrophosphorylase. *Biochemical Journal*, **320**, 493-498.
Sweetlove L.J., Last R.L. & Fernie A.R. (2003). Predictive metabolic engineering: a goal for systems biology. *Plant Physiol.,* 132, 420-426.
Sweetlove L.J. & Ratcliffe R.G. (2011) Flux-balance modeling of plant metabolism. *Frontiers in Plant Science*, **2**, 34.
Sweetlove L.J. & Fernie, A.R. (2013). The spatial organisation of metabolism within the plant cell. Annu Rev Plant Biology (in press)
Szecowka M., Heise R., Tohge T., Nunes-Nesi A., Vosloh D., Huege J., Nikoloski Z., Stitt M., Fernie A.R. & Arrivault S. (2013) Metabolic fluxes of an illuminated Arabidopsis rosette. *Plant Cell*, **in press**.
Tieman D., Taylor M., Schauer N., Fernie A.R., Hanson A.D. & Klee H.J. (2006) Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 8287-8292.
Tohge T., Mettler T., Arrivault S., Carroll A.J., Stitt M. & Fernie A.R. (2011) From models to crop species: caveats and solutions for translational metabolomics. *Frontiers in Plant Science*, **2**, 61.
Urano K., Kurihara Y., Seki M. & Shinozaki K. (2010) 'Omics' analyses of regulatory networks in plant abiotic stress responses. *Current Opinion in Plant Biology*, **13**, 132-138.
Vallino J.J. & Stephanopoulos G. (1993) Metabolic flux distributions in Corynebacterium-glutamicum during growth and lysine overproduction.. *Biotechnology and Bioengineering*, **41**, 633-646.
van Rijsewijk B.R.B.H., Nanchen A., Nallet S., Kleijn R.J. & Sauer U. (2011) Large-scale C-13-flux analysis reveals distinct transcriptional control of respiratory and fermentative metabolism in Escherichia coli. *Molecular Systems Biology*, **7**.
Viola R., Davies H.V. & Chudeck A.R. (1991) Pathways of starch and sucrose biosynthesis in developing tubers of potato (Solanum tuberosum L) and

seeds of Faba bean (Vicia faba L) - elucidation by C-13 Nuclear Magnetic Resonance spectroscopy. *Planta*, **183**, 202-208.
Vogel J.T., Walter M.H., Giavalisco P., Lytovchenko A., Kohlen W., Charnikhova T., Simkin A.J., Goulet C., Strack D., Bouwmeester H.J., Fernie A.R. & Klee H.J. (2010) SlCCD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. *Plant Journal*, **61**, 300-311.Weber A.P., Weber K.L., Carr K., Wilkerson C., Ohlrogge J.B. (2007). Sampling the Arabidopsis transcriptome with massively parallel pyrosequencing. *Plant Physiol.,***144**, 32-42.
Wheeler G.L., Jones M.A. & Smirnoff N. (1998) The biosynthetic pathway of vitamin C in higher plants. *Nature*, **393**, 365-369.
Wiechert W. (2001) 13C metabolic flux analysis. *Metabolic Engineering*, **3**, 195-206.
Wiechert W., Möllney M., Petersen S. & de Graaf A.A. (2001) A universal framework for 13C metabolic flux analysis. *Metabolic Engineering*, **3**, 265-283.
Wiechert W. & Noack S. (2011) Mechanistic pathway modeling for industrial biotechnology: challenging but worthwhile. *Current Opinion in Biotechnology*, **22**, 604-610.
Wiechert W. & Nöh K. (2005) From stationary to instationary metabolic flux analysis. In: *Advances in Biochemical Engineering/ Biotechnology* (ed U. Kragl), pp. 145-172. Springer-Verlag, Berlin.
Wiechert W., Schweissgut O., Takanaga H. & Frommer W.B. (2007) Fluxomics: mass spectrometry versus quantitative imaging. *Current Opinion in Plant Biology*, **10**, 323-330.
Williams T.C.R., Miguet L., Masakapilli S.K., Kruger N.J., Sweetlove L.J. & Ratcliffe R.G. (2008) Metabolic network fluxes in heterotrophic Arabidopsis cells: stability of the flux distribution under different oxygenation conditions. *Plant Physiology*, **148**, 704-718.
Williams T.C.R., Poolman M.G., Howden A.J.M., Schwarzlander M., Fell D.A., Ratcliffe R.G. & Sweetlove L.J. (2010) A Genome-Scale Metabolic Model Accurately Predicts Fluxes in Central Carbon Metabolism under Stress Conditions. *Plant Physiology*, **154**, 311-323.
Yizhak K., Benyamini T., Liebermeister W., Ruppin E. & Shlomi T. (2010) Integrating quantitative proteomics and metabolomics with a genome-scale metabolic network model. *Bioinformatics*, **26**, i255-i260.
Young J.D., Shastri A.A., Stephanopoulos G. & Morgan J.A. (2011) Mapping photoautotrophic metabolism with isotopically nonstationary C-13 flux analysis. *Metabolic Engineering*, **13**, 656-665.
Young J.D., Walther J.L., Antoniewicz M.R., Yon H. & Stephanopoulos G. (2008) An Elementary Metabolite Unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnology and Bioengineering*, **99**, 686-699.
Yuan J., Bennett B.D. & Rabinowitz J.D. (2008) Kinetic flux profiling for quantitation of cellular metabolic fluxes. *Nature Protocols*, **3**, 1328-1340.
Zamboni N. (2011) C-13 metabolic flux analysis in complex systems. *Current Opinion in Biotechnology*, **22**, 103-108.
Zamboni N., Fischer E. & Sauer U. (2005) FiatFlux - a software for metabolic flux analysis from C-13-glucose experiments. *Bmc Bioinformatics*, **6**, 8.
Zhao Z., Kuijvenhoven K., Ras C., van Gulik W.M., Heijnen J.J., Verheijen P.J.T. & van Winden W.A. (2008) Isotopic non-stationary (13)C gluconate tracer

method for accurate determination of the pentose phosphate pathway split-ratio in Penicillium chrysogenum. *Metabolic Engineering*, **10**, 178-186.
Zhu X.G., de Sturler E. & Long S.P. (2007) Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: A numerical simulation using an evolutionary algorithm. *Plant Physiology*, **145**, 513-526.

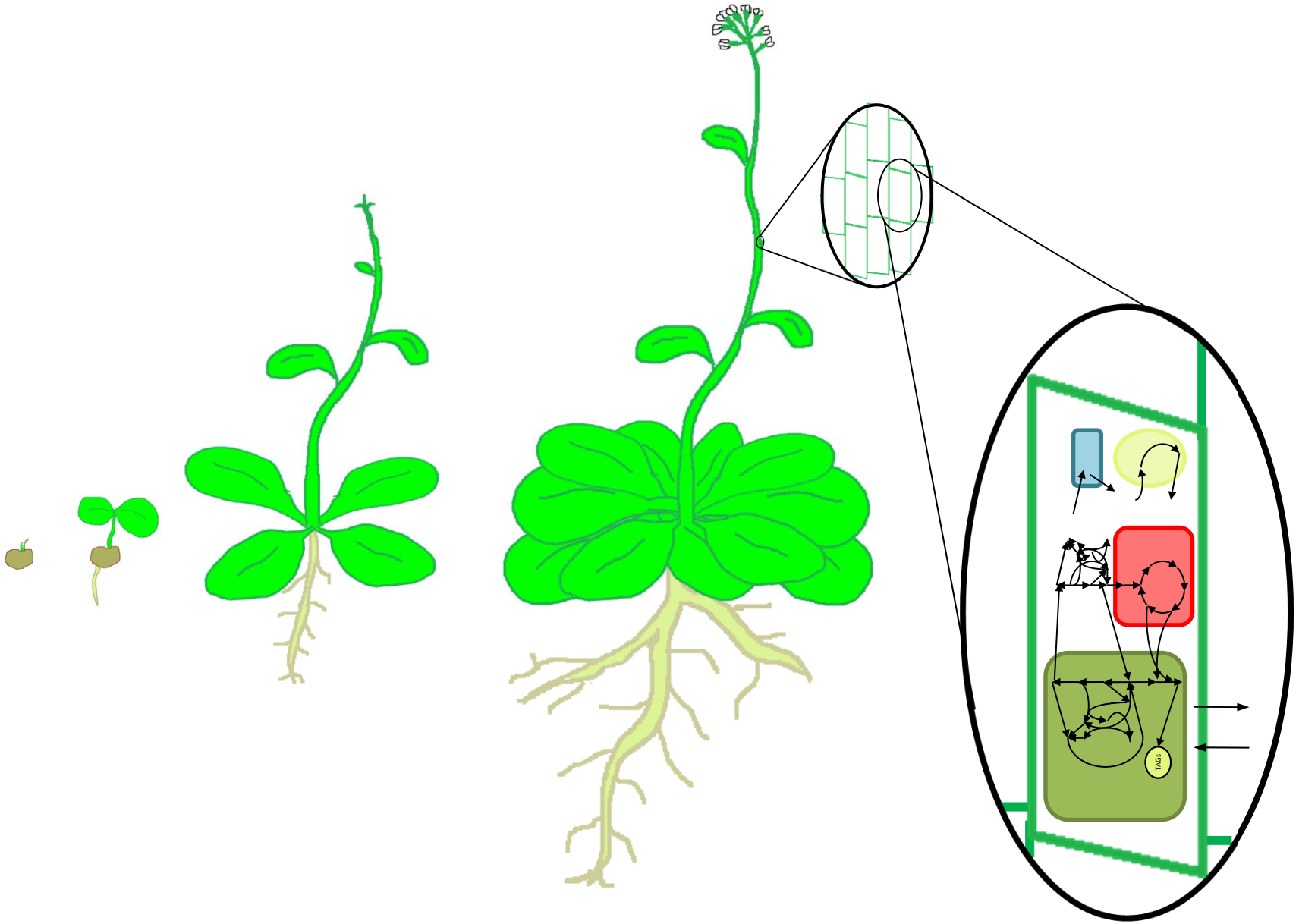
FIGURE LEGENDS

Figure 1. An illustration of the several layers of metabolic compartmentation in space and time. There are numerous tissues (e.g. roots, flowers and shoots) which perform vastly different metabolic functions. Even a single organ is comprised of many cell types (not pictured). Inside of an individual plant cell there is compartmentation of metabolism. The plastid is shaded in green, the mitochondria in red, the vacuole in blue and the peroxisome in light yellow. Over time plants proceed through a developmental program. Moreover diurnal cycles have a strong influence on metabolic pathways.

Figure 2. The different time scales of metabolic regulation. The characteristic time is essentially the time over which significant changes in which the concentrations of species change. The blue arrow is translational regulation. The dotted arrows represent regulation at the transcriptional and post-translational levels, including allosteric regulation of enzymes and hence fluxes and metabolite concentrations by metabolites that are not the direct substrate or product of an enzyme.

APPROACH FOR FLUX DETERMINATION*			
STATIONARY FLUX ANALYSIS		**INSTATIONARY FLUX ANALYSIS**	
a.k.a.: steady-state flux analysis		*a.k.a.: dynamic flux analysis, kinetic flux analysis*	
METHOD	**SOFTWARE**	**METHOD**	**SOFTWARE**
Stationary flux analysis	^{13}C Flux; FIAT; OpenFlux	Elementary metabolite units (EMU)	Not publically available
Elementary metabolite units (EMU)	Not publically available	Kinetic Flux Profiling (KFP)	Not publically available
SCOPE		**SCOPE**	
applicable for estimation of fluxes at branchpoints		very high resolution of fluxes isotopic steady-state not a prerequisite	

Table 1. Comparison of stationary and instationary flux profiling approaches including synonyms, methodologies, software availability and scope.* note all approaches in the Table require that the system under study is at metabolic steady state



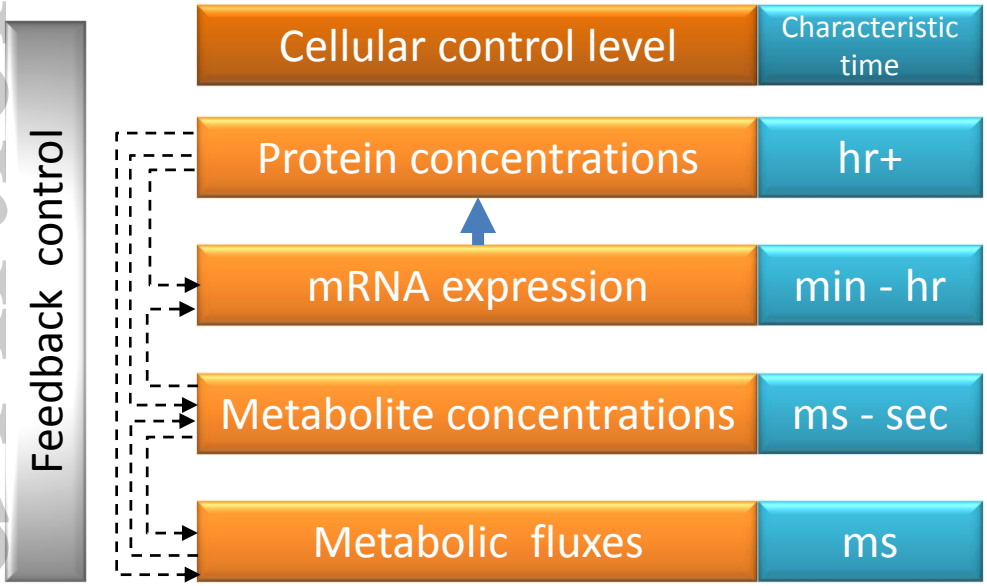


Figure 2