Biol. Rev. (2005), **80**, pp. 27–43. © *Cambridge Philosophical Society*
DOI: 10.1017/S1464793104006530 *Printed in the United Kingdom*

Revealing metabolic phenotypes in plants: inputs from NMR analysis

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(*Received* 6 *January* 2004; *revised* 25 *May* 2004; *accepted* 2 *June* 2004)

ABSTRACT

Assessing the performance of the plant metabolic network, with its varied biosynthetic capacity and its characteristic subcellular compartmentation, remains a considerable challenge. The complexity of the network is such that it is not yet possible to build large-scale predictive models of the fluxes it supports, whether on the basis of genomic and gene expression analysis or on the basis of more traditional measurements of metabolites and their interconversions. This limits the agronomic and biotechnological exploitation of plant metabolism, and it undermines the important objective of establishing a rational metabolic engineering strategy. Metabolic analysis is central to removing this obstacle and currently there is particular interest in harnessing high-throughput and/or large-scale analyses to the task of defining metabolic phenotypes. Nuclear magnetic resonance (NMR) spectroscopy contributes to this objective by providing a versatile suite of analytical techniques for the detection of metabolites and the fluxes between them.

The principles that underpin the analysis of plant metabolism by NMR are described, including a discussion of the measurement options for the detection of metabolites *in vivo* and *in vitro*, and a description of the stable isotope labelling experiments that provide the basis for metabolic flux analysis. Despite a relatively low sensitivity, NMR is suitable for high-throughput system-wide analyses of the metabolome, providing methods for both metabolite fingerprinting and metabolite profiling, and in these areas NMR can contribute to the definition of plant metabolic phenotypes that are based on metabolic composition. NMR can also be used to investigate the operation of plant metabolic networks. Labelling experiments provide information on the operation of specific pathways within the network, and the quantitative analysis of steady-state labelling experiments leads to the definition of large-scale flux maps for heterotrophic carbon metabolism. These maps define multiple unidirectional fluxes between branch-points in the metabolic network, highlighting the existence of substrate cycles and discriminating in favourable cases between fluxes in the cytosol and plastid. Flux maps can be used to define a functionally relevant metabolic phenotype and the extensive application of such maps in microbial systems suggests that they could have important applications in characterising the genotypes produced by plant genetic engineering.

Key words: metabolic flux analysis, metabolic networks, metabolite profiling, metabolomics, nuclear magnetic resonance spectroscopy, plant metabolism, stable isotope labelling.

CONTENTS

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I. INTRODUCTION

The success of the reductionist approach in biology can largely be attributed to the continuing development of techniques for identifying the molecular components of cells and for probing the interactions between them. The available methods vary enormously in scope and precision, and following the success in developing methods for the analysis of complete genomes, the emphasis in recent years has been on the development of complementary high-throughput, system-wide methods for the analysis of mRNA (transcriptomics: Eisen & Brown, 1999), proteins (proteomics: Zhu, Bilgin & Snyder, 2003) and metabolites (metabolomics: Nicholson, Lindon & Holmes, 1999; Fiehn, 2002; Weckwerth, 2003). These methods have greatly increased the flood of analytical data, and this is readily confirmed by a casual inspection of such publicly accessible databases as the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp), Swiss-Prot (http://www.ebi.ac.uk/swissprot/) and the Protein Data Bank (www.rcsb.org/pdb/).

Identifying the components of a biological system is an essential first step towards explaining biological function, but the true complexity of the system only emerges when the interactions between the components are taken into account (Palsson, 2000). This phenomenon is observable at all levels of biological organization – from the molecular level upwards – and it is a fundamental property of any network in which there are multiple interactions between the components (Newman, 2003). Cellular metabolism, which is the process that enables a cell to meet the fluctuating demand for energy and material, provides a good example of such an interacting network and indeed the structural complexity of the metabolic network appears to be essential for its biological role. However the precise relationship between the structural complexity of metabolic networks and their function remains a major challenge that merits further investigation.

Metabolism is based on the enzyme-catalysed interconversion of metabolites, and the resulting network of reactions and transport steps can be characterised in terms of the fluxes between different parts of the network. Several important questions arise about the relationship between the complexity of the network and its performance. For example, what are the properties of the network that allow it to maintain an appropriate flux distribution and thus to control resource allocation between competing metabolic demands? How does a robust network incorporate sufficient flexibility to permit changes in the flux distribution, for example in response to developmental cues or uncontrollable changes in external factors? What is the relationship between network performance and the need to avoid excessive fluctuations in metabolite concentration in a crowded intracellular environment with limited solvating power and constraints on water potential? What is the impact of changes in the structure of the network on fluxes and metabolite levels? It is not yet possible to provide complete answers to these questions, and one consequence of this can be found in the field of plant biotechnology where it is apparent that the current understanding of the relationship between the architecture of the plant metabolic network and the flux distributions it can support is insufficient to permit the routine implementation of rational metabolic engineering (Morandini & Salamini, 2003; Sweetlove, Last & Fernie, 2003; Kruger & Ratcliffe, 2004).

Two distinct strategies have emerged in the quest for a more detailed understanding of metabolic phenotypes. One approach is based on traditional metabolic analysis and involves characterising metabolic networks and the fluxes they support in even greater detail than before. This

involves the use of high-throughput system-wide methods for metabolite analysis, as well as techniques for mapping the intracellular fluxes in metabolic networks. In fact none of the available metabolomic techniques is capable of a complete quantitative analysis of all the metabolites in a tissue, and the available methods either provide a metabolite profile, in which a large number of metabolites are identified and quantified, or a metabolite fingerprint, in which a large number of metabolites are detected for the purposes of pattern recognition analysis (Fiehn, 2002; Sumner, Mendes & Dixon, 2003). The other approach is the construction of *in silico* genome-scale models of metabolic networks *ab initio*, using inputs from high-throughput technologies, particularly genomics, and constraints based on reaction stoichiometry, enzyme capacity and reaction directionality (Price *et al.*, 2003; Reed & Palsson, 2003). These genome-scale models are increasingly successful in capturing the phenotypic properties of bacterial metabolism (Palsson, 2002; Reed *et al.*, 2003) and it is clear that analysis of network structure itself can provide new insights into metabolism (Fell & Wagner, 2000; Jeong *et al.*, 2000). However, since it is not yet feasible to extend the construction of *in silico* genome-scale metabolic networks to plant cells, metabolic analysis remains the key to defining plant metabolic phenotypes and this is the approach that will be discussed in detail here.

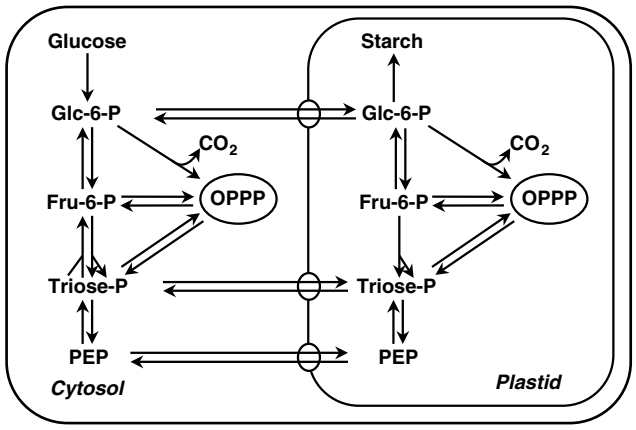

Fig. 1. A fragment of the metabolic network in a heterotrophic plant cell emphasising the subcellular duplication of some of the pathways of carbohydrate metabolism. Most of the steps are reversible and communication between the cytosol and the plastids is facilitated by transporters. The actual network in a particular system, for example the extent to which the complete oxidative pentose phosphate pathway (OPPP) occurs in both compartments, is likely to be both tissue- and species-dependent. Fru-6-P, fructose 6-phosphate; Glc-6-P, glucose 6-phosphate; PEP, phosphoenolpyruvate.

(1) Metabolic complexity in plants

Answering questions about network performance in plants is particularly difficult because of the structural complexity of the plant metabolic network (Kruger & Ratcliffe, 2004). Thus plant metabolism is characterised by its varied biosynthetic capacity, entailing the presence of multiple pathways for the synthesis of upward of 200 000 secondary metabolites across the plant kingdom (Sumner *et al.*, 2003), by extensive subcellular compartmentation, which imposes a requirement for a substantial number of metabolite transfers between compartments (Knappe, Flügge & Fischer, 2003), and by the existence of identical metabolic steps and even pathways in different compartments. Thus there is extensive duplication of the pathways of carbohydrate oxidation in the cytosolic and plastidic compartments of plant cells (Fig. 1; Neuhaus & Emes, 2000) and this is a major complication, for example, in analysing the operation of the oxidative pentose phosphate pathway (Kruger & von Schaewen, 2003). Similarly the pathways of folate-mediated one-carbon metabolism are distributed between, and to some extent duplicated in, the cytosol, the mitochondria and the plastids (Hanson, Gage & Shachar-Hill, 2000). This structural complexity is generally attributed to the autotrophic, poikilothermic and sessile life-style of plants, and indeed the *in silico* analysis of the uncompartmented metabolism in *Escherichia coli* indicates that greater complexity allows cells to function at near-optimal rates over a wider range of physiological conditions (Stelling *et al.*, 2002). Thus structural complexity is a fundamental property of the plant metabolic network and it presents a severe challenge for the construction of predictive models of plant metabolism (Kruger & Ratcliffe, 2004).

Despite its structural complexity, the building blocks of the plant metabolic network are exactly the same as those found in simpler systems (Fig. 1). Thus the network is constructed from a collection of metabolites that can be interconverted and relocated, sometimes irreversibly, through the action of a set of enzymes and transporters. In general such networks are characterised by a high level of connectivity and a correspondingly high number of ways (elementary flux modes) in which they can carry metabolic traffic (Stelling *et al.*, 2002). The subcellular compartmentation of plant cells adds to the structural complexity of the metabolic network and it greatly complicates metabolic measurements (ap Rees, 1987). However it is not a fundamentally different source of complexity, since increasing the number of network components by adding discrete compartmented pools of the same metabolite, and the transporters that link them, is equivalent in principle to adding further enzymes and metabolites to an uncompartmented network.

By contrast there are other features of metabolic networks that do lead to further levels of complexity. Thus many metabolites in central metabolism are substrates for more than one enzyme and they often act as effectors as well, regulating enzyme activities through binding interactions. Moreover the enzymes themselves may be substrates for other enzymes, permitting covalent modification and a change in catalytic activity. The effect of this is to establish a pattern of molecular cross-talk between different components of the network and to reduce the opportunities for segments of the network (which might equate to traditional pathways) to function in isolation. Furthermore the enzymes and transporters are expressed at controllable levels in specific compartments and membranes, and these levels are frequently responsive to a range of endogenous and external

factors. Thus the architecture of the network is not necessarily static and ultimately this flexibility has to be incorporated into realistic models of network performance. In this regard it is notable that the emerging second generation of *ab initio* models seek to incorporate expression profiling data into the analysis (Palsson, 2002).

(2) Metabolic analysis with NMR

Metabolic networks present two analytical challenges: the first is to identify and quantify all the relevant components, including their spatial location and temporal characteristics; and the second is to examine the operation of the network and the factors that influence the observed fluxes. There are several reasons why these apparently mundane tasks are the focus of renewed interest. First, as discussed above, there is a fundamental need for a more detailed understanding of the performance of the metabolic networks in all organisms, including plants. Secondly, the scope of metabolic analysis is increasing, with the development of high-throughput, system-wide methods for metabolite detection and sophisticated methods for metabolic flux analysis. Finally, metabolic analyses can be useful in functional genomics, offering strategies for deducing gene function (Raamsdonk *et al.*, 2001; Allen *et al.*, 2003) as well as potential future applications for constraining genome-scale models of the metabolic network (Palsson, 2002).

Accordingly this article describes the way in which a particular analytical technique, nuclear magnetic resonance (NMR) spectroscopy, can provide insights into the structure and function of plant metabolic networks. The contribution of NMR to this endeavour is based on the options it provides for detecting metabolites and for observing their interconversions. This leads to a wide range of applications for NMR in metabolic analysis, including important applications in the topical fields of metabolomics and metabolic flux analysis. The principles that underpin these two important applications are described in Sections II and III, and their contribution to the definition of plant metabolic phenotypes is discussed in Section IV.

II. METABOLITE DETECTION

NMR detects isotopes with non-zero nuclear magnetic moments, such as ^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{15}N and ^{31}P. The NMR signals from these magnetic isotopes have properties that are sensitive to the precise environment of the detected nucleus, and as a result NMR is a powerful tool for identifying chemical compounds. In fact all biologically significant chemical entities have at least one NMR signal and so NMR has the potential to record metabolic signals from samples of biological origin. More specifically NMR can be used to identify and quantify metabolites; to probe the cellular or subcellular location of these metabolites in favourable cases; and to measure the extent to which metabolites have been labelled after the incorporation of a magnetic isotope of low natural abundance, most commonly ^{2}H, ^{13}C or ^{15}N. Full details of the experimental procedures for implementing these metabolite detection strategies in plants and plant samples have been described elsewhere (Ratcliffe, 1994; Ratcliffe, Roscher & Shachar-Hill, 2001). Here the aim is to examine the main options that are available for the detection of plant metabolites, the scope and limitations of the various methods, and the steps that can be taken to maximise the usefulness of the approach.

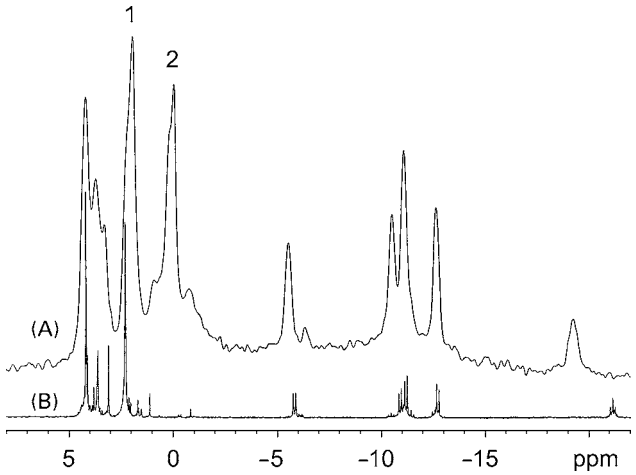

Fig. 2. Phosphorus nuclear magnetic resonance (NMR) spectra recorded (A) from a sample of maize root tips *in vivo*, and (B) from a perchloric acid extract of the same sample *in vitro*. The ^{31}P NMR spectra of plant tissues usually show signals from cytoplasmic and vacuolar inorganic phosphate (peaks 1 and 2), as well as from a range of the more abundant phosphate esters. Typically the latter include glucose 6-phosphate, phosphocholine, uridinediphosphoglucose (UDPG) and ATP. Extraction destroys any information about the subcellular origin of the signals, but it also greatly reduces their linewidth. This leads to increased spectral resolution, and to more detailed information about the metabolite profile of the sample. However while the detection threshold is lower in the extract than *in vivo*, it is still not low enough to allow every phosphorus metabolite to be detected. Adapted from Roscher *et al.* (1998) with the permission of the American Society for Biochemistry and Molecular Biology.

(1) Measurement options

(*a*) In vivo versus in vitro *detection*

NMR spectroscopy is non-destructive and it is frequently possible to record informative spectra from functioning cell suspensions, tissues and whole plants. This approach is invariably more demanding and time-consuming than recording a spectrum from an extract, because it is necessary to use an experimental arrangement that is compatible with the physical constraints of the NMR magnet and the physiological requirements of the tissue. It is often possible to solve this problem, but the *in vivo* spectrum is still likely to be inferior to the *in vitro* spectrum from a tissue extract recorded in the same time. In particular the *in vivo* spectrum is likely to be characterised by a poorer signal-to-noise ratio and worse resolution, both of which can limit the interpretation of the spectrum (Fig. 2). Despite this penalty, *in vivo*

spectroscopy is the method of choice when it can reveal information that might be lost on extraction, for example information on subcellular compartmentation or labile compounds, or when the intention is to follow the time dependence of the metabolite signals during kinetic (time-course) experiments. In fact for time-courses, the *in vivo* detection of a series of spectra from the same sample is generally a considerably more efficient and statistically reliable process than preparing multiple extracts from similar samples at different time points. Moreover in the *in vivo* experiment each sample acts as its own control making it easier to pinpoint phenomena that might otherwise be obscured by biological variability. For example it is easier to determine the exact sequence and timing of the onset of alanine, lactate and ethanol production in response to the imposition of hypoxia in an *in vivo* experiment than it is in a time-course that is reconstructed from a set of sampling experiments *in vitro* (Shachar-Hill, Pfeffer & Germann, 1996).

(*b*) *Identification* versus *fingerprinting*

The NMR spectrum of a tissue or tissue extract usually has signals from all the metabolites that contain the detected magnetic isotope, provided that each metabolite is present at a higher concentration than the detection threshold. Even for the most sensitive biologically important magnetic isotope, ^{1}H, the threshold for routine detection in an extract is no lower than $10\,\mu\mathrm{mol\,l^{-1}}$, implying that many low-concentration components of the metabolome will be undetectable. Thus NMR can only be expected to detect a subset of the metabolites in the sample (Fig. 2) and as such is best classified as a metabolite profiling technique (Ratcliffe & Shachar-Hill, 2001; Fiehn, 2002; Sumner *et al.*, 2003). In fact metabolite profiling of plant tissue extracts by ^{1}H and ^{13}C NMR is well established (Fan, 1996; Fan *et al.*, 2001; Bailey *et al.*, 2003; Defernez & Colquhoun, 2003; Le Gall *et al.*, 2003; Ward *et al.*, 2003), and its scope can be extended by inserting an in-line liquid chromatography step before the NMR analysis and by including a parallel analysis with mass spectrometry (Bailey *et al.*, 2000*a*, *b*). Liquid chromatography reduces the difficulties caused by overlapping signals in the NMR spectrum, as well as providing the possibility of concentrating minor components through solid-phase extraction, and mass spectrometry can provide complementary structural information. This strategy, which is analogous to the way in which chromatographic separation is coupled to mass spectrometry, takes the NMR approach further away from the metabolomic ideal of a high-throughput single-pass assay of the whole metabolome, but it does provide a method for obtaining high-quality analytical information on pre-selected groups of metabolites.

NMR can also be used as a metabolite fingerprinting technique in plant tissues (Lommen *et al.*, 1998; Noteborn *et al.*, 2000). In this approach there is no immediate attempt to assign the ^{1}H NMR signals to particular metabolites and instead the digitised spectrum is used as an input for a pattern recognition analysis. This allows comparisons to be made between the metabolic composition of different genotypes, or of the same genotype grown under seemingly identical or knowingly different conditions. In keeping with other metabolomic analyses (Roessner *et al.*, 2000; Sumner *et al.*, 2003) these fingerprinting analyses have revealed considerable variability in the metabolic composition of plants grown under supposedly identical conditions and this has the potential to be a major complication in the utilization of metabolic information for phenotyping purposes.

(*c*) *Time implications of the detection strategy*

The versatility of the NMR technique is such that it is possible for a metabolic NMR experiment to have a duration of anything between a minute and a week. Fortunately, if the concentration is not too close to the *in vivo* or *in vitro* detection threshold, metabolite detection can usually be achieved in a timescale of minutes, and it is on this basis that NMR is seen as a useful tool for high-throughput metabolomic applications (Sumner *et al.*, 2003). The actual time of the experiment depends on the sensitivity of the magnetic isotope, the concentration of the metabolite and the target signal-to-noise ratio in the final spectrum. In general it takes longer to record a spectrum for quantitative purposes, since such spectra need to be accumulated in a way that generates unbiased signal intensities. However this problem can be avoided in the routine analysis of similar samples, by calibrating the effect of the acquisition conditions on the intensities and using the resulting saturation factors to correct for the distortion of the spectrum. Moreover if it is also necessary to identify the metabolite that gives rise to the NMR signal, for example by exploring the relationship between the signals from different atoms in the same molecule, then further spectra will have to be recorded using different NMR detection schemes, and this will inevitably add time to the analysis.

(2) Loosening the constraints of sensitivity and resolution

It has always been desirable to maximise the sensitivity and resolution of an NMR experiment, but the current trend towards high-throughput analysis of potentially hundreds of metabolites puts an even higher premium on achieving this objective. In fact there have been continuous and spectacular improvements in the sensitivity and versatility of NMR ever since it was adopted as an analytical technique, and there are several options that can have a major influence on the suitability of NMR for plant metabolic analysis (Ratcliffe *et al.*, 2001).

(*a*) *Hardware options*

The two key hardware components that impact on sensitivity and resolution are the field strength of the magnet and the design of the probehead that accommodates the sample and generates the NMR signals. The NMR magnets currently available and suitable for metabolic analysis range in field strength (B_o) from 7 to 21 Tesla, corresponding to ^{1}H resonance frequencies of 300–900 MHz. Since the resolution, sensitivity of detection and signal-to-noise ratio in the

spectrum are proportional to B_o, B_o^2, and $B_o^{3/2}$, respectively, it is a great advantage to record spectra at the highest available field strength. However magnets with the highest field strengths are usually only available for macromolecular structure determination, being scarce and expensive, and so most metabolic analysis is conducted with spectrometers operating in the range 300–600 MHz. High magnetic fields are particularly beneficial for metabolite profiling applications, because the improved resolution leads to the definition of more signals, and while spectrometers at the upper end of this frequency range are preferred, 400 MHz spectrometers have been used effectively for metabolite fingerprinting and profiling by ^{1}H NMR (Raamsdonk *et al.*, 2001; Le Gall *et al.*, 2003).

Probeheads are available with a bewildering range of specifications but there are several features that can add substantially to sensitivity and thus potentially increase the scope of NMR as a metabolic profiling technique. First, scaled-down probes called microprobes allow spectra to be recorded from sample volumes as small as 50 μl, or even lower (Lacey *et al.*, 1999; Griffin *et al.*, 2002). These probes are optimal for samples that are only available in very small quantities and they give substantially better spectra than can be obtained from the same quantity of material diluted into the larger volume (500 μl) required for a conventional probehead. Secondly, the leading manufacturers have recently commercialised the concept of a cryogenic probehead (Styles *et al.*, 1984), in which sensitivity is markedly increased by lowering the thermal noise in the detection circuitry. Again expense mainly limits the utilization of this technology to the area of macromolecular structure determination, but these probes are becoming more widespread and indeed a recent analysis of alkaloid metabolism in cultured plant cells exploited the substantial gain in sensitivity obtained with a cryogenic probehead in a 500 MHz magnet (Hinse *et al.*, 2003). Finally, the implementation of several important NMR experiments, including the so-called inverse detection experiments that allow relatively insensitive isotopes such as ^{13}C or ^{15}N to be observed *via* the much more sensitive ^{1}H nucleus, requires probeheads that can generate pulsed magnetic field gradients. Indirect detection of plant metabolites *in vivo* has been attempted only rarely (Shachar-Hill *et al.*, 1996), but the application of these methods to plant extracts is well established, for example in the analysis of ^{15}N-labelled metabolites extracted from cell cultures (Mesnard *et al.*, 2000) and in the analysis of the metabolites in root exudates (Fan *et al.*, 2001).

(*b*) *Detection schemes*

The versatility of NMR as an analytical technique is greatly increased by the many options that exist for manipulating the detected signals. These manipulations increase the information content of the NMR spectrum, for example by introducing new signals, resolving overlapping signals, or enhancing intensities, and they greatly extend the scope of the technique as a tool for identifying metabolites and for probing metabolic events. Many of these manipulations carry a significant time penalty, making them unsuitable for routine high-throughput detection schemes, and they are more likely to be useful in detailed investigations of network function. Full details of the available options are given elsewhere (Ratcliffe *et al.*, 2001) and the discussion here is limited to a short description of two commonly encountered options: two-dimensional NMR and stable isotope labelling.

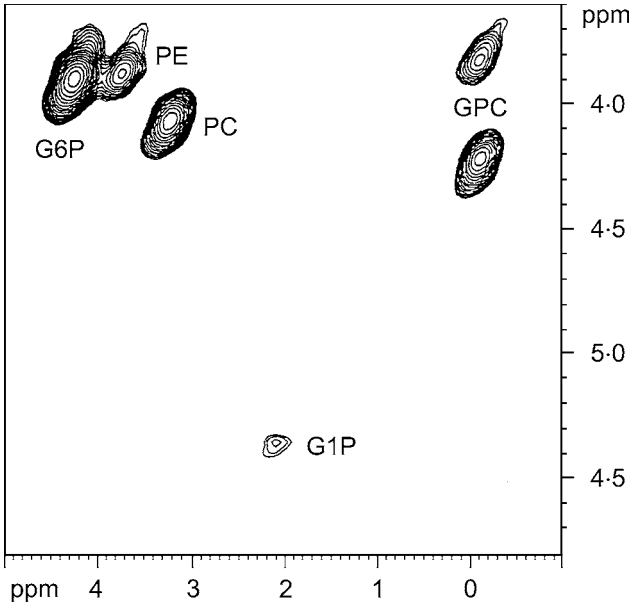

Fig. 3. Correlation between ^{1}H and ^{31}P nuclear magnetic resonance (NMR) signals in a two-dimensional NMR spectrum recorded from a sample of maize root tips *in vivo*. Each set of contours represents a signal from a metabolite in which there is a specific interaction between a phosphorus nucleus and a nearby hydrogen atom in the same molecule. The horizontal and vertical axes correspond to the ^{31}P and ^{1}H NMR frequencies of these two atoms, respectively, and the correlation between the two frequencies separates signals that overlap in the corresponding ^{1}H and ^{31}P one-dimensional spectra, providing a sound basis for the assignment of the spectra. The assignments are as follows: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; PC, phosphocholine; PE, phosphoethanolamine; GPC, glycerophosphocholine. Adapted from Roscher *et al.* (1998) with the permission of the American Society for Biochemistry and Molecular Biology.

(*i*) *Two-dimensional NMR.* Two-dimensional NMR methods are used to increase spectral resolution, to establish connections between signals for metabolite identification and other purposes, and to achieve sensitivity enhancement. The only penalties for the increased information content are a more complicated scheme for generating the spectrum and the need in many cases for longer data acquisition times. However two-dimensional NMR spectra can be run routinely, once the necessary parameters have been determined for the detection scheme to work optimally, and the experiments that are relevant to metabolic analysis are well within the capabilities of modern spectrometers.

Two-dimensional experiments exploit the interactions between the NMR-detectable isotopes in a molecule, and typically the spectra are presented as a contour plot with two frequency axes that correspond to the signals that are correlated in the experiment (Fig. 3). Homonuclear

correlation experiments generate spectra with identical frequency axes corresponding to the observed nucleus, most commonly ^{1}H; whereas heteronuclear correlation experiments produce spectra with two different frequency axes, one corresponding to ^{1}H and the other corresponding to a nucleus such as ^{13}C, ^{15}N or ^{31}P. Homonuclear correlation experiments, such as total correlation spectroscopy (TOCSY), are invaluable for analysing the ^{1}H spectra of complex mixtures, such as root exudates and cell extracts (Fan, 1996), because they allow subsets of peaks in the crowded one-dimensional ^{1}H spectrum to be linked and assigned to specific metabolites. Heteronuclear experiments are similarly informative when the extracted metabolites have been labelled with ^{13}C or ^{15}N. For example the indirect detection techniques, such as heteronuclear multiple quantum coherence (HMQC) spectroscopy, heteronuclear single quantum coherence (HSQC) spectroscopy and heteronuclear multiple bond coherence (HMBC) spectroscopy, improve metabolite identification by providing information on the relationship between the signals from two different nuclei, and they simultaneously increase the detection sensitivity by using the ^{1}H nucleus to report on the presence of ^{13}C or ^{15}N (Fan, 1996; Shachar-Hill *et al.*, 1996; Mesnard *et al.*, 2000). The implementation of metabolic two-dimensional NMR analyses, particularly *in vivo*, is more demanding than the simple one-dimensional experiments that are usually used for metabolite profiling and metabolite fingerprinting. However two-dimensional spectra can often provide a more complete analysis and the application of these informative but underutilized experiments in plant NMR spectroscopy is reviewed elsewhere (Ratcliffe *et al.*, 2001).

(*ii*) *Stable isotope labelling.* Several biologically interesting magnetic isotopes, particularly ^{2}H, ^{13}C and ^{15}N, have a low natural abundance (0.015, 1.1 and 0.37%, respectively), making it difficult to detect the corresponding NMR signals. The main exception to this generalisation occurs when a plant metabolite accumulates to relatively high concentrations, allowing the analysis of site-specific natural isotope fractionation (SNIF) by ^{2}H or ^{13}C NMR after extraction (Martin, Martin & Zhang, 1992) and the detection of natural abundance ^{13}C NMR signals *in vivo*, particularly at very high magnetic fields (Hinse *et al.*, 2003). However supplying a plant tissue with a ^{2}H-, ^{13}C- or ^{15}N-labelled metabolic precursor will generally lead to the redistribution of the label throughout the metabolic network and to the selective enhancement of the NMR signals from a range of metabolites. The extent of this redistribution depends on the nature of the precursor, the duration of the labelling experiment, and the turnover of the endogenous pools of metabolites and macromolecules, thus yielding useful information about the metabolic activity of the tissue. This technique of stable isotope labelling is widely used in NMR and it provides a powerful method for pathway delineation and flux analysis (see Section III) in plant metabolic networks (Roberts, 2000; Ratcliffe & Shachar-Hill, 2001; Kruger, Ratcliffe & Roscher, 2003).

An important aspect of the NMR analysis of ^{13}C-labelling experiments is that quantitative information about the labelling of particular carbon atoms is readily available

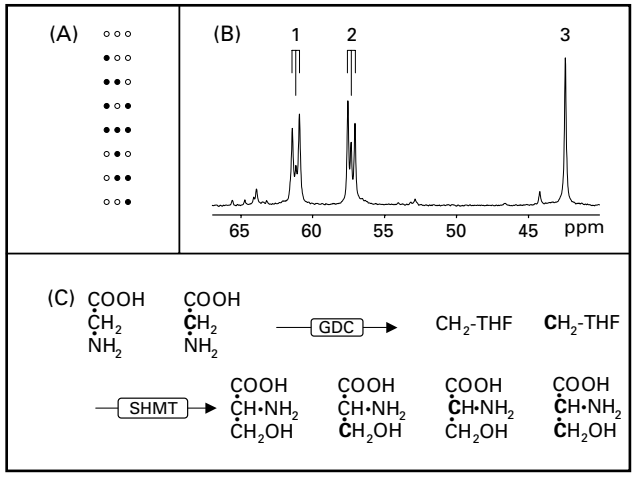

Fig. 4. Nuclear magnetic resonance (NMR) detection of ^{13}C-labelled isotopomers. (A) A labelled three-carbon compound such as serine can exist in a total of eight isotopomers, where ● and ○ represent the NMR-detectable ^{13}C and NMR-undetectable ^{12}C isotopes, respectively. (B) An *in vivo* ^{13}C NMR spectrum of maize root tips after an 18 h incubation with [2-^{13}C]glycine showing the presence of three isotopomers of serine. The assignments are as follows: 1, signals from the C3 position of serine, comprising a central singlet peak from [3-^{13}C]serine, and flanking that a doublet from carbon 3 of [2,3-^{13}C]serine; 2, signals from the C2 position of serine, comprising a central singlet peak from [2-^{13}C]serine, and flanking that a doublet from carbon 2 of [2,3-^{13}C]serine; 3, [2-^{13}C] glycine. Adapted from Hartung & Ratcliffe (2002) with the permission of Oxford University Press. (C) Metabolic scheme showing how four of the possible eight serine isotopomers arise from the combined action of glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT) on a mixture of [2-^{13}C]labelled and unlabelled glycine. GDC generates both labelled and unlabelled 5,10-methylene tetrahydrofolate (CH_2-THF); and then SHMT transfers the labelled or unlabelled methylene group to either labelled or unlabelled glycine.

(Fig. 4). Redistribution of exogenously supplied label will generally lead to a non-uniform distribution of the label in a selected metabolite, and the site-specific fractional enrichments can then be determined directly by a combination of one-dimensional ^{1}H and ^{13}C NMR analysis (for example, Edwards *et al.*, 1998) or by two-dimensional methods (Szyperski, 1995). The measured fractional enrichment of ^{13}C at a particular carbon atom represents the summed contribution of a set of isotopomers, each labelled with ^{13}C at the chosen carbon atom and with either ^{12}C or ^{13}C at all the other carbon atoms (Fig. 4). Thus a three-carbon compound has a total of $2^3 = 8$ isotopomers and importantly many of these can be measured directly by NMR. For example the metabolism of [2-^{13}C]glycine by the combined action of glycine decarboxylase and serine hydroxymethyltransferase yields a mixture of [2-^{13}C]-, [3-^{13}C]- and [2,3-^{13}C]serine and the ^{13}C NMR signals of these isotopomers are readily distinguished (Fig. 4; Prabhu *et al.*, 1996; Mouillon *et al.*, 1999; Hartung & Ratcliffe, 2002).

Stable isotope labelling of metabolic end-products can also be analysed by NMR and a retrobiosynthetic analysis

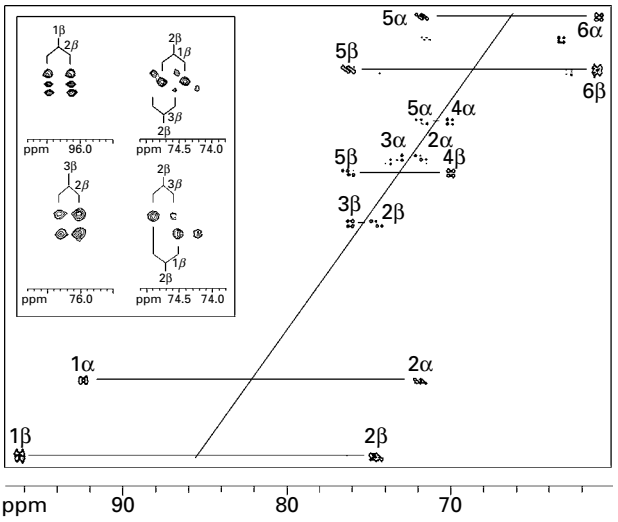

Fig. 5. Nuclear magnetic resonance (NMR) detection of ^{13}C-labelled isotopomers in a two-dimensional spectrum recorded with the INADEQUATE pulse sequence. Maize kernel cultures were grown on a medium supplemented with [U-$^{13}C_6$] glucose and the isotopomeric composition of the glucosyl moieties of extracted starch was determined after hydrolysis to glucose. The horizontal lines in the two-dimensional spectrum link pairs of signals that demonstrate the presence of isotopomers containing pairs of adjacent ^{13}C atoms labelled 1,2-, 2,3-, 4,5- and 5,6-. Since glucose exists in two anomeric forms in solution there are two sets of signals for each carbon atom (designated α and β). Expansion of some of the signals in the inset reveals fine structure that demonstrates the presence of both [1,2-^{13}C]glucose and [1,2,3-^{13}C]glucose. Adapted from Glawischnig *et al.* (2002) with the permission of the American Society of Plant Biologists.

can then be used to deduce the pathways and fluxes that led to the observed labelling (Bacher *et al.*, 1999). The analysis of the isotopomers of amino acids derived from labelled protein is particularly important, with applications in the retrobiosynthetic approach to pathway delineation (Glawischnig *et al.*, 2001) and metabolic flux analysis (Szyperski, 1995, 1998). Other metabolic end-products can be analysed in a similar way and the steady-state labelling of starch in developing maize kernels supplied with uniformly labelled glucose, sucrose or acetate has been analysed in detail (Glawischnig *et al.*, 2002). The latter study made extensive use of two-dimensional NMR techniques to identify the multiply labelled isotopomers of the glucose units present in starch (Fig. 5).

III. METABOLITE INTERCONVERSIONS AND FLUXES

As well as providing a method for identifying and quantifying plant metabolites, NMR can also be used to investigate the pathways that link them and the fluxes that occur between different parts of the network. These applications can be divided into those based on kinetic experiments in which the intensities of the metabolite signals are monitored over an extended time course; and those based on an analysis of the network in a metabolic and physiological steady state. Time-course experiments can be further divided into those using non-labelled precursors and those using metabolic substrates labelled with NMR-detectable stable isotopes.

(1) Time-course experiments

(*a*) *Non-labelled substrates*

Changes in metabolite levels brought about by changes in substrate supply, or a change in the physiological conditions under which the tissue or cell suspension is maintained, are readily detected using *in vivo* ^{1}H, ^{31}P, and to a lesser extent ^{13}C, NMR spectroscopy. Thus, in a typical *in vivo* plant NMR experiment the tissue or cell suspension is incubated with an exogenous substrate, and the uptake and metabolism of the substrate is monitored over a period of several hours or longer. The *in vivo* measurements are often complemented by *in vitro* NMR spectra recorded from extracts and the combined data can be used to probe the pathways by which the substrate is utilized. This approach, using ^{13}C and ^{31}P NMR, has been used extensively to analyse the metabolism of *Acer pseudoplatanus* cell suspensions and representative applications include investigations of glycerol (Aubert *et al.*, 1994), homoserine (Aubert *et al.*, 1998) and methanol (Gout *et al.*, 2000) metabolism.

Many of these studies are essentially qualitative, but in principle the spectroscopic data on the concentrations of intermediates and end-products could be used as inputs for a quantitative kinetic model of the time course of the metabolic response. This approach is not commonly used in the NMR analysis of plant metabolism, but an example is provided by an investigation of the factors limiting glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase (McNeil *et al.*, 2000). As part of this study, tobacco leaves were incubated with choline, and *in vivo* ^{31}P NMR was used to demonstrate that the rate of phosphocholine synthesis and its location in the cytosol were consistent with a detailed metabolic model of the system that had been developed on the basis of radiolabelling experiments with ^{14}C- and ^{33}P-labelled precursors.

(*b*) *Labelled substrates*

As discussed in Section II.2*b*(*ii*), NMR provides a convenient method for detecting and quantifying the redistribution of stable magnetic isotopes of low natural abundance in labelling experiments. This approach is used extensively for *in vivo* investigations of the pathways of primary and secondary plant metabolism (Roberts, 2000; Ratcliffe & Shachar-Hill, 2001) and it is largely based on the use of ^{13}C- and ^{15}N-labelled substrates. Taking the pathways of nitrogen metabolism as an example, ^{15}N-labelling is usually achieved through the provision of labelled nitrate or ammonium, although other substrates have been used, including ^{15}N-labelled amino acids (Hartung & Ratcliffe, 2002) and ^{15}N-labelled N_2 (Scharff *et al.*, 2003). These ^{15}N-labelled substrates provide a route into both primary metabolism, as

exemplified by studies of ammonium assimilation (Robinson *et al.*, 1991) and γ-aminobutyric acid (GABA) synthesis (Carroll *et al.*, 1994) in carrot cells, and secondary metabolism, as exemplified by investigations of nitrogen metabolism in hairy root cultures (Ford *et al.*, 1994; Ford, Ratcliffe & Robins, 1998).

As with the analogous experiments using non-labelled substrates, the emphasis in these time-course experiments is usually on a qualitative, or at best semi-quantitative, exploration of the metabolic network. By contrast, the data from similar investigations on micro-organisms and some animal tissues (Jucker, Lee & Shulman, 1998; Mason & Rothman, 2004) have been used to develop kinetic models of the redistribution of the label in the metabolic network and this leads to the definition of a number of intracellular fluxes. A good example of this procedure can be found in an analysis of the pathways of nitrogen assimilation in *Corynebacterium glutamicum* (Tesch, de Graaf & Sahm, 1999). While kinetic models are certainly feasible in plants (Morgan & Rhodes, 2002), the greater complexity of the network, particularly the need to discriminate between subcellular pools of the same metabolite, makes it difficult to extrapolate directly from the successful modelling of microbial metabolism. Moreover successful kinetic modelling depends on high-quality NMR data with good time resolution, which may be harder to achieve with plants than micro-organisms, and even then it may be difficult to obtain sufficient information to allow the accurate definition of fast steps that follow slow steps in the labelling pathway. By contrast, the analysis of steady-state labelling experiments in plant tissues (Kruger *et al.*, 2003) is both more straightforward and more informative, and currently this is the preferred approach for measuring intracellular metabolic fluxes from NMR data (Section III.2).

A further useful feature of the *in vivo* NMR spectra recorded during time-course experiments with either labelled or non-labelled substrates is that it is sometimes possible to distinguish specific pools of a metabolite directly. This capability hinges on the sensitivity of the NMR signals to differences in the local environments experienced by the metabolite (Ratcliffe & Shachar-Hill, 2001; Ratcliffe *et al.*, 2001) and in favourable cases this can lead to the detection of separate signals from the internal and external pools of the substrate, or the detection of separate signals from metabolites that are distributed between the cytoplasm and the vacuole (Fig. 2). Observations of this kind can provide a direct method for quantifying certain transport steps, for example the uptake rate of the substrate. NMR analysis of the metabolic redistribution of stable isotope labels can also shed light on the preferred pathway of certain intracellular transport steps, as in the demonstration by ^{13}C NMR of the importance of hexose phosphate uptake for the synthesis of starch in amyloplasts (Keeling *et al.*, 1988) and the demonstration by ^{2}H NMR of the export of carbon from chloroplasts in the dark in the form of hexoses rather than trioses (Schleucher, Vanderveer & Sharkey, 1998). In fact it now appears that it is maltose rather than glucose that is the principal exported carbohydrate from chloroplasts under these conditions (Weise, Weber & Sharkey, 2003), but the NMR demonstration of the insignificance of triose export in the dark remains an important observation. Quantitation of the unidirectional fluxes through certain intracellular transport steps also arises directly in the analysis of steady-state stable isotope labelling experiments (Section III.2).

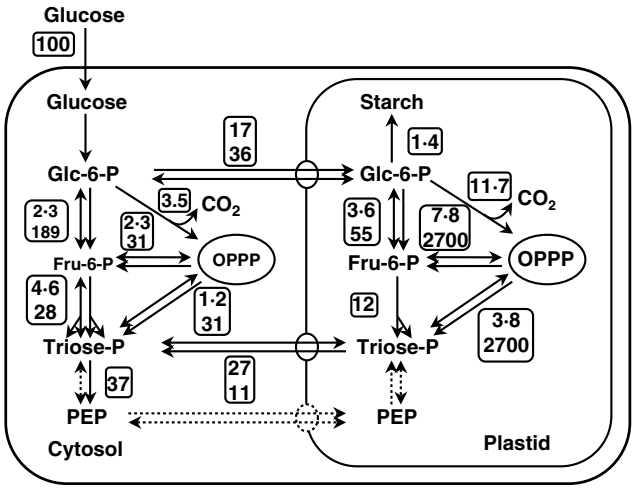

Fig. 6. Metabolic fluxes determined in maize root tips from a steady-state [2-^{13}C]glucose labelling experiment on maize root tips. Analysis of the redistribution of label leads to the definition of multiple fluxes, each expressed relative to a glucose uptake rate of 100. For the reversible reactions, the upper number is the net rate and the lower number is the exchange flux. The fluxes are mapped on to the same fragment of the metabolic network shown in Fig. 1 and because the network is incomplete the influx of glucose does not equal the efflux of label from the cytosolic glucose 6-phosphate pool. The dotted lines indicate that there is insufficient information to determine every flux in the network. Based on a provisional analysis of unpublished data by N. J. Kruger, R. G. Ratcliffe and A. Roscher.

A final important point about *in vivo* NMR time-course experiments is that it is often possible to make a detailed investigation of the response of the metabolic network to a specific perturbation, for example a change in the physiological conditions imposed on the tissue in the NMR magnet. This is a standard procedure in NMR studies of plant metabolism (Ratcliffe & Shachar-Hill, 2001; Ratcliffe *et al.*, 2001) and a good example is provided by the impact of anoxia on the pathways of intermediary metabolism (Roberts *et al.*, 1992).

(2) Steady-state experiments

(*a*) *Labelled substrates – principles of steady-state analysis*

In a steady-state NMR labelling experiment, the plant tissue or cell suspension is incubated with a ^{13}C-labelled substrate and the redistribution of the label is analysed by a combination of ^{1}H and ^{13}C NMR methods after the system has reached an isotopic steady state. The redistribution of the label reflects the underlying flow of material through the metabolic network, and the data on the fractional enrichments and isotopomer populations of the labelled metabolites can be used to construct a flux map of the network (Fig. 6). This approach to metabolic flux analysis was

developed in studies of micro-organisms and its utility is increased by the fact that the analysis can be based on complementary data inputs from both NMR and mass spectrometry (Szyperski, 1998; Wiechert, 2001; Wiechert *et al.*, 2001). Several features of the plant metabolic network complicate the extension of the steady-state stable isotope labelling method to plants (Roscher, Kruger & Ratcliffe, 2000) but the suitability of the method for analysing heterotrophic plant metabolism has been established in a number of studies that have been reviewed in detail elsewhere (Kruger *et al.*, 2003).

For the steady-state labelling experiment to be informative, the observed labelling must be determined by competing fluxes from substrates that contain both labelled and unlabelled components. In practice this means that the usual precursor for a steady-state labelling experiment is either a substrate that is labelled in some but not all carbon atoms, for example [1-^{13}C]glucose, [1,2-$^{13}C_2$]glucose or [2-^{13}C] glycerol, or a mixture of a uniformly labelled substrate with two or more carbon atoms and its unlabelled counterpart. It should be noted that while there has been considerable success in micro-organisms with a strategy based on a single labelling experiment with a diluted mixture of uniformly labelled glucose (Szyperski, 1998), the complexity of the plant metabolic network probably requires multiple labelling experiments if the intention is to define the fluxes through the central pathways of metabolism with comparable accuracy across the network (Schwender, Ohlrogge & Shachar-Hill, 2003).

After labelling, the targets for analysis can be any component of the system that is likely to report on the pathways of interest. Thus some flux analyses of plant metabolism have been based on the analysis of a small number of easily extractable metabolites and intermediates (Edwards *et al.*, 1998; Fernie *et al.*, 2001), while others have been based on the analysis of end-products of metabolism with slower turnover rates, such as natural products (Eichinger *et al.*, 1999) and storage lipids (Schwender *et al.*, 2003). Proteins, carbohydrates and nucleic acids can also be analysed after hydrolysis into their monomeric constituents. The isotopomeric analysis of the amino acids derived from labelled protein forms the basis of a powerful method for flux analysis in micro-organisms (Szyperski, 1998) and the first applications of this approach are beginning to appear in plant tissues (Glawischnig *et al.*, 2001; Schwender *et al.*, 2003). The importance of analysing the label distribution in hydrolysed starch was recognised rather earlier (Keeling *et al.*, 1988) and this is a routine feature of the procedures that have been developed to map the central pathways of plant metabolism (Dieuaide-Noubhani *et al.*, 1995; Rontein *et al.*, 2002).

Irrespective of the procedures for labelling and isotopomeric analysis, these experiments only give a valid flux map if the extract is prepared from a system that is in an isotopic and metabolic steady state. In other words all fluxes are constant and the distribution of the label among the analysed components is unchanging. The requirement for a metabolic steady state is difficult to satisfy in a growing system, but it can be relaxed to encompass a metabolic state that is changing sufficiently slowly to allow the maintenance of the corresponding isotopic steady state through turnover of the metabolic intermediates (Roscher *et al.*, 2000). The situation is further complicated by the fact that almost any plant system will be composed of a mixture of cells in different metabolic states, but this is an almost universal problem in analytical biochemistry and in the absence of technological solutions it is only possible to bear the problem in mind when interpreting the flux maps and using them as the basis for further investigation. The requirement for an isotopic steady state is easier to evaluate, and in general the faster the turnover of the labelled component the better the match with the prevailing metabolic state. Thus analyses based on the direct detection of low molecular weight metabolic intermediates have an advantage over those that depend on analysing macromolecules with low turnover, since for the latter it is more likely that the measured isotopic distribution will reflect the metabolic history of the sample over the labelling period rather than the actual metabolic state at the time of extraction.

(*b*) *Labelled substrates – applications to plants*

In its most advanced applications steady-state stable isotope labelling generates relatively large-scale flux maps in which forward and reverse fluxes are defined at multiple steps in the metabolic network (Fig. 6). To date, such maps have only been generated for a handful of plant systems, principally maize root tips (Dieuaide-Noubhani *et al.*, 1995), tomato cell suspensions (Rontein *et al.*, 2002) and cultured oilseed rape embryos (Schwender *et al.*, 2003), but several common pathway features are emerging.

First, the maps highlight the occurrence of substrate cycles in the metabolic network. The functional significance of these cycles is uncertain and the problem is only likely to be resolved through the analysis of the flux maps for a given genotype under a range of conditions or for genotypes with altered expression levels of the participating enzymes. Notable examples of these substrate cycles include: a cycle for the synthesis and degradation of sucrose, which has been estimated to consume 70 % of the available ATP in excised maize root tips (Dieuaide-Noubhani *et al.*, 1995) and 60 % in tomato suspension cells during exponential growth (Rontein *et al.*, 2002); and the commonly observed cycling between triose phosphate and hexose phosphate in the cytosol. In one NMR analysis of the latter cycle in transgenic tobacco lines, it was shown that the flux was sensitive to the endogenous level of fructose 2,6-bisphosphate, thus proving that pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFP), the enzyme responsible for the interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate is sensitive to physiologically relevant fluctuations of its effector *in vivo* (Fernie *et al.*, 2001).

Secondly the maps provide quantitative information on the transporters that link the principal metabolically active compartments in the plant cell. As indicated above (Section III.1*b*) labelling experiments have sometimes provided direct evidence for the identity of metabolites that cross the plastid envelope (Keeling *et al.*, 1988; Schleucher *et al.*, 1998), and the quantitative information on such processes in the flux maps of heterotrophic tissues represents a significant

Table 1. Metabolites and end-products that provide information on the subcellular compartmentation of plant metabolism in steady-state labelling experiments

Metabolite/end-product	Compartments	Key intermediates	References
Amino acids			1–3
alanine	cytosol	pyruvate	
leucine/valine	plastid	pyruvate	
phenylalanine/tyrosine	plastid	PEP/erythrose 4- phosphate	
aspartate	cytosol/mitochondrion	Phosphate	
glutamate/proline	cytosol/mitochondrion	2-oxoglutarate	
Carbohydrates			1, 4–7
starch	plastid	glucose 6-phosphate (G6P)	
sucrose	cytosol	G6P/fructose 6-phosphate	
Fatty acids			1
short/medium chain	plastid	acetyl CoA	
long chain	cytosol	acetyl CoA	

Analysis of the steady-state labelling of metabolites and end-products that are synthesised in specific subcellular compartments provides information on the labelling of the corresponding pools of key intermediates. This is the basis on which steady-state labelling experiments can reveal the subcellular compartmentation of the metabolic network. References: 1, Schwender *et al.*, 2003; 2, Edwards *et al.*, 1998; 3, Glawischnig *et al.*, 2001; 4, Keeling *et al.*, 1988; 5, Dieuaide-Noubhani *et al.*, 1995; 6, Fernie *et al.*, 2001; 7, Glawischnig *et al.*, 2002.

contribution to the characterisation of the elusive properties of organellar transporters *in vivo*. One interesting observation is that the plastid exchange processes in oilseed rape embryos are so fast that the cytosolic and plastidic hexose phosphate and triose phosphate pools are kinetically indistinguishable (Schwender *et al.*, 2003), indicating that the subcellular compartmentation of the pathways of carbohydrate oxidation in this system has less functional significance than would be thought from the known distribution of the relevant enzymes. By contrast the plastid exchange processes in other systems are less rapid and the flux map shows distinguishable fluxes through parallel pathways in the two compartments (Dieuaide-Noubhani *et al.*, 1995; Rontein *et al.*, 2002).

This ability to reveal the subcellular compartmentation of the metabolic network is the third interesting feature of the flux maps that have been published so far. For this to be possible it is necessary: (i) to be able to analyse the steady-state labelling of metabolites and end-products that are synthesised uniquely in one compartment (Table 1); and (ii) that none of the precursor intermediates should be in such fast exchange between the compartments that their subcellular pools become kinetically indistinguishable. Fortunately the list of potential metabolites for analysis is quite extensive and this approach to subcellular metabolism is a very promising aspect of the steady-state labelling technique.

While the flux maps generated by steady-state stable isotope labelling offer great promise for defining functionally useful metabolic phenotypes, it is also important to urge caution in their interpretation at this stage in their development. First, the procedure that leads to the calculated flux maps is a constraints-based procedure that depends on defining a stoichiometric map of the metabolic network that is believed to be present. Any errors in this network, or omissions arising from ignorance of the true network, will be carried over into the flux map and it may not be apparent from the fitting process that the defined network is erroneous (van Winden, Verheijen & Heijnen, 2001). Secondly, it is not immediately obvious which features of a flux map are likely to be of most interest in understanding the relationship between genotype and network performance. There is clearly a wealth of information in the large-scale flux maps, but only further experience will reveal their true value as a tool for phenotypic characterisation.

(*c*) *Magnetisation transfer*

Although steady-state metabolic flux analysis usually depends on the introduction of an isotopic label into the metabolic network, it is also possible in certain circumstances to deduce such fluxes without isotopic labelling using the NMR technique of magnetisation transfer (Brindle, 1988). In this experiment specific atoms within a metabolite are magnetically labelled during the NMR experiment, and the label is then transferred to a second metabolite through an enzyme-catalysed exchange process. This leads to changes in the NMR spectrum that can be used to calculate unidirectional exchange rates. The magnetic label is short lived (a time scale of seconds only) and the experiment is mostly useful for measuring the flux between certain phosphorylated metabolites. Full details of the applications of this *in vivo* method to plants are given elsewhere (Ratcliffe *et al.*, 2001), but in practice it is rarely used. Indeed there has been only one significant application of the method to plant metabolism in recent years – a study of the impact of temperature on the rates of ATP synthesis and hydrolysis, on the unidirectional fluxes between 3-phosphoglycerate and phospho-enolpyruvate, and on the rates of the reactions catalysed by UDP-glucose pyrophosphorylase, phosphoglucomutase and hexose phosphate isomerase in maize root tips (Roscher *et al.*, 1998). Ultimately the main limitations of

the magnetisation transfer experiment are the requirement for the measured rate constant to be comparable with the short lifetime of the magnetic label and the need for long acquisition times to generate data suitable for quantitative analysis (Roscher *et al.*, 1998). Thus this method of flux analysis lacks the generality of the steady-state stable isotope labelling approach and its application to metabolism is confined to a handful of specific exchange processes.

IV. UNDERSTANDING METABOLIC COMPLEXITY

Traditionally metabolic data have been used to provide insights into the operation of pathways by framing specific questions about segments of the network. Often the intention has been to provide an explanation for the observed response to a physiological or genetic perturbation, but as discussed elsewhere in relation to photosynthetic carbon metabolism (Kruger & Ratcliffe, 2004), the application of this strategy in transgenic plants has uncovered a functionally complex network of reactions and metabolic crosstalk in which metabolic perturbations can be propagated to remote corners of the network. Unfortunately the complexity of the system and the increasing volume of pertinent data are making it increasingly difficult to comprehend these effects, and so metabolic analysis, whether by NMR or other methods, will be increasingly dependent on pattern-recognition techniques that allow the comparison of large datasets and on modelling.

This trend is immediately apparent in metabolomics, where data reduction, based on the use of clustering techniques such as principal components analysis and hierarchical cluster analysis, is used extensively (Fiehn *et al.*, 2000; Raamsdonk *et al.*, 2001; Roessner *et al.*, 2001*a*; Sumner *et al.*, 2003). In fact data reduction is now an essential tool for the comparison and classification of metabolic datasets, allowing the definition of a metabolic phenotype based on the metabolome (Fiehn *et al.*, 2000; Fiehn, 2002). Metabolic flux analysis also depends on large datasets, either in the form of unlabelled/labelled metabolite time courses for kinetic analysis, or as sets of fractional enrichments and isotopomeric compositions for steady-state analysis. These datasets are used to produce kinetic models of the redistribution of the label or steady-state flux maps of the metabolic network. The kinetic models can be developed further to include information on the kinetic properties of the enzymes catalysing the observed flux (McNeil *et al.*, 2000; http://www.hort.purdue.edu/cfpesp/models/models.htm), and both kinetic models and steady-state flux maps provide immediate insights into the operation of the network.

Ultimately the aim of metabolic analysis must be to provide an efficient predictive tool for understanding the performance of the metabolic network (Sweetlove *et al.*, 2003; Kruger & Ratcliffe, 2004). This has yet to be achieved in plant metabolism, and so the following sections discuss the phenotypic value of the current approaches to metabolite detection and flux analysis, before concluding with some comments on genome-scale network analysis.

(1) Metabolomics

The development of metabolomics as an arm of functional genomics has been reviewed (Sumner *et al.*, 2003; Weckwerth, 2003) and its current prominence is based on two considerations. On the one hand is the idea that the metabolome, as arguably the ultimate biochemical expression of the genome, should be able to serve as a metabolic phenotype that might shed light on gene function (Fiehn *et al.*, 2000; Fiehn, 2002). On the other hand is the realisation that there are limitations to the extent to which gene expression analysis and proteomics can be expected to give insights into metabolic capability and performance (Buckhout & Thimm, 2003). Thus there appears to be no necessary correlation between the transcriptome and network performance (ter Kuile & Westerhoff, 2001); and protein levels are only an approximate guide to the activities of enzymes whose levels may in any case exercise little control over the distribution of flux in a pathway (Fell, 1997). These arguments have led to the rapid acceptance of metabolite profiling and metabolite fingerprinting as valid experimental approaches (Raamsdonk *et al.*, 2001; Sumner *et al.*, 2003) and in the light of this it is interesting to consider the relevance of these approaches to the problem of understanding the complexity of plant metabolic networks.

The most common use for metabolomics is in distinguishing plants of different genotypes, or plants subjected to different treatments, on the basis of a statistical analysis of the metabolome. This application is well established (Lommen *et al.*, 1998; Fiehn *et al.*, 2000; Noteborn *et al.*, 2000; Roessner *et al.*, 2001*a*; Roessner, Willmitzer & Fernie, 2001*b*) but does not in itself give much insight into the functional complexity of the plant metabolic network. Indeed as stated elsewhere while 'the first steps have been made to generate biological hypotheses from metabolomic datasets … such steps must be extended by better use of statistics to gain significant, rather than clustered, information' (Weckwerth & Fiehn, 2002).

A more functional application of metabolomic data is to be found in the technique known as functional analysis by co-responses in yeast (FANCY; Raamsdonk *et al.*, 2001). This method uses metabolite data to calculate metabolite co-responses (essentially the change in the ratio of a pair of metabolite NMR signal intensities in response to a perturbation) and then compares the results for a mutant of a gene with unknown function with mutants of genes with known functions. On the basis of this comparison it is then possible to propose a function for the gene on the assumption that genes with similar functions will produce similar metabolite co-responses. While FANCY does not address metabolic complexity directly, and appears not to have been applied to plants so far, it has the potential to identify genes that will ultimately need to be included in genome-based models of metabolism.

Another route to functional information may be to use metabolite profiling data in pairwise transcript – metabolite correlation analyses (Urbanczyk-Wochniak *et al.*, 2003). This method has been applied to potato tubers, revealing strong correlations between the RNA profile and certain nutritionally important metabolites, and it has been

proposed that this method could be useful in identifying candidate genes for the rational modification of the composition of the tuber.

It has also been proposed (Weckwerth & Fiehn, 2002) that it might be possible to discover metabolic pathways using a method based on pair-wise metabolite correlation plots (Arkin, Shen & Ross, 1997; Samoilov, Arkin & Ross, 2001). This method would be based on a series of metabolomic snapshots after a perturbation of the system followed by correlation of the changes in pairs of metabolites to elucidate their relative positions in the network. The practicality of the method has yet to be demonstrated in a plant system, and the existence of parallel pathways and separate pools of the same metabolite in different subcellular compartments may cause insuperable difficulties.

(2) Metabolic flux analysis and metabolic phenotypes

In contrast to the metabolomic approach, flux analysis generates quantities that are directly related to the performance of the plant metabolic network. In particular it has been established that the analysis of steady-state ^{13}C-labelling experiments is an effective method for determining the flux distribution through the compartmented pathways of primary metabolism in plant cells. Although restricted to the analysis of heterotrophic metabolism, because the substrate for photosynthesis contains only one carbon atom (Roscher *et al.*, 2000), steady-state analysis is a powerful alternative to the kinetic analysis of labelling time courses (Morgan & Rhodes, 2002), provided that the experimental system satisfies, or is close to (Hellerstein & Neese, 1999), the fundamental requirement for an isotopic and metabolic steady-state prior to analysis. The kinetic analysis of complex networks is more difficult, but there is scope for combining the two approaches by using time-course experiments to provide complementary flux information for intracellular steps on the periphery of the network. This combined approach has apparently not been implemented in plants so far, but it could further improve the definition of the fluxes in large-scale maps.

The conclusion from the large-scale mapping of microbial fluxes is that the technique provides a quantitative tool for assessing the metabolic impacts of physiological and environmental change, as well as a method for comparing the metabolic performance of different genotypes (de Graaf *et al.*, 1999; Marx *et al.*, 1999; Sauer *et al.*, 1999). The expectation is that the flux maps obtained for plants will be similarly informative, providing insights into network robustness and highlighting potential targets for metabolic engineering (Kruger & Ratcliffe, 2004). In fact at this early stage in the application of large-scale flux mapping to plants, no system has been analysed under a sufficiently wide range of experimental conditions to demonstrate the full utility of the approach. However there has been one study in which flux maps were determined for a cell suspension at several points in the culture cycle and it was possible to show that the relative fluxes through glycolysis, the tricarboxylic acid cycle and the pentose phosphate pathway were unaffected by progression through the cycle, whereas the anabolic fluxes were more variable (Rontein *et al.*, 2002). Small-scale studies of fluxes through segments of the network have also shown responses to physiological (Edwards *et al.*, 1998) and genetic (Fernie *et al.*, 2001) changes so it seems likely that further insights into the performance of the plant metabolic network will follow as the number of large-scale flux maps increases.

Metabolic flux analysis provides a direct read-out of network performance, and large-scale flux maps would appear to be a useful manifestation of the metabolic phenotype of a plant. It has been suggested elsewhere that the metabolite profile itself could serve as a metabolic (Roessner *et al.*, 2001*a*, *b*) or biochemical (Roessner, Willmitzer & Fernie, 2002) phenotype; while in another view it has been suggested that constraints-based modelling, with inputs from genomic, transcriptomic and ultimately metabolomic datasets, might provide a good basis for defining the metabolic phenotype (Palsson, 2002). However neither of these alternative definitions of the phenotype addresses the primary function of the network in supporting the metabolite fluxes that sustain the organism directly, and so a definition in terms of the fluxes themselves may be more appropriate. It could be argued that the robustness of the network (Stelling *et al.*, 2002) might limit the usefulness of the fluxes as a phenotyping tool and that the flux distribution will be relatively constant in comparison with large fluctuations in the metabolome (Raamsdonk *et al.*, 2001). However it is clear that plant cells do support markedly different flux distributions under different environmental and developmental conditions; while at the same time it has been found that the metabolic profile can vary substantially in identical plants grown under identical conditions (Noteborn *et al.*, 2000; Roessner *et al.*, 2000; Sumner *et al.*, 2003). So on this basis it can be concluded that flux maps provide a functionally relevant and practically achievable phenotyping tool.

(3) Genome-scale modelling

In parallel with metabolite profiling and flux mapping, genomic constraints-based models are being developed with the same objective of providing a framework for understanding the functional complexity of metabolic networks (Palsson, 2002; Förster *et al.*, 2003; Reed *et al.*, 2003). In their present form these modelling approaches do not require analytical data on metabolites or their interconversions, but instead rely on genomic, and increasingly, transcriptomic information to reconstruct the network. This is an intriguing approach, with the great advantage that it may be capable of generating fully predictive models without the detailed information on molecular interactions and kinetic mechanisms that is required for a mechanistic model of the network (Price *et al.*, 2003). A further advantage is the potential to reconstruct much larger metabolic networks than can be tackled by kinetic and steady-state modelling approaches without a large number of labelling experiments.

The first step is to build a complete genome-derived description of the architecture of the metabolic network, and then to use physical and chemical constraints, such as conservation laws and stoichiometry, to define the allowable flux distributions. Two types of prediction are possible with

such genome-based models. First, optimum modes of operation for desired outcomes, for example maximal growth or the synthesis of a particular metabolite, can be defined for different experimental conditions, for example growth on alternative carbon sources or with limited oxygen. Secondly, the impact of altering the network architecture by genetic or other interventions can be evaluated. Genome-based modelling has been largely developed in microbial systems and a network that contains over 900 metabolic reactions has been constructed and constrained for *E. coli* (Reed *et al.*, 2003). Earlier versions of this model were used to make successful predictions about the annotation of the genome and about the outcome of a range of known metabolic gene deletions. Currently these models are being extended to include information on gene expression as a further constraint on how the network may be functioning, and it is envisaged that metabolite levels will provide sufficient further constraints to arrive at a functional representation of the cell (Palsson, 2002). In fact it has already been demonstrated that network analysis can lead to physiologically sensible conclusions about red blood cell metabolism (Wiback & Palsson, 2002), so it would seem likely that a genome-based predictive model of the relatively simple metabolic networks in micro-organisms could be within reach.

Attempts are also being made to infer qualitative metabolic patterns from transcript data alone (Ruuska *et al.*, 2002). Analysis of the expression patterns for more than 3500 *Arabidopsis thaliana* genes during the switch from starch to oil and protein accumulation in developing seeds revealed that the expression of many genes coincided with the changes in metabolism. On this basis it was possible to infer the shift from starch synthesis and cytosolic metabolism of phosphenolpyruvate (PEP), to oil production and the plastid uptake and metabolism of PEP, from the patterns of gene expression, allowing the qualitative definition of a time-dependent flux network (Ruuska *et al.*, 2002). At the same time it was observed that all the genes for a pathway were not necessarily co-regulated and that there was surprisingly little change in gene expression in a mutant with a metabolic phenotype that resulted in greatly reduced accumulation of oil. Thus transcript analysis is informative, but as discussed elsewhere (Buckhout & Thimm, 2003), it is probably incapable of providing a sufficiently complete description of network performance on its own.

V. CONCLUSIONS

(1) The complexity of the plant metabolic network is a major obstacle to understanding and predicting its performance. Metabolic analysis appears to be the key to developing predictive tools, but with the emphasis on measurements that allow the detection of multiple metabolites and fluxes rather than the characterisation of individual steps in isolation.

(2) NMR permits the detection of many plant metabolites simultaneously, *in vitro* and *in vivo*, and it can be used in metabolite profiling and metabolite fingerprinting applications. Mass spectrometry is currently used more extensively for this purpose than NMR, but the lower sensitivity of NMR is offset by the speed and convenience of the analysis, and by the information it provides on compound identification.

(3) NMR analysis of stable isotope labelling experiments provides a powerful approach for the measurement of metabolic fluxes. Steady-state analysis is particularly informative and leads to the generation of flux maps for heterotrophic central carbon metabolism, including discrimination between cytosolic and plastidic fluxes. NMR and mass spectrometry provide complementary information for these analyses and the flux maps are sensitive to various factors, including genotype and the conditions imposed on the organism during the labelling experiment.

(4) Metabolic phenotypes can be defined in terms of metabolic composition or in terms of the fluxes supported by the metabolic network. Both approaches can be informative about gene function, but flux maps have the advantage that they address the primary function of the metabolic network, providing an incentive for further applications of such maps in plants.

(5) The construction of metabolic networks on the basis of annotated genomes and transcript analysis has developed into a powerful tool for the *in silico* analysis of microbial metabolism. However it has not yet been applied to plant metabolism, and potential difficulties are the need to include parallel pathways operating in subcellular compartments and the incomplete annotation of plant genomes, particularly for the genes of the pathways of secondary metabolism.

VI. ACKNOWLEDGEMENTS

Our research is supported by the Biotechnology and Biological Sciences Research Council (R.G.R.) and by the National Science Foundation and the United States Department of Agriculture (Y.S.H.). We thank N.J. Kruger (University of Oxford) and J. B. Ohlrogge (Michigan State University) for a critical reading of the manuscript and stimulating discussions.

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