NMR and plant metabolism
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Recent advances in NMR methodology offer a way to acquire a comprehensive profile of a wide range of metabolites from various plant tissues or cells. NMR is a powerful approach for plant metabolite profiling and provides a capacity for the dynamic exploration of plant metabolism that is virtually unmatched by any other analytical technique.

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Abbreviation
NMR nuclear magnetic resonance

Introduction
Nuclear magnetic resonance (NMR) spectroscopy can be used to analyse the composition of tissues both in vivo and in various extracts. NMR has its origin in the net magnetic moment or spin of an atomic nucleus that has an odd atomic mass and/or an odd atomic number [1]. Common nuclei that exhibit such magnetic properties are the highly abundant isotopes 1H (99.98% in nature) and 31P (100% in nature) and 15N (0.37% in nature). The widespread use of NMR to analyse plant metabolism and its compartmentation has been reviewed recently [1,2,3••]. We have chosen to focus on restricted recent examples to convince the reader that NMR is a powerful approach for plant metabolite profiling [1••].

NMR spectra
NMR signals (i.e. resonance) are observed when a sample is irradiated with pulses of radiofrequency electromagnetic radiation in a strong magnetic field. Each nucleus within a molecule experiences a slightly different magnetic field because of its distinct chemical environment and absorbs energy at a slightly different frequency. The separation of these resonance frequencies from an arbitrarily chosen reference is called the chemical shift (δ), which is calculated on the basis of the relationship:

\[ \delta = \frac{(\text{resonance frequency of the sample } [\text{vs}] - \text{resonance frequency of the reference } [\text{vr}])}{(\text{vr}.10^6)} \]

δ, which is independent of the field strength of the magnet, is a unitless number that is expressed in parts per million (ppm). The result of an NMR analysis of a tissue is a spectrum, that is, a plot of intensity (area) against chemical shift in which each signal occurs at a characteristic energy (Figure 1). NMR experiments using 13C-labelled compounds to decipher a metabolic pathway are usually hampered by the low sensitivity of the 13C nucleus (which is four-fold lower than that of the 31P nucleus). This problem can be overcome by using the cyclic j-cross polarisation technique, which allows the indirect detection of 13C nuclei coupled to 1H nuclei by exploiting the high NMR sensitivity of protons [5]. The signal provided by the sensitive protons theoretically increases the signal/noise ratio by a factor of 64 over that of the 13C signal. It is also possible to enhance the 13C-signal using the nuclear Overhauser effect (i.e. a change in the intensity of a spectroscopic signal caused by the irradiation of another nucleus during the NMR experiment) [6•].

NMR signal intensities are important analytically because at a given pulse sequence (signals are elicited by pulses of radiofrequency irradiation) they can be related to the content within the tissue of the molecules that produce the signals. On the other hand, the chemical-shift effect ensures that many metabolites, such as glucose, glucose-6-P and ATP, can be identified on the basis of their characteristic NMR signal patterns observed either in vivo (in a non-invasive way) or in vitro (in a perchloric extract). Moreover, the strength of NMR for tackling metabolism lies in the fact that isotopic labels in different positions of different compounds can be simultaneously measured.

In vivo NMR methods are frequently used to provide information on the absolute concentrations of the more abundant metabolites, including sucrose, glucose-6-P and inorganic phosphate, and on how these concentrations change during biochemical transformation. These methods eliminate the need for extractions and sample preparation procedures. Several devices exist that are used to maintain a living system in a physiologically viable and controllable state [1,2,3••]. These include the use of small leaf pieces that are infiltrated with perfusion medium, cell suspensions at high cell density and root tips. These systems entail maintaining the supply of oxygen and various nutrients, including sugars, and the removal of waste products such as ethanol. By modifying the composition of the circulating medium (e.g. by introducing phosphate or amino acids, sucrose starvation or removing oxygen) it is possible to perturb the metabolism of the living system and to monitor the spectral changes caused by these changes simultaneously, thereby obtaining several successive spectra from the same sample. For example, aerobic and anaerobic conditions could be alternated at intervals of as short as 2 min. Examination of intact cells or tissues by NMR, however, gives relatively poor separation of signals (i.e. allows more overlapping signals), loss of coupling multiplicities (i.e. loss of J-coupling) and relatively poor signal-to-noise ratios. Hence, the detection and quantification
of signals, especially of less intense signals, are typically inferior to those achieved using extracted tissue samples. For this reason, spectroscopy in vivo is usually supported by spectra taken from extracts of the cells or tissues being investigated (i.e. from perchloric extracts that are devoid of paramagnetic cations such as manganese). Such extract spectra have sharp lines and hence overlapping of signals from different elements is considerably reduced and coupling multiplicities are seen. In addition to one-dimensional NMR spectra from plant extracts, a wide range of NMR-spectroscopic methods that generate two-dimensional spectra of different nuclei have now been developed. Indeed, a typical metabolite contains more than one element that is detectable by NMR.

A pulse-chase experiment utilising in vivo $^{13}$C-NMR techniques. Series of NMR spectra registered during the incorporation of histidine by heterotrophic culture sycamore cells. The spectra were recorded at 20 °C on a Bruker AMX 400, WB, spectrometer, which was equipped with a 25-mm probe tuned at 161.9 MHz. They are the result of 900 scans (1 h). The signal-to-noise ratio was maximised using a specific perfusion arrangement [13*], which optimised the homogeneity of the analysed samples. Cells (10 g wet weight) were placed on a porous plate near the bottom of a 25-mm NMR tube. The porous plate was crossed by a central output glass tube; an inlet tube and a safety output tube were positioned 2 cm above the surface of the sedimented cells. The nutrient medium was circulated though the cells via a peristaltic pump attached to these tubes and recycled in a well-oxygenated external reservoir. The conditions for $^{13}$C-NMR acquisition utilised 70-µs pulses (90°) at 5.6-s intervals and a sweep width of 20.73 kHz. Broad-band decoupling at 4W during acquisition and 0.5 W during delay were applied using the Waltz sequence. Spectra were referenced to hexamethyldisiloxane at 2.7 ppm. The cells incubation medium contained 1 mM KCl, 5 mM KNO$_3$, 0.5 mM MgSO$_4$, 0.5 mM Ca(NO$_3$)$_2$, 0.1 mM KH$_2$PO$_4$, and 4 mM glucose. The pH of the external medium was regulated to 6.0. Histidine (100 µM) was added at time 1 h; a chase was started at time 16 h (see Figure 2) after the rinsing of perfused cells with a nutrient medium devoid of histidine. The total height of the 12 sucrose peaks is not shown. Citrate (cit), malate (mal) and glutamate (Glu) are also identified. The profile of soluble metabolites present in the reference spectrum of sycamore cells shows that sucrose is the most abundant compound. Quantification assays show that its concentration was circa 80 µmol/g cell wet weight. The incorporated histidine appears as six pairs of peaks corresponding to the six carbon atoms of the molecule. Except for carbon C5, the right peak in each pair corresponds to histidine present in an acidic compartment (i.e. the vacuole at pH 5.7) and the left one to histidine present in an alkaline compartment (i.e. the cytoplasm at pH 7.5). Carbon C5 splits in the opposite direction according to pH shifts. cyt-His, cytoplasmic histidine; vac-His, vacuolar histidine.
Furthermore, atoms of the same element within molecules are chemically non-equivalent and therefore worth distinguishing from one another by NMR. The type of additional information provided by 2D spectra and how it is interpreted is carefully described by Fan [7].

The various NMR spectroscopy techniques offer, therefore, some unique ways to decipher metabolic networks in plants, to probe the metabolic response of tissues or cells to physiological [2,8–10,11•–13•] and chemical [14•] perturbations, to follow the fate of a stable-isotope-labelled molecule along a metabolic pathway [15–24,25•,26•], to visualise the unpredictable changes in metabolism reported in genetically engineered plants [3••], and to analyse the metabolic dialogue between plants and their microbial symbionts ([27]; see also Update).

NMR is also a useful technique for the determination of intracellular pH in a variety of tissues or cells, pH determination by NMR is based on the dependence of the chemical shift of various endogenous molecules (i.e. inorganic phosphate, organic acids and amino acids) on intracellular pH [3••,13•] (Figures 1,2). 31P-NMR, for example, can discriminate vacuolar Pi (pH~5.5) from cytoplasmic Pi (pH~7.5), and this technique allows accurate and non-invasive studies of trans-vacuolar proton movements. The usefulness of Pi as a 31P-NMR probe for the measurement of pH in cells is, however, hampered by its sensitivity to ionic strength, its low and varying concentration during metabolism (e.g. during Pi starvation) and the large line widths of cellular peaks. (The line widths of intracellular peaks originated from the natural heterogeneity of cytoplasmic and vacuolar pHs within plant cells. The broader the signal, the more difficult it is to define its shape and extent.) Consequently, it is almost impossible to characterise distinct pools of Pi within the cytoplasm. For example, 31P-NMR cannot be utilised to study trans-mitochondrial or trans-plastidial proton movements (which involve a ΔpH of less than 0.2 units). To solve these problems, Pietri et al. [28•] have designed a new series of non-toxic uncharged α- and β-aminophosphonates in which alkoxyl groups are linked to the phosphorus atom. This new class of highly sensitive 31P-NMR pH indicators, which are permeable to cell membranes and exhibit a low sensitivity to ionic strength, allow the investigation of a large range of pKa values and offer the maximum NMR sensitivity at a given pKa value.

**Two-dimensional phosphorus NMR exchange spectroscopy**

There are numerous reactions in plant cells in which metabolites such as ATP or glucose 6-P turnover rapidly; their rate of turnover can be quantified by the saturation transfer NMR technique [3••,8]. This is the technique of choice for measuring the forward and backward rates of a reaction driven in situ by an enzyme and, therefore, for measuring unidirectional fluxes through the enzyme in situ in cells or tissues at steady states. Indeed, for any enzymatic reaction involving the translocation of a nucleus with a spin, transfer of magnetism directly reflects transfer of mass. Metabolites of interest are labelled by orienting their magnetic nuclei using radio waves. Two-dimensional NMR exchange spectroscopy has been used in an elegant way to study unidirectional fluxes through several of the enzymes of central metabolism (e.g. the activities of ATPases, phosphoglyceromutase, enolase, phosphoglucomutase and UDP-glucose pyrophosphorylase) in hypoxic maize root tips [29]. This method can potentially allow the simultaneous monitoring of several unidirectional enzymatic reactions at steady states in intact cells. This is a distinct advantage over previous one-dimensional saturation transfer NMR experiments in which only single exchange reactions were observed. Using this strategy, Roscher et al. [29] have shown that ATP turnover and glycolytic flux increase with temperature up to the point at
which oxygen availability limits respiratory rate (i.e. until hypoxia). During the course of hypoxia they observed a net flux through phosphoglucomutase and UDP-glucose pyrophosphorylase toward carbohydrate synthesis. Such a situation leads to a net production of pyrophosphate, which appears to be of the same order of magnitude as the flux needed for PPI-dependent phosphofructokinase to operate in the glycolytic direction.

Conclusions
Non-destructive and non-invasive NMR can be used to investigate the metabolism of plants. This method allows the identification of molecules and ions in tissues or cells as well as in various extracts, the determination of the absolute concentrations of the more abundant mobile metabolites, the measurement of the change in concentration of key molecules during biochemical transformations, and the measurement of unidirectional fluxes in intact cells or tissues at steady state. In addition, NMR can reveal unexpected information, including the discovery of novel compounds (Figure 3) that would escape detection by other analytical methods. Undoubtedly, this method will continue to decipher metabolic networks under changing physiological conditions in an increasingly sophisticated way. Research in plant biology has been revolutionised over the past decade by the creation of transgenic plants with the intention of causing a specific perturbation along a metabolic pathway. There would seem to be considerable scope for using in vivo NMR to visualise, in a single snapshot, the metabolites induced by the introduction of a foreign gene into a plant genome. Finally, rapid developments are occurring at the interface between NMR spectroscopy and NMR imaging [30]. Indeed NMR imaging, which allows repeated imaging of the same specimen (e.g. a hypocotyl or a root), has a great potential for the study of various physiological processes, growth and development, water flow and environmental effects.

Acknowledgements
We would like to thank Dr Elizabeth Gout for her unflagging collaboration. We would also like to thank Dr Claude Roby for his kind interest.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


Roberts explains in an elegant way in which NMR has been used to obtain quantitative information about fluxes in metabolic networks.

Figure 3

Representative in vitro $^{31}$P-NMR spectra for perchloric acid extracts of sucrose-supplied sycamore cells (bottom spectrum) and cells starved of sucrose for various periods (12-120 h). Cells harvested from the culture medium (9 g) were rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and incubated at zero time in sucrose-free culture medium. The spectra recorded at 20 °C are the results of 1024 transients (1h). Note the disappearance of hexoses-P and the steady accumulation of phosphorylcholine during the course of sucrose starvation. The accumulation of phosphorylcholine, which exhibits a remarkable metabolic inertness, is attributable to a massive intracellular membrane degradation (i.e. autophagy). In fact, this phosphodiester is derived from phosphatidylcholine degradation via the transient accumulation of glycerylphosphorylcholine. Fatty acids released during the course of polar lipid degradation are utilised to fuel the remaining mitochondria with respiratory substrates (for more details see [31]). Fru-6-P, fructose 6-P; Glc-6-P, glucose 6-P; GPC, glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine; GPG, glycerylphosphorylglycerol; GPI, glycerylphosphorylinositol; Man-6-P, mannose 6-P; P-Cho, phosphorylcholine; P-EA, phosphorylethanolamine; PGA, 3-phosphoglycerate.
Gas chromatography coupled to electron-impact quadrupole mass spectrometry is a powerful technology for establishing rapidly, and in a reliable and sensitive way, the metabolite profile of a single plant extract. Metabolite profiling provides a direct link between a gene sequence and the function of the metabolic network in plants.


Nuclear Overhauser-enhanced 13C-spectroscopy is developed to study the time course of sucrose inflow into the hypocotyl of castor bean seedlings.


Arbuscular mycorrhizal fungi are obligate symbionts that colonise the roots of the majority of crop plants. 13C-NMR is an ideal technique for following the fate of 13C-glucose through the fungus metabolic network. In this article, the authors propose an elegant model for major fluxes of carbon in arbuscular mycorrhizal fungi in the mycorrhizal state. According to this model, lipids are actively synthesised by the fungus within the roots (i.e. intraradically) from 13C-glucose and are stored or exported to the extraradical mycelium. There, they are stored or metabolised to produce glucose the precursor of trehalose, a major storage disaccharide commonly found in fungi.


13C-NMR is a useful technique for determining the intracellular pH of a variety of tissues or cells. pH is determined on the basis of the dependence of the endogenous inorganic phosphate (Pi) chemical shift on intracellular pH. The intracellular pHs of Pi-deprived cells were measured using methylphosphonate as a non-metabolisable pH probe. The combined use of 13C- and 31P-NMR to analyse changes during anaerobic stress in higher plant cells led to the following major conclusions: first, the proton-releasing metabolism of ATP was at the origin of the cytoplasmatic acidosis established in cells immediately after the imposition of anoxia, and second, the proton pump of the plasmalemma did not operate under anaerobic conditions.

Aubert S, Pallett KE: Combined use of 13C and 31P-NMR to analyse the mode of action and the metabolism of the herbicide isoxaflutole. Plant Physiol Biochem 2000, 38:517-523. The authors of this paper describe the use of 13C-NMR (many herbicides including isoxaflutole are fluorinated molecules) to follow the metabolic fate of isoxaflutole in plant cells. 13C-NMR was used to characterise the effects of isoxaflutole on plant metabolism.


25. Mouillon J, Aubert S, Bourguignon J, Douce R, Rébéflé F: Glycine and serine catabolism in non-photosynthetic higher plant cells: their role in C1 metabolism. Plant J 1999, 20:197-205. Using the 13C-NMR technique and various 13C-labeled substrates (e.g. 2-[13C]-serine and [2-13C]glycine), Mouillon et al. have shown that serine metabolism in plants (a cytosolic enzyme) is essentially connected to the glycin cycle through the mitochondrial glycine decarboxylase/serine hydroxymethyltransferase enzymatic system. This serine-glycin cycle therefore involves reactions that take place in both the cytosolic and mitochondrial compartments. Indeed in most organisms, the single carbon involved in folate-dependent processes is derived from the β-carbon of serine with the concomitant formation of glycine. Assuming that for each utilisation of one C1 unit there is the production of one glycine, it is clear that glycine catabolism via mitochondrial GDC-SHK coupled reactions is necessary.


In this article, new highly sensitive \( ^{31} \text{P} \) NMR pH indicators (i.e. uncharged \( \alpha \)-aminophosphonates) have been designed for analysing pH modifications during biological processes. Aminophosphonates could be of great utility in enhancing our understanding of trans-mitochondrial and trans-plastidial proton movements.

