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Nuclear Magnetic Resonance and Plant Metabolic Engineering

Yair Shachar-Hill

Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, New Mexico 88001

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Nuclear magnetic resonance (NMR) can be used to measure metabolite levels and metabolic fluxes, to probe the intracellular environment, and to follow transport and energetics nondestructively. NMR methods are therefore powerful aids to understanding plant metabolism and physiology. Both spectroscopy and imaging can help overcome the unique challenges that plants present to the metabolic engineer by detecting, identifying, quantifying, and localizing novel metabolites *in vivo* and in extracts; revealing the composition and physical state of cell wall and other polymers; allowing the identification of active pathways; providing quantitative measures of metabolic flux; and testing hypotheses about the effects of engineered traits on plant physiological function. The aim of this review is to highlight recent studies in which NMR has contributed to metabolic engineering of plants and to illustrate the unique characteristics of NMR measurements that give it the potential to make greater contributions in the future. © 2002 Elsevier Science

INTRODUCTION

Compared to microorganisms, plants present unique challenges to the metabolic engineer. Plant metabolism is more complex both because of the existence of several metabolically active subcellular compartments and because of large metabolic fluxes through pathways unique to plants. There are many genes of unknown functions in plant genomes, suggesting that more unique features will emerge. Furthermore, the heterogeneity of metabolism within plant tissues is only beginning to be analyzed at the level of gene expression and metabolite analysis. For these and other reasons, our understanding of plant metabolism is substantially less complete than is the case for microorganisms and mammals. Our imperfect knowledge of how gene expression is regulated and how proteins are targeted and processed posttranslationally is also a hindrance to rational engineering of plant metabolism. Therefore methods that can analyze metabolic fluxes in detail, survey metabolism in general, and monitor metabolite compartmentation and cellular energetics are especially desirable for plant metabolic engineering (ME). Nuclear magnetic resonance (NMR) provides a toolbox of such methods.

A number of reviews of NMR spectroscopy and imaging of plant systems have appeared recently (Roberts, 2000; Köckenberger, 2001a,b; Bligny and Douce, 2001; Pfeffer *et al.,* 2001; Ratcliffe and Shachar-Hill, 2001; Ratcliffe *et al.,* 2001); between them they cover both technical and biological aspects of plant NMR. Other recent articles review the applications of NMR to biotechnology in general (Keifer, 1999; Hemminga, 2000; Lens and Hemminga, 2001) and a recent article by Weichert (2001) reviews the uses of ^{13}C in metabolic engineering. Here the aim is to draw attention to applications of NMR to plant ME and to point out the advantages and limitations of NMR for the metabolic engineer.

NMR signals can be detected from the nuclei of many isotopes. ^{1}H, ^{13}C, ^{15}N, and ^{31}P are the most widely used for biological NMR spectroscopy, whereas most NMR imaging uses signals from ^{1}H. NMR methods are relatively insensitive, so only signals from compounds present at relatively high levels (concentrations of at least tens of micromoles per liter) can be detected in spectra. For imaging, levels a thousand times higher are needed, so most images are of the water, though the most abundant ions and metabolites can also be imaged. Since metabolic engineering often results in the accumulation of relatively high concentrations of metabolites, this insensitivity is often not as restrictive for compound detection and identification as it is in other areas of biochemistry. However, in the case of metabolic networks, the measurement of metabolite levels and of isotopic labeling is often restricted to metabolic endproducts rather than intermediates. This in turn guides, for example, the kind of modeling for which NMR can provide the required data.

As well as the information used for compound identification and detection and to build and test metabolic models, NMR provides unique chemical insight into cell wall and other solid-state compounds, can be used to obtain "metabolic fingerprints" and metabolite profiles, and gives unique information on energetics and subcellular compartmentation. The relationship of different NMR methods to the information obtained is illustrated schematically in Fig. 1. In the following pages the uses of each of these types of information are illustrated by

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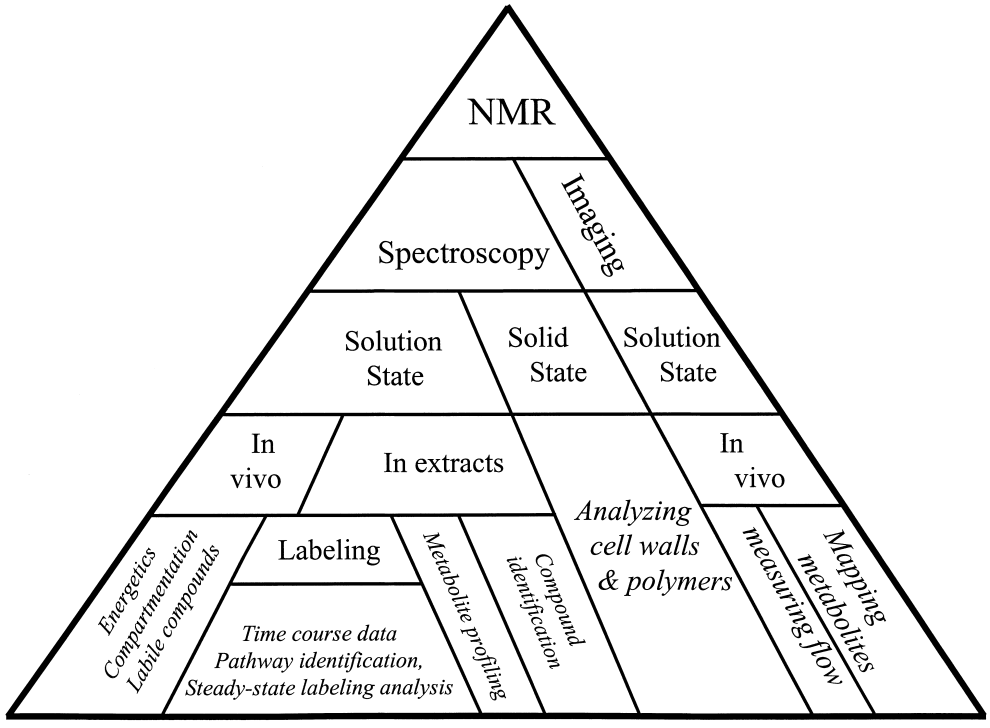

FIG. 1. Illustration of different NMR methods and the information they provide for plant metabolic engineering. The diagram subdivides the types of NMR measurements (plain text) and connects them with the types of information they yield (in italic). Reading from top to bottom the diagonal lines separate methodologies (spectroscopy versus imaging; solution versus solid state; *in vivo* versus extracts; labeled versus unlabeled tissue). The information available from the measurements is positioned underneath the methods that are best suited or most commonly used to obtain them. Thus for example: NMR spectroscopy in solution taken of extracts of unlabeled tissues is generally suited to compound identification in transgenic plants. The relative size of horizontally separated categories indicates which type of NMR measurement has been more commonly used in plant metabolic engineering to date (for example, solution state > solid state, compound identification > metabolite profiling).

recent studies. Some of the ME studies described are ones in which the main aim was to achieve a practical goal, and in others ME methods were used primarily to further our understanding of plant metabolism and physiology.

DETECTING AND IDENTIFYING METABOLICALLY ENGINEERED PRODUCTS

NMR is a convenient method for confirming the presence of known biomolecules and for assigning the chemical structure of novel ones. Spectra give detailed structural information on the number of, chemical environment of, and connectivity between atoms in a molecule, and one- and two-dimensional spectroscopic methods are routinely used in the chemical sciences for these reasons. For metabolic research additional advantages include the ability to measure compounds in crude extracts or *in vivo* and the nondestructive nature of NMR. Thus several different spectra or images giving different sorts of information can be obtained from the same sample, and other chromatographic or spectroscopic methods can subsequently be applied to the same material.

As an analytical spectroscopy, NMR has been used to confirm the identity of various compounds introduced into plants through metabolic engineering. Such compounds include glycine betaine, which is an important target in plant ME for improving stress tolerance (Alia *et al.,* 1998; Holmstrom *et al.,* 2000; Moghaieb *et al.,* 2000). In these studies the presence of glycine betaine was confirmed and its levels could be measured in extracts of metabolically engineered tomato, tobacco, and alfalfa. In fact NMR was employed in identifying low-molecular-weight metabolites accumulated in some of the first ME efforts aimed at improving the stress tolerance of plants. These include the production of mannitol by transgenic tobacco (Tarczynski *et al.,* 1992) and of pinitol, a cyclic sugar alcohol, by tobacco transformed with a gene from the halophyte mesembryanthemum (Vernon *et al.,* 1993). Other ME efforts employing NMR include at least one study in which the chemical structure of a metabolically engineered oligomer with a range of molecular weights was confirmed. In that case NMR was used to analyze

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fructan produced in tubers of potato plants expressing a levan sucrase transgene (Rober *et al.,* 1996). This study shows that NMR can help in efforts to alter the levels of storage carbohydrates and lipids in crop plants, an important area of plant ME.

NMR is diagnostically convenient in detecting known, expected compounds in crude extracts of transgenic plants, but in fact this can frequently be accomplished by other means without much greater effort. However, the detection and identification of unexpected or novel metabolic products by engineered plant varieties is often far harder by other means, and NMR is generally the method of choice, alone or with mass spectrometry for determination of molecular structure and stereochemistry. Novel compounds produced in metabolically engineered plants that have been characterized by NMR include *trans*-resveratrol-3-*O*-β-D-glucopyranoside in transgenic alfalfa, which was shown to confer resistance to attack from a fungal pathogen (Hipskind and Paiva, 2000), and two novel anthocyanins in the flowers of transgenic lisianthus (Markham, 1996). In a different study, in which ME was used to investigate sterol metabolism, NMR was used in structure determination of sterols from mutant, wild-type, and transgenic *Arabidopsis* lines (Gachotte *et al.,* 1995).

The NMR spectroscopy in these studies was employed after tissue extraction, but some compounds are less easy to extract in their native state, either because of low solubility or chemical or biochemical lability or because they exist in supramolecular aggregates. The characterization of compounds by NMR *in vivo* can therefore be extremely valuable. A significant example of this is the detection of polyphosphates in the leaves of transgenic potatoes (van Voorthuysen *et al.,* 2000). Here ^{31}P *in vivo* NMR spectra confirmed the presence of polyphosphate through its unique resonances. ^{31}P *in vivo* NMR spectra of plant tissues typically reveal the levels of ATP, cytoplasmic and vacuolar P_i, UDPG, and hexose monophosphates; they also allow direct measurement of pH in cytosolic and vacuolar compartments. Such spectra are therefore informative about energetics and the intracellular environment. In the study by van Voorthuysen and co-workers the introduction of a gene for polyphosphate kinase affected carbohydrate partitioning, and the NMR confirmations of polyphosphate and other changes in P metabolism were important in interpreting the results.

Other metabolically engineered compounds like waxes can be extracted but their analysis by mass spectrometry and other means is often destructive. Lardizabal and coworkers used *in vivo* NMR to assess the composition and state of wax in *Arabidopsis* seeds expressing several introduced genes of jojoba wax biosynthesis (Lardizabal *et al.,* 2000).

DETERMINING ENGINEERED CHANGES IN CELL WALL STRUCTURE AND OTHER POLYMERS

Compounds in the solid-state can also yield informative NMR signals and the applications of solid-state NMR to plant biology in general are reviewed elsewhere (Ratcliffe *et al.,* 2001). Solid-state NMR spectra give unique chemical and physical insight into structure, composition, and dynamics of polymers, aggregates, and amorphous solids, whereas diffraction methods and other spectroscopies generally yield much more limited information about such materials than about crystalline solids or solutions. This makes solid-state NMR extremely relevant for plant ME in two areas: the modification of cell walls and the production of polymers such as bioplastics.

In recent years there has been a growing interest in the production in plants of bioengineered biodegradable plastics and other chemical feedstocks. The large-scale production of polyhydroxyalkanoate-based polyesters from bacteria has been demonstrated (Gorenflo *et al.,* 2001) and the advantages of producing these plastics in plants have attracted attention (Hanley *et al.,* 2000). Recently Bohmert and co-workers (2000) demonstrated substantial yields of polyhydroxybutyrate polymer in the plastids of *Arabidopsis* plants expressing the transgenes for a three-enzyme pathway to polyhydroxybutyrate. This field is likely to continue to be one of active study in coming years, with a concomitant interest in methods that reveal the structure and composition of the products. Since NMR is an established tool for studying polyhydroxyalkanoate and other polymers (Sudesh *et al.,* 2000) it has the potential to contribute significantly to the development of this promising area of plant ME.

A significant set of targets for ME of plant cell wall biochemistry has been the alteration of enzyme activities involved in ripening (for a review see Brummel and Harpster, 2001). In an interesting solid-state NMR study of cell walls in tomato fruit, Fenwick and co-workers studied ripening in one of the first transgenic food plants (Fenwick *et al.,* 1996). Solid-state NMR spectra from this study, in which resonances from cellulose versus pectin can be distinguished (see Fig. 2), allowed the authors to separately analyze the mobility of the cellulose and pectin cell wall components. Such spectra were used to examine the relaxation rates of the hydrogens attached to the different carbons, which allowed detailed inferences to be made about changes in the local mobility of the pectin during ripening in the fruit of wild-type versus engineered tomato varieties. The study showed that the expression of an antisense sequence for polygalacturonase reduced the softening of the cell wall by selective effects on the rigidity of the pectin, but did not do

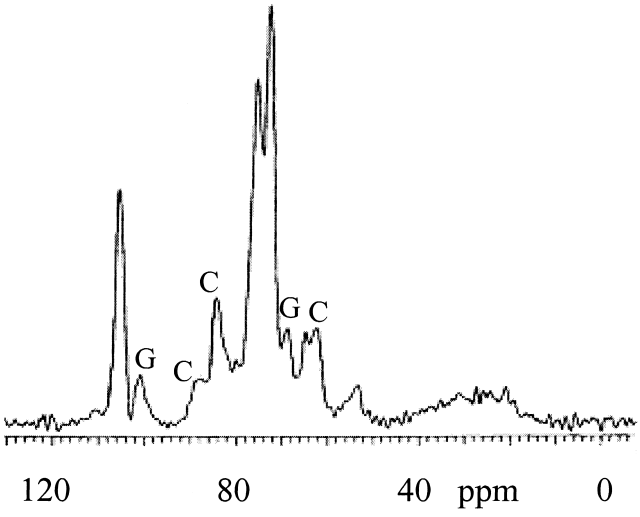

FIG. 2. Solid state ^{13}C NMR spectrum (cross polarization magic angle spinning) of cell walls from ripe tomato. The resonances labeled C are from cellulose and those labeled G are from galacturonal. Unlabeled peaks are from more than one compound. The broad lines typical of solid-state spectra make spectroscopic overlap a much more serious challenge than in solution-state NMR. In this study the authors used spectroscopic editing and relaxation methods to derive information about the state and mobility of different cell wall components. Adapted from Fenwick *et al.* (1996) with permission of the publisher.

so as much as might have been expected from the almost complete loss of polygalacturonase activity (Fenwick *et al.,* 1996).

The analysis of cell wall structure and dynamics is also important in plant ME because of the potentially enormous value of wood and fiber products with altered physical properties. Lignin metabolism is a prime determinant of the physical characteristics of wood and its metabolism has thus become a significant ME target (Sederoff *et al.,* 1999). Solid-state NMR is an established tool for investigation of lignins and other wood components (Gil and Neto, 1999) since peaks in solid-state NMR spectra reveal the chemical structure (abundance and linkages of different chemical groups within cell walls), whereas the measurement of NMR relaxation rates gives insight into the proportion of crystalline versus amorphous material and into the rigidity and dynamics of the material. In ME studies to date, however, most applications of NMR have involved the use of solution-state NMR with extracted lignins and their precursors.

In one such study on engineering of cell wall metabolism, Kajita and co-workers (1997) used NMR to analyze the structure of a novel lignin in transgenic tobacco having reduced expression of 4-coumarate:coenzyme A ligase. Altered lignin composition was also engineered into tobacco plants through the repression of cinnamyl alcohol dehydrogenase and cinnamoyl-CoA reductase (Ralph *et al.,* 1998, 2001b). The slowed conversion of cinnamyl aldehyde to alcohol in this system also allowed the chemical condensation of monomers into lignin to be analyzed (Kim *et al.,* 2000). In these studies NMR was used to determine the structure of the lignin and the products of condensation reactions. NMR was used to demonstrate that in the lignin of an *Arabidopsis* mutant deficient in ferulate 5-hydroxylase, with or without transgenic overexpression of the same gene, there were dramatic changes in the proportions of the different groups (Marita *et al.,* 1999). In an investigation of methylation steps in lignin production, NMR was used to identify the products that accumulated in transgenic poplar tissue having 90% less caffeoyl-coenzyme A *O*-methyltransferase than does the wild type (Meyermans *et al.,* 2000). The identification of the accumulating metabolites was important in assigning the *in vivo* function of a key enzyme in lignin biosynthesis. In the same system NMR was used to demonstrate the incorporation of 5-hydroxyconiferyl alcohol, a phenol not normally incorporated into lignin (Ralph *et al.,* 2001a).

It may be expected that solid-state NMR will become increasingly important as ME of lignin and other wood and fiber products moves from the exploration of basic mechanisms to the development and optimization of viable products.

NMR MEASUREMENTS OF ISOTOPE LABELING FOR ANALYZING METABOLIC FLUXES

Central to rational metabolic engineering is the use of models of metabolism (Weichert, 2001; Morgan and Rhodes, this issue). Such models are routinely used in ME of microorganisms to identify constraints on metabolism and therefore to choose sensible targets for engineering. Models of metabolic flux can broadly be classified as "kinetic" models that explain and predict changes in either isotopic labeling patterns or metabolite levels following a perturbation or the introduction of labeled precursor and "steady-state" models that address labeling patterns and flux balances that do not change with time. NMR can contribute information needed by both types of model: *in vivo* NMR allows the efficient collection of time course data following labeling and *in vitro* NMR analysis of extracts allows isotopomer analysis and the accurate determination of fractional enrichments. However, because of the relative insensitivity of NMR, labeling in metabolic intermediates that are present at low levels is not easily measured so that the information needed for kinetic models is generally obtained from radiolabeling experiments rather than NMR.

In recent years there has been much more progress in the application of NMR to steady-state modeling. In such studies, NMR is used to analyze label distribution in

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metabolic end-products that have reached isotopic steady state after growth in the presence of ^{13}C-labeled precursors (Weichert, 2001). This is an exciting and active field of research in metabolism and in ME of microorganisms, and applications to plants have also begun to appear. Analyzing metabolic networks and quantifying fluxes through them in plants is challenging compared to microorganisms for the reasons mentioned in the Introduction, and steady-state modeling faces the additional challenge that true isotopic steady state is far harder to achieve in plants than in actively growing microorganisms. Only by making assumptions about "pseudo-steady state" has it proved possible to apply this approach to understanding metabolic fluxes in plant tissues. See Roscher *et al.* (2000) for a review of theoretical and practical aspects. Notable studies include the work of Dieuaide-Noubhani and co-workers (1995), who analyzed labeling in a range of low-molecular-weight metabolites in crude extracts of maize root tissue and were able to determine the relative fluxes though more than 20 transformations in central metabolism.

Recently Glawischnig and co-workers (2001) have applied to maize kernels an approach related to the powerful method that Szyperski and co-workers developed in bacteria (Szyperski, 1995). In this method, relative fluxes in central carbon metabolism are deduced from the ^{13}C labeling patterns in the amino acids of hydrolyzed proteins from cells exposed to multiply labeled glucose. In the study by Glawischnig and co-workers, information was obtained on the metabolic pathways by which amino acids are synthesized with some quantitation of relative fluxes. The work of Glawischnig and co-workers did not report whether isotopic steady state was achieved in the proteins, and this remains a challenge for plant ME work if the full power of the Szyperski method is to become available.

In a significant recent study ME methods and steady-state modeling analysis were applied to low-molecular-weight metabolites to critically test hypotheses about the regulation of glycolysis in plants (Fernie *et al.,* 2001). In this study high-resolution ^{13}C and ^{31}P NMR spectra of extracts of tobacco tissue from plants engineered to express a heterologous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase were made. NMR was used to measure ^{13}C label redistribution in hexoses. Together with measurements of the levels of phosphorylated glycolytic/gluconeogenic intermediates, these data were used to assess the regulatory function of fructose 2,6-bisphosphate thus addressing directly a longstanding question in plant metabolism. Figure 3 shows ^{13}C NMR spectra from that study that reveal the isotopic labeling patterns in sugars. The relative labelings in different carbon positions within the same sugars and between different sugars were used with a flux model to deduce the relative fluxes through glycolysis,

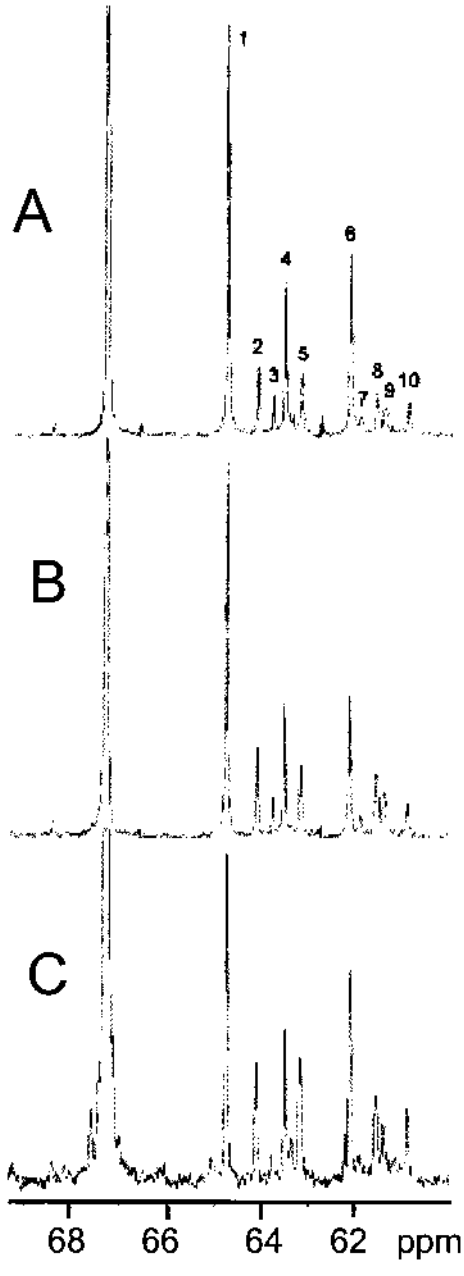

FIG. 3. ^{13}C NMR spectra of extracts of wild-type (A) and transgenic (B and C) callus tissue incubated for 12 h in 50 mM [1-^{13}C]glucose. The strong signal at the left is a reference compound, the labeled peaks are from the labeled positions C1 and C6 of glucose, fructose, and sucrose: 1, β-fructopyranose C1; 2, β-fructopyranose C6; 3, α-fructopyranose C1; 4, β-fructofuranose C6; 5, sucrose fructosyl C1; 7, α-fructopyranose C6; 8, β-glucose C6; 9, α-glucose C6; 10, sucrose glucosyl C6. The authors used such spectra in quantitative analysis of relative fluxes between triose and hexose and among hexoses and sucrose. The differences in labeling patterns between wild-type and transgenic tissue allowed direct testing of hypotheses about the regulation of these key fluxes in plant carbohydrate metabolism. Adapted from Fernie *et al.* (2001), with permission of the publisher.

gluconeogenesis, sucrose metabolism, and glucose consumption.

TESTING HYPOTHESES AND MODELS OF ENGINEERED ALTERATIONS IN METABOLISM

As well as providing data for model building, NMR measurements of compartmentation and energetics can be used to independently test metabolic models. Independent tests of model assumptions and predictions are particularly important since models of metabolic networks involving multiple subcellular compartments and/or multiple branch points are typically underdetermined by the results of labeling and metabolite pool size experiments. For example, most efforts to engineer glycine betaine production in plants (see above) have met with rather limited success, since the buildup of glycine betaine is modest compared with species that naturally accumulate this osmoprotectant. Therefore metabolic modeling based mainly on radiolabeling data has recently been used to identify the metabolic constraints that limit accumulation (McNeil *et al.,* 2000a,b). To test the predictions of this model in terms of subcellular compartmentation and metabolite accumulation rates, *in vivo* ^{31}P NMR was used to measure compartmentation and levels of key phosphorylated intermediates (McNeil *et al.,* 2000b). This type of NMR application is as yet little used in plant ME, a situation that may change as the use of metabolic modeling increases in the coming years.

SURVEYING AND PROFILING METABOLIC EFFECTS OF ENGINEERING

There is currently significant interest in metabolite profiling—measuring the levels of a range of metabolites—as a way of analyzing the perturbations caused by engineered alterations in metabolic systems. Mass spectrometry is currently the most widely used method for profiling because of its sensitivity and ability in combination with chromatographic separations (GC and HPLC) to measure the levels of scores of metabolites from a single extract. There are three ways in which NMR can contribute to profiling. One is the coupling of NMR spectroscopy with separation methods, such as HPLC, with or without subsequent mass spectrometry, in which an NMR spectrum is taken of each chromatographic peak. These are referred to as "hyphenated NMR" methods (see Wilson *et al.,* 2000; Gavaghan *et al.,* 2001; for reviews). Development of this approach has been led by Nicholson and others, and applications to plants, though not yet to plant ME, have recently been presented (Wilson *et al.,* 1999; Bailey *et al.,* 2000; Lommen *et al.,* 2000).

The second approach with potential to aid in ME is the use of NMR spectra as "metabolic fingerprints," wherein the focus is not on assigning all the peaks but on comparing control with transgenic tissues in order to ascertain whether there are significant differences in the levels of any detectable metabolite (Lommen *et al.,* 1998). This approach has been used to address the issue of "substantial equivalence," an important one in ME of food plants. The term substantial equivalence refers to the question of whether foods derived from metabolically engineered species are nutritionally and otherwise equivalent to the nonengineered varieties. To determine this, Noteborn and co-workers (2000) used NMR fingerprinting of transgenic tomato fruit. The results are interesting as an exploration of the approach and also serve to highlight one of the challenges facing plant ME, the plasticity of plant metabolic networks which leads to much larger variability in the accumulation of metabolites than is usually encountered in microorganisms under defined conditions.

The third way in which NMR can contribute to metabolite profiling is through the application of multinuclear multidimensional spectroscopic methods applied to crude extracts. This approach has the potential to identify and quantify multiple metabolites directly, although the number of metabolites is generally in the low dozens rather than over a hundred, as is often possible by mass spectrometry of similar extracts (see Ratcliffe and Shachar-Hill, 2001, and Ratcliffe *et al.,* 2001, for review). With further developments in NMR methods and automated spectral analysis and pattern recognition, the power of this approach will doubtless increase.

IMAGING METABOLITE DISTRIBUTIONS

Underutilized in plant ME and indeed in plant biology in general is NMR imaging (MRI). This field was reviewed recently by Köckenberger (2001a,b). The ability of NMR imaging to generate maps of the distributions of biochemicals in plant tissues has been demonstrated for both primary and secondary metabolites (Meininger *et al.,* 1997; Metzler *et al.,* 1995). Maps of the distribution of amino acids and sugars in seedlings and of alkaloids in a tropical liana were generated in these studies. Because of tissue heterogeneity and variable expression of transgenes in plants this approach may prove useful in optimizing and understanding the accumulation of ME products. Indeed NMR imaging of gene expression patterns in intact tissues has recently been demonstrated (Louie *et al.,* 2000). In that study a reporter gene whose product generated contrast in NMR images by catalyzing the activation of an exogenously supplied MRI contrast reagent was employed to reveal the location of active transcription of the transgene.

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Metabolite transport, both of precursors and of products, is important in plant ME in ways that it is not in engineered microorganisms, and NMR microscopy can provide detailed *in vivo* images of the flows and labeling patterns of plant metabolites in intermediate and long-range transport processes (Verscht *et al.,* 1998). The most obvious area of potential applicability for these methods to plant ME efforts is in efforts to engineer carbon allocation (see above).

CONCLUDING REMARKS

NMR provides an array of spectroscopic and imaging methods. Some of these, notably the detection and structure determination of engineered products, are already used regularly if not ubiquitously in plant ME. The extent to which these and other NMR methods are employed in plant ME in the coming years depends as much on the directions that prove most active in plant ME as on the advantages and limitations of the NMR techniques themselves. A likely increase in the use of some NMR methods, such as applications of NMR analysis of steady-state labeling patterns, may be inferred from the ways in which NMR is now being used in microbial ME research. Other NMR methods such as solid-state spectroscopy are well developed technically but will be increasingly applied when ME of wood and fiber products and other insoluble polymers becomes more important. Other NMR methods, like metabolite profiling and metabolite imaging, will probably find widespread applications when the methodologies become more routine and are validated against existing methods. NMR is perhaps most strikingly underutilized in plant ME when it comes to testing metabolic and physiological hypotheses *in vivo*. Since this is the most established area of plant NMR, its relative neglect by metabolic engineers is interesting, and the situation will change to the extent that plant ME is increasingly used to probe regulatory processes in plants.

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