Conclusions

Preventing oxidative stress or reducing the level of the reactive molecules appears to be a promising approach to obtain plants with diverse tolerance to abiotic stress. Engineering crop plants that can cope with oxidative molecules could have a broad application in agriculture. The examples discussed in this article show that there are several pathways that can be used to obtain stress-tolerant plants. Because there are still many stress-activated genes with unknown functions, future experiments might discover further pathways that lead to reduced levels of reactive molecules. Therefore, as yet unidentified stress-induced genes could have the potential to engineer plants with improved stress tolerance.

References


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Functional imaging of plants by magnetic resonance experiments

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Microimaging based on magnetic resonance is an experimental technique that can provide a unique view of a variety of plant physiological processes. Particularly interesting applications include investigations of water movement and spatially resolved studies of the transport and accumulation of labelled molecules in intact plant tissue. Some of the fundamental principles of nuclear and electron magnetic resonance microimaging are explained here and the potential of these techniques is shown using several representative examples.

Only a few techniques make it possible to map physical and chemical parameters in intact, living plants. Imaging methods based on magnetic resonance are among the most versatile techniques within this group. The information that is available from the use of nuclear magnetic resonance (NMR) techniques includes the in vivo distribution of metabolites, water flow in the vascular conduits and physical properties such as water diffusion and relaxation mechanisms in different cellular compartments. In addition, electron paramagnetic resonance (EPR) techniques can be used to detect free stable radicals in plant tissue.

Noninvasive images of virtual transverse sections

Several nuclides, such as 1H, 13C, 15N and 17O, have angular momentum (nuclear spin) and a magnetic moment. These two nuclear properties are a prerequisite for any NMR experiment. In microimaging experiments with plants, images are frequently formed from the dominant signal of the protons bound in the water molecule. However, it is also possible to detect protons in metabolites with much lower concentrations or to use 13C nuclei in an imaging experiment. The principles underlying the detection of nuclei by NMR are summarized in Box 1. In essence, the application of a strong magnetic field creates a weak sample magnetization, which can be manipulated by irradiating with appropriate radio-frequency pulses. The sample magnetization can then be detected through an induction of a weak voltage in a coil placed around the sample. The frequency components of the time-dependent signal can be extracted by Fourier analysis and represented in a spectrum.

If the sample consists of one type of nuclei, such as protons bound in the water molecule, and a homogeneous external magnetic field is applied, there is only one frequency component in the spectrum (Fig. 1a). The signal can be spatially encoded by exploiting one of the most fundamental principles of magnetic resonance, which states that the resonance frequency is proportional to the interacting local magnetic field. Therefore, if a magnetic field gradient is applied across the sample, the local magnetic field becomes spatially nonuniform, and the detected signal...
Box 1. Nuclear magnetic resonance

An externally applied magnetic field $B_0$, created with either a superconductive magnet or an electromagnet, interacts with the nuclear magnetic moments. As a result, the nuclei split into different populations, which occupy different energy levels. In the classical description of nuclear magnetic resonance, the magnetic dipoles are depicted as vectors that precess (rotate) with a typical frequency $\omega$ around the axis of the applied magnetic field $B_0$ (a). In Fig. I, the nuclear dipoles are represented by just eight vectors. They are either aligned parallel (five) or antiparallel (three) to the applied magnetic field. A weak sample magnetization ($M_{\text{equilibrium}}$), depicted by a red vector in the diagram, arises from the unequal distribution between the two energy levels. The initial distribution can be pushed away from the equilibrium state through the application of radio-frequency pulses that induce transitions between the energy levels and synchronize the precession of the magnetic dipoles (b). They precess now in phase and a detectable magnetization ($M_{\text{detectable}}$) is created perpendicular to the axis of the applied magnetic field. In Fig. I, $M_{\text{equilibrium}}$ is rotated into the plane perpendicular to the axis of the applied field by a ‘90°-radio-frequency pulse’. The precessing sample magnetization creates a small electromotive force in a detection coil after its manipulation by an appropriate sequence of radio-frequency pulses. This signal is amplified and digitized. After the application of a radio frequency pulse, the sample magnetization returns to the initial equilibrium by relaxation (c). Although there are several different processes contributing to relaxation, frequently the time dependence of the sample magnetization can be described by two phenomenological first-order equations. The rate of return of the sample magnetization after excitation to the initial equilibrium state is mediated by dissipative processes and is characterized by the time constant $T_1$. This process is called longitudinal relaxation. The decrease of detectable sample magnetization by a loss of phase coherence is described by the time constant $T_2$ and the process is called transverse relaxation. In Fig. I, this loss of phase coherence is represented by the difference in the orientation of the magnetic dipoles (black vectors) compared with their orientation after the radio-frequency pulse (red vectors). Note that owing to the loss of phase coherence between the individual magnetic dipoles, the resulting magnetization $M_{\text{detectable}}$ is attenuated.

Consists of a range of frequencies. From this signal, a one-dimensional projection of the sample can be derived by Fourier transformation (Fig. 1b). High magnetic field strengths and large magnetic field gradients in three dimensions are exploited in NMR microscopy to achieve a high spatial resolution, typically 10 µm to a few hundred µm (for a comprehensive introduction, see Ref. 1). NMR microimages of plant organs with such high resolution show a wealth of anatomical details.

With particularly favourable plant material, a cellular resolution can be obtained (Fig. 2). A variety of anatomical and morphological NMR microscopy studies have been carried out with plants1–5. Recent applications include the investigation of fruit6 and bulb7 development, and the absorption of water in a resurrection plant8. Furthermore, NMR microimaging has also been used to study root growth9 and gravitropism10.
properties of the compartments. In addition, water molecules or protons exchange between these compartments either by chemical processes or by molecular diffusion. It is one of the interesting potentials of NMR experiments that they make it possible to measure the different relaxation rates and bulk fractions of the compartments and to analyse exchange processes between them. Such an approach was used to investigate the exchange of water between the apoplast, cytoplasm and vacuoles in apple fruit tissue. One particular problem often arises in NMR experiments with plants because there are numerous small, air-filled intercellular spaces within the plant tissue. Air and water have different magnetic susceptibilities and large internal magnetic field gradients can occur at the air–water interface. Diffusional motion of the nuclei within these field gradients contributes to the non-recoverable loss of detectable magnetization and the transverse relaxation in plant tissue can therefore be particularly fast. Several attempts have been made to analyse the NMR signal attenuation owing to susceptibility gradients within the sample and to extract additional information of the tissue properties. Depending on the experimental parameters chosen in an NMR microimaging experiment, it is possible to create contrast in the images, which reflects the variation of the relaxation parameters within the plant tissue. Variations in the nuclear spin density (e.g. the water concentration) within the tissue also contribute to the image contrast.

Relaxation processes reflect the environment of water

There is a variety of interactions between the magnetic moments of the observed nuclear spins and the surrounding nuclei and electrons. These interactions between nuclear spins and their environment play an important role in relaxation processes (Box 1). Through these interactions, it is possible to probe the physicochemical properties of the spin environment using NMR relaxation measurements. For instance, such measurements can be used to measure the viscosity of a solution or the presence of paramagnetic ions such as manganese. Plant tissue, which consists of vacuoles, cytoplasm and extracellular spaces, is a heterogeneous multicompartment system. The signal from the water protons can relax with different rates for each compartment depending on the physicochemical diffusional (randomly oriented) and translational (directed) motion of water molecules. Translational motion of water in plants occurs in phloem sieve tubes and xylem vessels when water is flowing within these specialized cell structures. The flow velocity is encoded in a phase shift of the signal by the use of pulsed magnetic field gradients. It is possible to measure both the xylem and phloem water flow in such experiments. Therefore, this technique offers an interesting approach to studying the dependence of phloem water flow on phloem loading and to evaluating the Münch theory of pressure-driven water flow and assimilate transport through the tiny structures of the sieve tubes and sieve plates. This technique is equally important in the investigation of the mechanism and driving forces of xylem water flow, a subject that has been the focus of dispute over the past few years. Noninvasive NMR microimaging could provide a interesting tool to analyse water flow under in vivo conditions, particularly in light of recent experiments that have demonstrated that changes in the conductivity of xylem vessels correlate with changes in salt concentration in the xylem sap.

Figure 3 shows two examples of flow-sensitive NMR microimaging measurements. Bidirectional water flow was clearly observed, with water moving from the roots to the cotyledons within the xylem vessels and from the cotyledons with roots embedded in natural or artificial soil.

Fig. 1. Spatial encoding of the nuclear magnetic resonance signal. (a) A spectrum of a tube filled with water. Only one resonance line at one frequency is detected in the spectrum. An arrow indicates the direction of the applied external magnetic field. (b) A spectrum acquired from the same sample after a magnetic field gradient was superimposed on the external magnetic field across the sample. The gradient is represented by the ramp of arrows. On the left-hand side of the tube, the gradient adds to the external main field and, on the right-hand side of the tube, the gradient decreases the external main field. Correspondingly, the resonance frequency of nuclei will be increased at the left-hand side of the sample and decreased at the right-hand side. There is now a range of frequencies within the spectrum, yielding a projection of the sample perpendicular to the applied magnetic field gradient.

Fig. 2. High-resolution nuclear magnetic resonance microimage of Basella rubra. Field of view is 5.5 mm with a nominal resolution of 21 μm × 21 μm and a virtual slice thickness of 800 μm; the acquisition time was 30 min. The parenchyma is resolved in a cellular pattern owing to differences in water proton relaxation at the cell walls and membranes, and to the presence of air-filled intercellular spaces. The vascular bundles appear dark in the centre of the plant stem. Scale bar = 1 mm.
The water flow in the xylem is clearly recognizable. Scale bar = 2 mm. The field of view in the flow-sensitive experiment is 6 mm, with an in-plane resolution of 47 \( \mu \text{m} \times 47 \mu \text{m} \); the acquisition time was 4.5 h. Reproduced, with permission, from Ref. 25. (b) A \(^1\text{H}\) NMR image through a stem of a tomato plant overlaid with an image of a flow-sensitive NMR experiment. The water flow in the xylem is clearly recognizable. Scale bar = 2 mm. The in-plane resolution in the flow-sensitive experiment is 117 \( \mu \text{m} \times 117 \mu \text{m} \); the acquisition time was 17 min. Modified, with permission, from Ref. 28.

Fig. 3. (a) A \(^1\text{H}\) nuclear magnetic resonance (NMR) image through the hypocotyl of a six-day-old castor bean overlaid with an image of a flow-sensitive NMR experiment (detail of original image). The two different scales (yellow–red and blue) indicate the bidirectional water flow in the seedling. Scale bar = 2 mm. The field of view in the flow-sensitive experiment is 6 mm, with an in-plane resolution of 47 \( \mu \text{m} \times 47 \mu \text{m} \); the acquisition time was 4.5 h. Reproduced, with permission, from Ref. 25. (b) A \(^1\text{H}\) NMR image through a stem of a tomato plant overlaid with an image of a flow-sensitive NMR experiment. The water flow in the xylem is clearly recognizable. Scale bar = 2 mm. The in-plane resolution in the flow-sensitive experiment is 117 \( \mu \text{m} \times 117 \mu \text{m} \); the acquisition time was 17 min. Modified, with permission, from Ref. 28.

Investigating the distribution of metabolites

As well as using the dominant \(^1\text{H}\) resonance of water, it is possible to form images from the much smaller resonance lines arising from protons bound in different metabolites. Frequently, resonance lines from different chemical groups can be distinguished by their typical chemical shift. This shift in resonance frequency can again be explained by the fundamental principle that the resonance frequency of a nuclear spin is proportional to the local magnetic field. The different chemical shifts are caused by differences in the shielding of the external magnetic field by surrounding electrons and nuclei. The resonance lines of higher concentrated sugars such as sucrose or amino acids in plants are particularly suitable for spectroscopic NMR experiments. In spectroscopic NMR-imaging experiments, a map displaying the distribution of a metabolite in a plant is derived from a set of spatially resolved \(^1\text{H}\)-spectra. This technique was used to investigate the compartmentation of aromatic oil compounds in fennel mericarps. A map of the sucrose distribution in the hypocotyl was acquired in experiments with six-day-old castor bean seedlings (Fig. 4). The spatially resolved spectra of the plant tissue can be calibrated during post-processing by using spectra of reference solutions, and the metabolite concentration can be derived quantitatively. Using this procedure, it was possible to measure the sucrose concentration within the phloem quantitatively and non-invasively in the castor bean seedling.

There is a triangular relationship in an NMR imaging experiment between the spatial resolution, the time required for the acquisition of the data and the sensitivity achieved in the detection of a particular metabolite. For instance, improving the spatial resolution in all three dimensions by a factor of two would take 64 times longer if the same sensitivity is to be maintained for the detection of a particular metabolite. The sensitivity for the detection of metabolites also depends on the relaxation properties of the tissue. Rapid transverse relaxation of the magnetization causes line broadening of the resonance lines and signal overlap. Because of signal overlap, resonance lines are often not well enough resolved in spectra acquired by \textit{in vivo} one-dimensional spectroscopy to enable their unambiguous identification. This is especially a problem for plants: air in the intercellular spaces causes susceptibility problems and large line broadening. The use of two-dimensional spectroscopic techniques (e.g. correlation spectroscopy) offers a clear advantage for the identification of metabolites via their corresponding resonance lines. Two-dimensional spectroscopy can also be spatially resolved by linking it with imaging techniques (Fig. 5), with the
disadvantage of a further increase in the required experimental time. In principle it is also possible to combine spectroscopic imaging experiments with techniques for diffusion weighting. Medical applications of diffusion ordered spectroscopy have been used to study the compartmentation of metabolites in various tissues\(^\text{19}\). 

**In vivo studies of dynamic processes using stable isotope tracers**

The stable isotope \(^{13}\text{C}\) is ideally suited for use as a tracer in biological systems because of its low natural abundance (1.1%). Metabolic pathways in plants have been investigated by supplying \(^{13}\text{C}\)-position-labelled precursors in various spectroscopic experiments with excised tissue such as leaf discs or root tips, or with perfused cell cultures. However, owing to the low sensitivity of \(^{13}\text{C}\)-NMR experiments, it is difficult to resolve \(^{13}\text{C}\)-labelled tracers spatially in intact plants. One way to enhance the sensitivity of NMR experiments to \(^{13}\text{C}\) nuclei is to use indirect detection techniques. In these experiments, polarization is transferred between the protons and \(^{13}\text{C}\)-nuclei within a chemical group, exploiting the chemical bond between them\(^\text{41}\). Experiments with six-day-old castor bean plants showed that it is possible to observe the accumulation of \(^{13}\text{C}\)-labelled sucrose in the intact hypocotyl by acquiring spatially resolved and chemically selective maps of the \(^{13}\text{C}\)-labelled sugar\(^\text{42}\) (Fig. 6).

In addition to \(^{13}\text{C}\), \(^{2}\text{H}\)-labelled water has been used to investigate the uptake and exchange of water in plant tissue. Recently, directly detected sodium images were acquired from the hypocotyl of six-day-old castor bean seedlings and the uptake and accumulation of sodium was observed\(^\text{44}\).

**EPR imaging visualizes the distribution of radicals in plants**

Electrons also have an angular momentum and a magnetic moment, which can be exploited in experiments analogous to those using NMR. In particular, it is possible to detect the unpaired electrons of organic radicals or paramagnetic ions such as manganese in an EPR experiment. Applications of EPR to biological systems frequently use weak magnetic fields in which electrons resonate at frequencies at the lower end of the microwave range (L band; 0.3–1.2 GHz). Spatial encoding of the EPR signal is achieved by applying magnetic field gradients to the sample in a similar way to NMR imaging. Organic free radicals play a role in the defence processes of many plants during fungi infection. In sycamore (Acer pseudoplatanus), these radicals are located within a reaction zone between the healthy, functional sapwood and decaying, microbially colonized, tissue\(^\text{46}\). In the EPR image in Fig. 7, the infection area is clearly visible in the healthy sapwood (R.B. Pearce et al., unpublished).

**Conclusion and perspectives**

Magnetic resonance microimaging is an underused technique in plant physiological research. There are several reasons for this. First, access to costly high field strength magnets equipped with magnetic field gradients is sometimes difficult to arrange if experiments require long acquisition times. Second, and more important, there is probably a lack of awareness of the potential use of these techniques in plant science. However, several experiments have shown that microimaging techniques provide unique spatially resolved information of water...
motion and metabolite distribution in plants. This article was intended to give some representative examples.

These techniques can now be used in conjunction with genetically transformed plants to study changes in metabolic pools or changes in water movement arising from altered protein expression. Interesting possible examples include the response of phloem sucrose concentration to modifications of sucrose carrier expression or the response of xylem water flow to alteration of the expression of membrane aquaporins. In addition to these existing techniques, there are several interesting directions that could be explored further in collaborations between plant biologists, chemists and physicists. For instance, gene expression could be visualized in microimaging experiments using NMR contrast agents that respond to the expression of reporter genes. Recently, an NMR contrast agent based on a gadolinium complex that is modified by the action of β-galactosidase has been used in animal systems.46

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References
Honours for Plant Scientists in 2001

David C Baulcombe (Sainsbury Laboratory, J ohn Innes Centre, Norwich, UK) and Dale Sanders (University of York, UK) have been elected Fellows of the Royal Society. David Baulcombe laid the corner stone to our current understanding of the connection between plant virology, gene silencing and disease resistance. Dale Sanders has consistently fortified our knowledge on plant and fungal membrane transport systems and signal transduction.

Clark J. Lagarias (University of California, Davis, CA, USA), Christopher B. Field (Carnegie Institution of Washington, Stanford, CA, USA) and Patricia C. Zambryski (University of California, Berkeley, CA, USA) have been elected members of the National Academy of Sciences. Clark Lagarias has made outstanding contributions to the field of chemistry and biochemistry of the photoreceptor phytochrome. Christopher Field developed large-scale manipulative experiments and simulation models to explore the ecosystem responses to interacting global changes, and Patricia Zambryski has concentrated her research on floral differentiation and plasmodesmata structure and function.

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