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Intact plant MRI for the study of cell water relations, membrane permeability, cell-to-cell and long-distance water transport

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Abstract

Water content and hydraulic conductivity, including transport within cells, over membranes, cell-to-cell, and long-distance xylem and phloem transport, are strongly affected by plant water stress. By being able to measure these transport processes non-invasively in the intact plant situation in relation to the plant (cell) water balance, it will be possible explicitly or implicitly to examine many aspects of plant function, plant performance, and stress responses. Nuclear magnetic resonance imaging (MRI) techniques are now available that allow studying plant hydraulics on different length scales within intact plants. The information within MRI images can be manipulated in such a way that cell compartment size, water membrane permeability, water cell-to-cell transport, and xylem and phloem flow hydraulics are obtained in addition to anatomical information. These techniques are non-destructive and non-invasive and can be used to study the dynamics of plant water relations and water transport, for example, as a function of environmental (stress) conditions. An overview of NMR and MRI methods to measure such information is presented and hardware solutions for minimal invasive intact plant MRI are discussed.

Key words: Cell compartments, diffusion, flow conducting area, hydraulic conductivity, phloem, stress imaging, $T_2$ relaxation, xylem.

Introduction

Long-term growth and crop yield are considerably reduced compared with maximum attainable yield due to water stress. This topic is turning into an enormous social and environmental problem due to the potential impacts of climate change on rainfall patterns and temperature extremes. Several abiotic stresses are united by the fact that at least part of their detrimental effects on plant performance is caused by disruption of plant water status (Verslues et al., 2006). Water content and hydraulic conductivity, including transport within cells, over membranes, from cell-to-cell, and long-distance xylem and phloem transport, is therefore key information in studying the effect of water stress. On the cell and tissue level, aquaporins, water channel-forming proteins, are of eminent importance in defining hydraulic conductivity (Verkman, 2000; Javot and Maurel, 2002; Tylerman et al., 2002; Tournaire-Roux et al., 2003; Lee et al., 2005). In xylem and phloem the flow conducting area and the resistance within the vessel or tracheid connections determine hydraulics (Sperry et al., 2006).

By being able to measure transport processes in relation to the plant (cell) water balance it will be possible explicitly or implicitly to examine many aspects of plant function and plant performance. This is key information to validate biophysical functional-structural plant models based on integrated carbon and water allocation and functions. Such models are in use to address water stress-induced effects and growth limitations (Daudet et al., 2002; Tardieu, 2005). In addition, such models are used to count for the contribution of plant evapotranspiration and carbon exchange within global atmospheric circulation models (Sellers et al., 1997).

Transport within intact plants is difficult to measure because only a few techniques are suitable. In the phloem, but also in the xylem and the surrounding tissue, complex
and fragile gradients in pressure and osmotic potential exist that are easily disturbed by invasive experimentation (Verkman, 2000; Steudle, 2001; Koch et al., 2004). On the cell level, the cell pressure probe has been proven to be very valuable to measure water (and solute) membrane permeability, either diffusional (\(P_d\)) or under hydrostatic or osmotic pressure gradients (\(P_o\)) (Tomos and Leigh, 1999; Verkman, 2000). On the tissue level, the pressure bomb and root pressure probe techniques allow water potential and tissue hydraulics to be measured (Henzler et al., 1999). These techniques have been applied to excised roots, leaves, and other pieces of a plant and are clearly very informative but destructive. Hydraulic conductivity has been studied by the use of the high pressure flow meter (Tyree et al., 1995). That method can only be applied to excised parts as well.

For several decades heat tracer methods were used to measure the mass flow in xylem. Up to now they have been used in several ecophysiological investigations, and have led to acceptable results if precautions against potential sources of errors are taken (Smith and Allen, 1996). A general problem is that heat dissipation probes do not truly integrate velocity along the probe length. Furthermore, the placement of the sensor itself is a source of errors, particularly for the heat-pulse method (Clearwater et al., 1999). Calculations of mass flow rates from sap velocities obtained by heat pulse techniques require a reliable estimate of sap-conducting surface area. Measurement of the active surface, in general, is very difficult. In addition, it is known that shrinking and swelling of the stem occur periodically (Sevanto et al., 2002), and that day/night rhythm may be accompanied by changes in sap-conducting surface area (Windt et al., 2006). All these factors can result in substantial underestimation of the actual sap flow.

A very promising and attractive method for providing detailed, non-invasive, and quantitative information on water transport and water balance in intact plants is Magnetic Resonance Imaging (MRI). MRI was considered to include NMR Microscopy or MRM, the high spatial resolution analogue of MRI. MRI is, in essence, spatially resolved Nuclear Magnetic Resonance (NMR). NMR is a non-invasive and non-destructive technique that has a large number of anatomical and physiological applications to plant cells, plant organs, and living plants (Walter et al., 1992; Ratcliffe, 1994; Shachar-Hill and Pfeffer, 1996; Chudek and Hunter, 1997; Ishida et al., 2000; Köckenberger, 2001; Ratcliffe et al., 2001). \(^1\)H NMR has long been used for the characterization of the physical state of water in plant tissue and for the non-invasive measurement of molecular displacement such as flow and diffusion in plants (MacFall and Van As, 1996).

Although well known from (bio-)medical applications, MRI in plant research is still far from being a routine tool. Dedicated hardware is required in order to image intact plants or even trees. MRI systems that are used for plant studies have often been developed for other purposes, such as medical imaging. As a result, many machines have been used with horizontal bore superconducting magnets with cylindrical geometry of the magnetic field gradient coils. In such systems plants have to be placed horizontally instead of vertically. Fitting the shoot or roots of a plant through the narrow cylindrical bore of a gradient set can be stressful and damaging for the plant. Better solutions for minimal invasive studies on intact plants, with a (potted) root system and extended shoot (leaves), require special hardware: open access magnets, open access gradient and detector coil systems, and climate control. Dedicated MRI equipment and methods are now available to study cell water balance, cell-to-cell, phloem and xylem transport in (large) potted plants. An overview of NMR and MRI methods and hardware to measure such information is presented here.

**NMR and MRI basics related to plant (cell) structure**

Some basics of NMR and MRI and the information available by NMR directly related to water in plant (cell) structures is first briefly introduced. For more extended descriptions of the NMR technique and MRI strategies see one of the many excellent recent textbooks (Callaghan, 1993; Levitt, 2001) and the reviews cited in the Introduction.

Water molecules contain two protons (\(^1\)H), which are spin-bearing nuclei. In a strong magnetic field (created by a magnet) aligning the magnetic moments of such nuclei results in a weak sample magnetization, which can be manipulated by applying time-dependent magnetic field pulses at the proper frequency (radio frequency or rf pulses). The component of the sample magnetization perpendicular to the main magnetic field induces a weak induction voltage in a detector coil placed around the sample. The time-dependent induction signal can be analysed into frequency components by Fourier transformation, resulting in a frequency spectrum. In a homogeneous main magnetic field \(B_0\), equal spins (e.g. protons of the water molecules) have identical Larmor precession frequency or resonance frequency, and a single resonance line in the frequency spectrum is observed. When a well-defined constant magnetic field gradient \(G\), \(G=\partial B_0/\partial r\), is created within the magnet, identical spins at different positions along this gradient have different resonance frequencies, because the resonance frequency is proportional to the local magnetic field experienced by the spins. \(G\) can be created in three independent directions \(x\), \(y\), or \(z\), or combinations thereof. In this way spins can be uniquely spatially encoded. This is the basis for NMR imaging.

Position labelling by magnetic field gradients can be performed in a variety of ways (Callaghan, 1993).
Depending on the actual method used, the process of position labelling will take some time and acquisition of the signal occurs at a certain time $TE$ (echo-time) after the excitation of the spin system. During that time, the observed signal will decay according to the transverse or $T_2$ relaxation process: $S(TE) = A_0 \exp(-TE/T_2)$. $A_0$ is the signal amplitude directly after excitation, and is a direct measure of the amount of spins under observation in the detector coil. In imaging it is primarily a function of the spin density. In order to obtain a full two-dimensional image of $N \times N$ pixels, the sequence has to be repeated $N$ times for position encoding in the second direction. If the repetition time $TR$ is long enough, the spin system has restored equilibrium along the magnetic field direction: $TR > 3T_1$, where $T_1$ is the signal decay time in the longitudinal or main magnetic field direction. If $TR < 3T_1$, the effective signal amplitude, $A_{\text{eff}}$, does not represent the spin density in each pixel uniquely, but depends on a combination of the spin density and the relaxation time $T_1$: $A_{\text{eff}} = A_0(1-\exp(-TR/T_1))$. As a result, NMR image intensity usually depends on a combination of these parameters, reflecting spin density, $T_1$, and $T_2$. In addition to these parameters, diffusion behaviour of the molecules can also contribute to the contrast (see below) (Duce et al., 1992; Callaghan et al., 1994a; Xia, 1995; Edzes et al., 1998).

Methods are available by means of which quantitative images are obtained that represent each of these parameters separately. Multiple Spin-Echo (MSE) MRI (Edzes et al., 1998) and Inversion recovery (IR) MSE MRI (Donker et al., 1996) are examples of such sequences. In MSE a series of images is obtained during the decay of the signal after the first excitation rf pulse. Single parameter images can now be processed from the MSE-experiment by assuming a mono-exponential relaxation decay as a function of $n \times TE$ in each picture element or pixel. Here $n$ is the image number acquired during the signal decay. The resulting images are: signal amplitude ($A_0$), $T_2$, and $T_1$ (the latter in the case of Inversion Recovery (IR-)MSE MRI). In addition, images representing the diffusion coefficient, $D$, and flow can be obtained (see below). In this way a combination of anatomical and physiological (functional) information is obtained, referred to as functional imaging. Some examples of single parameter images are presented in Fig. 1.

**NMR relaxation times and compartments**

A variety of interactions between the magnetic moments of the observed spins and the surrounding nuclei and compartments are represented in NMR images. For instance, $T_2$ images reflect the transverse relaxation time, which is closely related to the water content of the tissue. $T_1$ images provide information about the longitudinal relaxation time and are sensitive to the paramagnetic properties of the tissue. The signal intensity in these images is directly related to the concentration of spins with a short $T_1$ or $T_2$ relaxation time, which is typical for water molecules. Conversely, spins with a long $T_1$ or $T_2$ relaxation time, such as those found in lipids or proteins, will contribute less to the signal. This contrast in signal intensity allows for the differentiation of different tissue types and the detection of pathologies.

**Fig. 1.** (A) Single parameter images of a 3 mm slice through the trunk of a poplar tree (1.8 m total length), obtained at 0.7 T. These images have been calculated based on a mono-exponential fit per pixel of the signal decay as a function of decay time $n \times TE$. a: Signal amplitude or spin density image, b: $T_2^{-1}$ image, c: $T_2$ image. (B) Single parameter images from a slice of the stem of *Ricinus* pant. a: Signal amplitude or spin density image, b: $T_1$ image, c: $T_2$ image. In-plane resolution is around 100×100 μm². Both examples demonstrate the sensitivity of $T_2$ to tissue structure or cell size. Amplitude images show water content times tissue density. Images by courtesy of C Windt.
electrons contribute to the relaxation times $T_1$ and $T_2$. These interactions make it possible to probe the physicochemical properties of the spin environment using NMR relaxation measurements. The protons in water molecules experience an intramolecular dipolar interaction between the two proton spins within one and the same water molecule, as well as an intermolecular interaction with protons of neighbouring water molecules. Both interactions fluctuate when the molecules rotate or translate. When the rotation correlation time of the molecules is short, as is the case for free water molecules ($\tau_c \approx 10^{-12}$ s), both $T_1$ and $T_2$ are equal and relatively long ($\approx 2$ s). Water close to macromolecules or to solid surfaces generally have slower tumbling rates ($\tau_c \approx 10^{-12} - 10^{-10}$ s), which leads to a reduction in both relaxation times. In addition, exchange of protons between water and other molecules, such as sugars, proteins, and other macro-molecules, also influences (shortens) the relaxation times (Hills and Duce, 1990).

The signal from water in plant tissue, containing among others vacuoles, cytoplasm, cell walls, and extracellular spaces, decays with different relaxation times. In biological systems multi-exponential relaxation is therefore normally observed and the different decay times (relaxation times) observed could be used to obtain information on the relative proportions of water in different environments or compartments (Belton and Ratcliffe, 1985). In plant tissue the different relaxation times can be assigned more or less uniquely to either water in the vacuole (longest $T_1$ and $T_2$), cytoplasm ($T_1 > T_2$, both shorter than vacuolar $T_1$ and $T_2$), or cell wall/extracellular space ($T_2$ depends strongly on the water content in this compartment, and ranges from about 5 ms up to hundreds of ms), respectively (Snaar and Van As, 1992; Van Dusschoten et al., 1995). In leaves, water in chloroplasts can be discriminated as well (McCain, 1995). Within these compartments diffusive exchange results in single exponential behaviour.

Proton exchange over the plasmalemma and the tonoplast membrane affects the observed relaxation times. Due to the effect of exchange, which depends on the difference in the relaxation times of water in the exchanging compartments, $T_1$ and $T_2$ results are, in general, different, even for the number of observed exponentials. Differences in $T_1$ for the different compartments are relatively small, and exchange between the compartments results in averaging over the compartments. Therefore $T_1$ values relate to water content more directly. Differences in $T_2$ are more pronounced, and exchange over membranes only results in partial averaging (depending on the size of the compartments and membrane water permeability). Cell compartments, therefore, can best be discriminated based on $T_2$ values (Snaar and Van As, 1992; Van Dusschoten et al., 1995).

Because of the relatively poor spatial resolution, most pixels within an image will contain information that originates from different subcellular compartments or even different cells. This is called the partial volume problem. In general, the low $S/N$ per pixel in imaging (typically in the order of 10–50) does not allow multi-exponential fitting, which might provide a way to resolve subcellular information. Such information is available from non-spatially resolved NMR measurements, with a much higher $S/N$ (1000 or higher). Therefore the information presented in images mostly is called ‘apparent’: e.g. apparent $T_2$, $T_{2,app}$, or apparent $D$, $D_{app}$. In images, the $S/N$ can be enhanced by summing up the signal of pixels containing identical information (same tissue). In this way subcellular information of that tissue can be obtained (Scheenen et al., 2002).

$T_2$, cell size, and (tonoplast) membrane permeability

Membrane permeability in plant cells (as well as in many other systems, for example, red blood cells (Regan and Kuchel, 2000, 2002)) has been determined using the NMR relaxation times of intracellular water protons based on the Conlon–Outhred technique (Conlon and Outhred, 1972; Stout et al., 1978; Ratković and Bačić, 1980; Snaar and Van As, 1992; Zhang and Jones, 1996; Donker et al., 1999). The disadvantage of this technique is that it needs the introduction of paramagnetic ions (such as Mn$^{2+}$), which can pass membranes, or other MRI contrast agents (that cannot pass membranes) in high, non-physiological concentrations. A non-invasive approach is based on the principle that the observed transverse relaxation time $T_2$ of water in a confined compartment such as a vacuole can be described as a function of the bulk $T_2$, $T_{2,\text{bulk}}$, of the water and the probability that water molecules reach the membrane and lose magnetization at the membrane, either by a direct interaction with the membrane (acting as a sink for relaxation) or by passing the membrane and entering a compartment with a (much) shorter relaxation time (Brownstein and Tarr, 1979; van der Weerd et al., 2001). The probability to reach the membrane is defined by the diffusion time and thus directly related to the compartment radii. No evidence has been found that membranes themselves act as a relaxation sink (McCain, 1995; van der Weerd et al., 2002a). The net loss of magnetization in a vacuole therefore depends on the membrane water permeability of the tonoplast and the effective relaxation in the cytoplasm (van der Weerd et al., 2002b; L van der Weerd, JEM Snaar, FJ Vergelt, H Van As, unpublished data). As a result the observed relaxation time depends, in addition to $T_{2,\text{bulk}}$, to the radii of the compartment along the $x$, $y$, and $z$ directions ($R_{x,y,z}$) and the net loss of magnetism at the compartment boundary, the
so-called magnetization sink strength \( (H) \) (van der Weerd et al., 2001):

\[
\frac{1}{T_{2,\text{obs}}} = \frac{H}{R_x + R_y + R_z} + \frac{1}{T_{2,\text{bulk}}} \tag{1}
\]

\( H \) is linearly related to the actual membrane permeability (van der Weerd et al., 2002b; L van der Weerd, JEM Snaar, FJ Vergelt, H Van As, unpublished data). A simple common-sense approach is sufficient to explain the effect of compartment properties on relaxation. The intermembrane distances \( (R) \) and the bulk diffusion coefficients \( (D) \) determine the average diffusion time of a water molecule to cross a compartment \( (t_{\text{diff}}=R^2/(2D)) \). The relaxation rate \( T_{2,\text{bulk}} \) in that compartment determines the chance that the molecule still bears magnetization once it reaches the membrane. If that is the case \( (2D/R^2) < T_{2,\text{bulk}}^{-1} \), the membrane permeability determines the exchange rate to the next compartment, and thereby influences the relaxation rate; if the above condition does not apply, the membrane permeability is of no consequence for the NMR signal and equation 1 does not hold.

An example of this relation based on MRI results is shown in Fig. 2 for cells in the apex zone of the stem of intact maize and pearl millet plants. In intact pearl millet plants, \( H \) in cells in this zone has been shown to change during osmotic stress experiments, in contrast to the same plants, intact maize and pearl millet plants. In intact pearl millet plants were used for microscopic sections to determine the cell dimensions for maize (filled triangles) and pearl millet (filled diamonds) cells in maize plants where no changes were observed during osmotic stress experiments, in contrast to the same plants, intact maize and pearl millet plants. In intact pearl millet equation 1 does not hold.

Permeability is of no consequence for the NMR signal and rate; if the above condition does not apply, the membrane permeability determines the exchange rate to the next compartment, and thereby influences the relaxation rate; if the above condition does not apply, the membrane permeability is of no consequence for the NMR signal and equation 1 does not hold.

Some care must be taken into account by using \( T_{2,\text{obs}} \) from images. The observed \( T_2 \) value in images with respect to its value in non-imaging NMR depends on a number of contributions that relates to details of the image experiment and plant tissue characteristics (Rofe et al., 1995; Edzes et al., 1998):

\[
\frac{1}{T_{2,\text{obs}}} = \frac{1}{T_2} + \frac{1}{T_1} + \frac{1}{T_2^o}
\]

Here \( T_2 \) represents the original \( T_2 \) of the liquid in the tissue, which is also observed in non-imaging mode experiments. \( T_3 \) and \( T_2^o \) originate from diffusion effects.

In plants, especially in leaves and woody tissue, small air spaces in the order of a few \( \mu \)m up to \( 100 \mu \)m are present. Due to the difference in the magnetic permeability of air and tissue/water, local magnetic field gradients, \( g_{\text{zr}} \), originate. Displacement through these local gradients due to diffusion results in a reduction of the observed \( T_2 \) value:

\[
\frac{1}{T_1} \propto \gamma^2 g_{\text{zr}}^2 T_2 E^2 D
\]

Here \( \gamma \) is the gyromagnetic ratio, which is a constant for each type of nuclear spin. \( D \) is the (self-)diffusion coefficient of water in the tissue. The strength of these field inhomogeneities is proportional to the applied magnetic field (Lüdecke et al., 1985). Minimizing this effect can be achieved by imaging at relatively low magnetic field strength, \( B_0 \), and at short \( TE \) values (Donker et al., 1996, 1997, 1999). In non-spatially resolved \( T_2 \) measurements this \( TE \) value can be chosen to be very short (in the order of 200–400 \( \mu \)s whereas in imaging mode \( TE \) is in the order of a few ms or longer.

In addition to the effect of local field gradients, diffusion through the repeatedly applied position encoding magnetic field gradients contributes to the observed relaxation time:

\[
\frac{1}{T_2} = \gamma G^2 \delta^2 \left( 1 - \frac{4\delta}{3TE} \right) D
\]

Here \( 2\delta \) is the duration of the applied gradient. In practice, \( G \) and \( \delta \) are dictated by the choice of pixel resolution, image size, and actual \( TE \). To image relaxation times in the order of 1 s, as a rule of thumb a lower limit of 100 \( \mu \)s for the pixel size for objects of 1–2 cm diameter can be used (Edzes et al., 1998). At smaller pixel sizes the diffusive attenuation in the position-encoding gradient becomes the leading contribution to the observed transverse relaxation time, and the information available from the actual \( T_2 \) is lost and mixed up with (restricted) diffusion information and equation 1 no longer holds.

From equation 1 it is clear that, for a proper interpretation of \( T_2 \) measurements in terms of membrane permeability, it is necessary to know the cell dimensions, or more precisely the dimensions of the vacuole. This information can be obtained by NMR as well by

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig2.png}
\caption{Relation between the relaxation time \( T_2 \) as observed by MRI and the cell dimensions for maize (filled triangles) and pearl millet (filled diamonds) cells of different internodes of the apex zone of the stem. Following the MRI measurements on the intact plants, the same plants were used for microscopic sections to determine the cell dimensions. After van der Weerd et al., 2001 (van der Weerd L, Claessens MMAE, Eldé C, Van As H. 2002. Nuclear Magnetic Resonance imaging of membrane permeability changes in plants during osmotic stress. Plant, Cell and Environment 25, 1538–1549) and reproduced by kind permission of Blackwell Publishing.}
\end{figure}
(restricted) diffusion measurements, which, in addition, gives access to membrane permeability of water over longer distances, resulting in cell-to-cell transport.

**Diffusion, restricted diffusion, cell (compartment) dimension and cell-to-cell transport**

All molecules in a fluid are subject to Brownian motion. The extent of this motion depends on the temperature and the viscosity of the fluid, which are incorporated in the bulk diffusion coefficient of the fluid. When an ensemble of molecules is followed in time, the root mean square distance travelled, \( \sigma \), increases with time as long as no boundaries are encountered, according to the Einstein relation:

\[
\sigma = \sqrt{2Dt}
\]  

(5)

\( D \) can be measured by NMR using a so-called pulsed field gradient (PFG) experiment. In this experiment a sequence of two magnetic field gradient pulses of duration \( \delta \) and equal magnitude \( G \) but opposite sign (or equal sign but separated by an inverting rf pulse) label the protons as a function of their position. If the spins remain at exactly the same position the effect of the gradient pulses compensates each other. However, as soon as translational (displacement) motion occurs, the gradients do not exactly compensate each other any more, resulting in attenuation of the signal amplitude. The amount of this attenuation is determined by \( \delta \) and \( G \) of the gradient pulses, and by the mean translational distance travelled during the interval \( \Delta \) between the two pulses. The NMR-signal amplitude \( S(G) \) normalized to the signal amplitude \( S(0) \) at \( G=0 \) for free diffusion at a given \( \Delta \) is given by (Stejskal and Tanner, 1965; Norris, 2001):

\[
\frac{S(G, \Delta)}{S(0, \Delta)} = \exp \left( -\gamma^2 \delta^2 G^2 D \left( \Delta - \frac{\delta}{3} \right) \right)
\]  

(6)

By measuring \( S(G, \Delta) \) as a function of \( G, D \) can be obtained. The distance travelled depends on the bulk diffusion coefficient of the fluid in the compartment and \( \Delta \) (equation 5 with \( t=\Delta \)). If water experiences a barrier to diffusion, for example, a cell membrane, the cell dimensions determine the maximum displacement (in case of an impermeable barrier) or to what extent it is hindered (semi-permeable membrane). For restricted diffusion equation 6 no longer holds and becomes dependent on the geometry and size of the compartment and the permeability of the surrounding membrane (Callaghan et al., 1999; Norris, 2001; van der Weerd et al., 2002b; Regan and Kuchel, 2000, 2002).

To extract the information on size and membrane permeability, diffusion coefficients have to be measured as a function of the diffusion labelling time, or observation time, \( \Delta \), the time between the two gradient pulses (for a tutorial on this subject see Sen, 2004). By varying \( \Delta \) the distance over which the spins can diffuse freely and to what extent they can pass over the membrane, which restricts the diffusion process, are observed. The probability to pass the membrane is a direct measure of the water permeability of the surrounding membrane (Regan and Kuchel, 2000). At longer values of \( \Delta \), water molecules can even travel from cell to cell. An example of a theoretical curve of \( D \) versus \( \Delta \) for diffusion in a confined geometry is presented in Fig. 3 for a number of different values of the water membrane permeability. In the first region, at short diffusion times, free diffusion is observed. In the next region the diffusion becomes restricted, but the averaging of local properties over a large enough distance does not yet occur. In the last region, at long diffusion times, hindered diffusion is observed, which is defined by the permeability of the system, and for plants reflects cell-to-cell transport. The effective permeability \( P \) can now be estimated. For diffusion through a geometry consisting of a series of semi-permeable membranes (thin walls) separated by a distance \( d \), Crick (1970) obtained:

\[
D_{\text{inf}}^{-1} = D_0^{-1} + (Pd)^{-1}
\]  

(7)

or

\[
P = \frac{(D_{\text{inf}}D_0)}{(D_0 - D_{\text{inf}})d}
\]  

(8)

\( D_{\text{inf}} \) is the \( D \) value in the limit of \( \Delta \) to infinity. If \( D_0, D_{\text{inf}}, \) and \( d \) are known, \( P \) can be obtained. This is easily done for \( D(\Delta) \) information as presented in Fig. 3, where \( D_{\text{inf}} \) is reached. In practice, \( D(\Delta) \) is obtained over a smaller

![Fig. 3. Simulated behaviour of \( D(\Delta) \) as a function of \( \Delta \) in a system with a series of compartments separated by semi-permeable membranes (thin walls) at a distance of 10 \( \mu \)m, for different values of the permeability coefficient \( P \) (in m s\(^{-1}\)) of the membranes. For \( P=0 \) fully restricted diffusion is observed. At increasing \( P \) values \( D_{\text{inf}} \) becomes higher. Plot by courtesy of T Sibgatullin.](image)
range of $\Delta$ values and a procedure is needed to extract this information based on a limited range of $D(\Delta)$ values.

In the limit of short $\Delta$, $D$ depends linearly on the square root of the diffusion time. The slope of this dependence is determined by the surface-to-volume ratio ($S/V$) of the water-containing compartment, irrespective of whether these compartments are connected or disconnected (Sen, 2004), according to the equation

$$\frac{D(\Delta)}{D_0} = 1 - \frac{S}{V} \frac{4}{9\sqrt{\pi}} \sqrt{D_0 \Delta}$$

(9)

For a sphere $S/V=3/R$, where $R$ is the radius of the sphere.

In the limit of long diffusion time ($\Delta >> R^2/D_0$) and fully restricted diffusion (impermeable wall) the apparent diffusion coefficient varies inversely with $\Delta$ according to Einstein’s equation

$$D(\Delta) = \frac{R^2}{2\Delta}$$

(10)

$R$ can thus be obtained from the slope, the maximum value of $\sigma$ to be travelled in the confinement. However, semi-permeable membranes will result in deviations from this dependence (Fig. 3). If semi-permeable membranes are present $D$ versus $\Delta$ curves can be described by the following equation (Anisimov et al., 1998; Valiullin and Skirda, 2001)

$$D(\Delta) = D_{eff1}(\Delta) \left(\frac{D_0 - D_{inf}}{D_{eff1}(\Delta) + D_0}\right) + D_{inf}$$

(11)

where

$$D_{eff1}(\Delta) = \left(\frac{D_0 D_{eff2}(\Delta)}{D_0 - D_{eff2}(\Delta)}\right)$$

(12)

and

$$D_{eff2}(\Delta) = \left(\frac{D(\Delta) - D_{inf}}{D_0 - D_{inf}}\right)$$

(13)

An example of experimental data is given in Fig. 4. This figure also illustrates that the experimental range of $\Delta$ values is limited by the relaxation times $T_2$ and $T_1$ and $D_{inf}$ is hard to obtain experimentally. $D_{eff2}(\Delta)$ (equation 13) behaves in much the same way as $D(\Delta)$ for the case of a geometry enclosed by an impermeable membrane (cf. equation 10). The effect of the permeable wall is removed and it results in a good estimate of $D_0$ at short $\Delta$ values (Fig. 4). However, due to the limited range of $t_d$ the dependence $D(\Delta)\sim \Delta^{-1}$ is not yet observed (Fig. 4, circles). Rescaling according to equation 12 ($D_{eff1}$) enlarges the range of diffusion time (to the shorter $\Delta$ values) where the dependence according to equation 10 is observed. It is very useful when the long diffusion times are unavailable experimentally. As a result parameter $R$ can be determined (cf. equation 10), and can be used to fit the experimental data $D(\Delta)$ versus $\Delta$ according to equation 11 resulting in $D_0$ and $D_{inf}$. Alternatively, $D_0$ can be estimated from equation 9 at short $\Delta$ values, if experimentally available. $P$ can now be calculated from $D_0$, $D_{inf}$, and $R (= d$, see equation 8).

$P$ will include the permeability of the tonoplast, plasmalemma, walls and plasmodesmata of neighbouring cells. At long observation times cell-to-cell transport becomes visible. Therefore $P$ is not identical to $H$ as obtained from $T_2$ measurements (equation 1). The value of $d$ as a result of the diffusion measurements can directly be used to obtain $H$ from the $T_2$ measurements. A complication is the effect of the geometry of the confined compartment. Equation 8 assumes plan-parallel membranes, which might be a good assumption for an array of cells in one dimension (the magnetic field gradient direction). The result of $H$ from equation 1 depends on the actual geometry of the compartment: for a sphere, the first term in equation 1 becomes $3H/R$, for a cylindrical geometry it becomes $2H/R$. This problem can be overcome by measuring $D$ in different directions, which is possible by applying magnetic field gradients in different directions.

Some first results are now available. By combining the results of $T_2$ and $D$ measurements on water in apple parenchyma (Granny Smith) $H$ was found to be around $1 \times 10^{-5}$ m s$^{-1}$ ($T_{2, obs}=1.25$ s and $R=86$ $\mu$m). Under the assumption of parallel planes $P=2.9 \times 10^{-6}$ m s$^{-1}$ (TA Sibgatullin, PA de Jager, FJ Vergeldt, AV Anisimov, E Gerkema, H Van As, unpublished results). For Cox apple parenchyma cells a tonoplast water membrane permeability of $P_d=2.44 \times 10^{-5}$ m s$^{-1}$ was reported (Snaar and Van As, 1992) based on the Conlon–Outhred method.
In maize roots, Anisimov et al. (1998) found a higher value of $P$: around $5 \times 10^{-5}$ m s$^{-1}$. Recently values were obtained of $P$ in excised roots of normal and osmotically stressed maize and pearl millet plants: $P$ was found to be around $3 \times 10^{-5}$ m s$^{-1}$ for both normal and stressed maize, whereas $P$ was around $9 \times 10^{-5}$ m s$^{-1}$ for normal pearl millet plants and $3 \times 10^{-5}$ m s$^{-1}$ for stressed plants (TA Sibgatullin and H Van As, unpublished data).

By time-dependent diffusion coefficient measurements combined with MRI the size and the membrane permeability of, for example, the vacuoles in vacuolated plant tissue have been measured, even spatially resolved within single pixels of an image (TA Sibgatullin, FJ Vergeldt, E Gerkema, H Van As, unpublished results).

As stated above, in imaging, $D_{app}$ is normally observed. In tissue with (large) vacuolated cells $D_{app}$ will mainly reflect $D$ of vacuolar water. In other tissue it can become more complex (van der Toorn et al., 2000). Correlated diffusion-$T_2$ measurements have been developed that allow unambiguously to relate $D$ and $T_2$ values of different water-containing cell compartments, even within single pixels in images.

**Correlated diffusion-$T_2$ measurements**

Water in different cell compartments can best be discriminated on the basis of the differences in relaxation behaviour ($T_2$) and (restricted) diffusion behaviour. By combined relaxation and diffusion measurements, together with a recently developed efficient and stable two-dimensional fitting procedure based on a Fast Laplace Inversion algorithm (Venkataramanan et al., 2002; Hürlimann et al., 2002), two-dimensional correlation plots between $D$ and $T_2$ can now be generated, which greatly enhance the discrimination of different water pools in subcellular compartments. In this way, an unambiguous correlation between relaxation time and compartment size can be obtained, resulting in a general approach to quantify water in the different cell compartments. This approach is very promising in non-spatially resolved measurements (Qiao et al., 2005), and has been shown to obtain sub-pixel information in images as well (Van Dusschoten et al., 1996; for recent plant applications H Van As, FJ Vergeldt, CW Windt, unpublished data).

**Flow, xylem and phloem hydraulics**

The method that has been most successful in providing detailed, non-invasive information on the characteristics of water transport in the xylem, as well as in the phloem of intact plants, is MRI [for overviews see MacFall and Van As (1996) and Köckenberger (2001)]. Both non-imaging and imaging methods have been developed and applied to plants. Several groups have used different MRI methods. Most of them are based on (modified) Pulsed Field Gradient (PFG) methods, either by using a limited number of PFG steps or by (difference) propagator approaches (Callaghan et al., 1994b, see below). Also flow measurements based on uptake and transport of (paramagnetic) tracers have been used (Link and Seelig, 1990; Clearwater and Clark, 2003).

The first (non-imaging) method to measure xylem water transport in plants was presented some 20 years ago (Van As and Schaafsma, 1984; Reinders et al., 1988a, b; Schaafsma et al., 1992). Based on that method a (trans) portable NMR bioflowmeter has been developed with a U-shaped permanent magnet (open access from one side) and an openable, hinged, rf coil. It has been used in greenhouse situations on intact plants (Van As et al., 1994). By the applied method averaged linear flow velocity and flux (volume flow) are obtained, and the ratio results in the effective flow conducting area. However, to interpret the data in terms of averaged flow velocity, calibration is needed: the results depend on the actual flow profile. Flow in a single xylem vessel, in general, is assumed to be laminar, but velocities within the different xylem vessels in a stem will be different, depending on vessel diameter. So the flow profile within the total cross-section of a stem is therefore not known *a priori*. In addition, xylem and phloem flow can not be discriminated by that method, the sum of both is observed.

To quantify xylem and phloem flow accurately, one needs to determine both the direction of the flow, and the actual flow profile, from which the flow velocity, the flux, and the flow conducting area can be obtained. At the same time, diffusing water molecules (stationary water in cells) and flow have to be discriminated. These goals can best be obtained by the use of PFG techniques. To quantify the unknown displacement-behaviour of an observed ensemble of spins correctly, one has to measure the NMR-signal $S(G)$ as a function of $G$. By applying a Fourier Transform on $S(G)$ as a function of $G$, the propagator $P(R, \Delta)$ is obtained (cf. Fig. 5). $P(R, \Delta)$ presents the probability that a spin at any initial position is displaced by a distance $R$ in time $\Delta$. For flow, dividing the displacement axis $R$ by $\Delta$ results in the flow profile $P(v)$. This type of measurement is referred to as displacement or propagator imaging and has been applied to measure flow in many porous systems, including plants (for some introductions and reviews see Callaghan et al., 1999; Fukushima, 1999; Mantle and Sedesman, 2003; Stapf and Han, 2005).

The propagator for free, unhindered, diffusing water has a Gaussian shape, centred around $R=0$ (Fig. 5). The root mean square displacement, $\sigma$, of diffusing protons,
observed by NMR, is proportional to the root of $\Delta$ times $D$ (equation 5). $\sigma$ is directly related to the width of the Gaussian distribution (Fig. 5). By contrast, the mean displacement $r$ of flowing protons is linearly proportional to $\Delta$ itself:

$$r = v_{av} \Delta$$ (14)

where $v_{av}$ is the average flow velocity of the flowing protons. The labelling time between the two PFGs has to be long (in the order of 150 ms or longer) to discriminate between slow (phloem) flow and diffusion ($D$ in the order of $2 \times 10^{-9} \text{ m}^2 \text{s}^{-1}$) (Scheenen et al., 2001). At lower velocities $\Delta$ has to be increased further (Fig. 5).

A crucial step in the quantification of the flow is to discriminate stationary and flowing water. The fact that the propagator for stationary water is symmetrical around zero is used to separate the stationary from the flowing water. The signal in the non-flow direction is mirrored around zero displacement and subtracted from the signal itself: $r = v_{av} \Delta$.

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Hardware considerations

In many cases dedicated hardware is required in order to image intact plants. Normally, only small parts of the plant (e.g. stem, a leaf, petioles, seed pots, fruit stalk, etc) will be chosen for study. If so, an optimal signal-to-noise ratio, $S/N$, is obtained by optimizing the radius of the rf coil, $r$, with respect to that part of the plant to be measured. The smaller $r$, the higher $S/N$. The best approach is to construct rf detector coils that closely fit that part of the

Fig. 5. Simulated propagators for a system consisting of 75% stationary water and 25% flowing water, with a laminar flow profile. $D$ is $2.2 \times 10^{-9} \text{ m}^2 \text{s}^{-1}$. The mean linear average flow velocity is 0.2 mm s$^{-1}$ (typical for phloem). (a) $\Delta=15 \text{ ms}$ ($\sigma=8.1 \text{ um}$, $r=3.0 \text{ um}$); (b) $\Delta=100 \text{ ms}$ ($\sigma=21 \text{ um}$, $r=20 \text{ um}$); (c) $\Delta=1000 \text{ ms}$ ($\sigma=66 \text{ um}$, $r=200 \text{ um}$). The arrow indicates the signal of the flowing water, that emerges from the Gaussian shaped signal centred on $R=0$ of diffusing water. Reprinted from Scheenen TWJ, Vergeldt FJ, Windt CW, de Jager PA, Van As H. Microscopic imaging of slow flow and diffusion; a pulsed field gradient stimulated echo sequence combined with turbo spin echo imaging. *Journal of Magnetic Resonance* 151, 94–100, copyright 2001, with kind permission from Elsevier.
plant or tree to be imaged (Scheenen et al., 2002, Windt et al., 2006).

Also, when the distance between the magnetic field gradient coils is made smaller, higher gradient strengths can be obtained. This is important to obtain a high spatial resolution, fast switching of gradients, and to measure low flow velocities: small displacements require a high G value to be detected.

Another way to increase S/N is to use higher field strength, $B_0$. However, for plant tissues with extracellular air spaces, this results in increased susceptibility artefacts, as discussed above. These artefacts can be overcome by increasing maximum imaging gradients, but this would result in a decrease in S/N and loss of $T_2$ information (see equations 3 and 4). At higher $B_0$ the effective (and bulk) $T_2$ is (much) shorter than at lower field strength, limiting the number of measurable images during the signal decay, and resulting in a lower sensitivity to extract the permeability information from $T_2$ measurements (equation 1).

Some hardware solutions for intact plant NMR and MRI are presented in Figs 7 and 8. In Fig. 7a and b a low field (0.7 T) imaging system based on an open access electromagnet is shown. A comparable (permanent) magnet has been used by Utsuzawa et al. (2005) to study xylem cavitation due to pine wilt disease. On top of the magnet a climate chamber for environmental control is placed (Fig. 7b). Maximal access into the centre of the magnet is obtained by use of plan parallel gradient plates (Fig. 7a). To maximize the filling factor a (solenoid) rf coil is wrapped directly around the stem of the plants. A solenoid coil results in about a factor 2 to 3 more sensitivity than Helmholtz or birdcage coils of the same diameter. This configuration allowed large plants, up to a size of two metres, to be placed upright easily in the NMR

\[ \text{Fig. 6. Quantitative NMR flow images of xylem water moving upwards through the hypocotyl of Ricinus communis (a)–(d), and phloem water moving downwards to the roots (e)–(h). Shown are the volume of stationary water per pixel (a, e), the flow conducting area per pixel (b, f), the average linear velocity per pixel (c, g), and the average volume flow per pixel (d, h). The xylem flow images were calculated from a single flow imaging measurement; the phloem flow images were constructed from seven consecutive individual flow imaging measurements. The differences in the amounts of water per pixel shown in (a) and (e) are due to differences in image matrix size (128×128 versus 64×64), slice thickness (3 mm versus 6 mm) and scaling. After Peuke et al., 2006 (Peuke AD, Windt C, Van As H. 2006. Effects of cold-girdling on flows in the transport phloem in Ricinus communis: is mass flow inhibited? Plant, Cell and Environment 25, 15–25) and reproduced by kind permission of Blackwell Publishing.} \]

\[ \text{Fig. 7. Magnet (a) and climate control unit on top of magnet (b) of a 0.7 T imager based on an electromagnet. The two plan parallel gradient coils plates on top of the poles of the magnet (one is seen as a dark plate in the centre of the magnet, see arrow) results in maximum access to the centre of the magnet (a).} \]
magnet. Placement of plants can be undertaken without causing much stress to the subject, other than the stress that is caused by moving and handling it. Potentially stressful actions like mounting an rf coil or, when necessary, the removal of a branch or leaf, can be undertaken well in advance.

In Fig. 8 part of a dedicated intact plant 3 T MRI system is shown. The superconducting magnet has a 50 cm vertical free bore (Fig. 8a). One of the gradient coils is able to be opened. It consists of four parts and can easily be mounted around the stem of a plant or trunk of a tree (Fig. 8b, c). An rf coil (4 cm inner diameter) that consists of two parts completes the fully openable construction. Inside the bore of the magnet the climate is controlled by use of a remote climate control unit. This 3 T MRI system provides an excellent and unique infrastructure for MRI on intact plants and trees.

Future of functional plant MRI

The combination of dedicated NMR/MRI equipment and $T_2$, diffusion and propagator flow MRI methods is now available to measure and quantify cell, tissue, phloem and xylem hydraulics routinely in intact plants up to a size of several metres and over periods of weeks. Plant responses in hydraulics and water content of the different tissues can now be studied as a function of changes in environmental conditions. The method has already been applied to study day–night rhythms in flow and flow conducting area in the stem of large potted plants (Windt et al., 2006), dynamics in phloem in response to stem cold girdling (Peuke et al., 2006), root cooling (inducing xylem air embolism, follow refilling and functionality of xylem; Scheenen, 2001), root anoxia and long dark periods, and the flow (and changes therein) in the stalk of a tomato truss during a 5-week period of fruit development (CW Windt et al., unpublished results). Flow MRI has been proven to be quantitative in terms of volume flow. The additional information on effective flow conductive area is totally new. First results demonstrate surprising, unexpected dynamics in this parameter (Windt et al., 2006) which may result in a better understanding of the mechanism and regulation of long-distance transport. This information is very difficult to measure, or cannot be measured at all, in intact plants using other techniques. Flow MRI can be of help in understanding the results obtained by heat pulse methods, which are routinely used in field situations. First comparisons between flow MRI and (modified) heat pulse methods have very recently been made in our laboratory. Clearly, flow MRI teaches us some striking lessons about sap flow of importance for the interpretation of other methods, improving their reliability.

By contrast to the measurement of long-distance transport (xylem and phloem), MRI methods to study hydraulics on the membrane and tissue level based on diffusion measurements have not yet been demonstrated to be quantitative. First results indicate that MRI can at least be used to monitor changes in hydraulics within a single plant and that these MRI methods are sensitive for aquaporin functioning. All tissues, independent of position, are accessible by MRI, in contrast to, for example, the pressure probe technique. At the moment it has to be demonstrated that both $P_d$ and $P_f$ can be obtained by MRI. In the next step it would be necessary to corroborate MRI results by results obtained by more established and quantitative methods for hydraulic conductivity, for example, by high-pressure flow meter or by cell pressure probe. For this purpose, the development of MRI image guided cell pressure probe measurements will be of great interest, as well as combining pressure bomb or high pressure flow meter and MRI.

Functional intact plant imaging by MRI offers exciting new possibilities for the non-invasive physiological mapping of intact plants. Functional MRI is expected to
become a powerful tool to characterize functionality of aquaporins to be studied. In this way it will contribute to resolve the role of aquaporins in plant water balance, hydraulics and stress tolerance (Verkman, 2000; Javot and Maurel, 2002; Tyerman et al., 2002; Tournaire-Roux et al., 2003; Lee et al., 2005). Over-expression plants, knock-out plants or anti-sense plants show different aquaporin gene expression patterns compared with wild-type plants and may be of great help in understanding the relationship between aquaporin gene expression and function. NMR imaging can elucidate in vivo gene functionality in tissue hydraulics at different levels and thereby bridge the gap between gene expression and function.

Currently MRI in plant science is still far from being a routine tool. Some reasons for this could be the high costs of MRI equipment, the difficult theoretical fundamentals on which the technique is based, the horizontal orientation of most of the standard imaging set-ups, the limited accessibility (for large plants) of the magnetic field’s iso-centre of most set-ups, and the specific (climatic) requirements which have to be met when measuring intact plants. It is to be expected, however, that relatively cheap imaging set-ups based on permanent magnet systems will soon become available (Rokitta et al., 2000; Haishi et al., 2001).

For NMR flow and hydraulic measurements to be applicable in situ (greenhouses, field situations), quantitative non-spatially resolved (non-imaging) methods with specifically designed magnets are being developed. The basic principles and components are now available to develop (reasonably priced) NMR plant flow-meters, as are specially designed magnets (Raich and Blümich, 2004). We can also think about small NMR or MRI detectors by making use of rather small magnets, as presented by Blümich and others (Blümich et al., 2002).

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