Nuclear Magnetic Resonance Study of Spin Relaxation and Magnetic Field Gradients in Maple Leaves

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ABSTRACT ¹H Nuclear magnetic resonance techniques were used to measure the distributions of spin-spin relaxation times, T₂, and of magnetic field gradients in both the chloroplast and nonchloroplast water compartments of maple leaves (Acer platanoides). Results showed that encounters between water molecules and membranes inside chloroplasts provide an inefficient relaxation mechanism; i.e., chloroplast membranes interact weakly with water molecules. Gradient measurements indirectly measured the sizes of chloroplasts by showing that water in the chloroplasts is confined to small compartments a few μm in diameter. A comparison between measured gradients and gradients calculated for a model leaf indicated that chloroplasts are somewhat more likely to occupy positions along cell walls adjacent to air spaces, but also they may be found in the interiors of cells.

INTRODUCTION

For nuclear magnetic resonance (NMR) purposes, a leaf may be regarded as a system of water-filled compartments and air spaces. Its ¹H NMR spectrum is dominated by the signal from water. But, unlike the single, narrow peak that is the characteristic spectrum of bulk water, or the broad, asymmetric peak from the leaves of most plants, the NMR spectrum of a Norway maple (Acer platanoides) shade leaf presents two resolved peaks of approximately equal amplitude. The peaks are signals from water in different compartments; one is the signal from water inside the chloroplasts (chloroplast water), while the other is from water in all other leaf compartments (nonchloroplast water) (McCain and Markley, 1986). Relative peak intensities have been used to measure the fraction of leaf water allocated to the chloroplasts (McCain et al., 1988). Also, NMR images have been obtained that show the different spatial distributions of chloroplast water and of nonchloroplast water (McCain et al., 1993).

A maple shade leaf contains cells of several types and shapes arranged in a highly ordered structure. Typically, the cells are 10–30 μm in diameter. The chloroplasts are smaller compartments (diameter 2–3 μm) located inside the cells and concentrated midway between the outer leaf surfaces. Chloroplasts contain about half the total leaf water, while air spaces make up about 20% of leaf volume (McCain et al., 1988, 1993; McCain and Markley, 1992).

When separate peaks can be resolved in an NMR spectrum, it is possible to measure separate spin-spin relaxation times, T₂. Such measurements may provide information about the sizes of water compartments and the compositions of their walls, as well as the architecture of nearby air spaces that are responsible for magnetic field gradients.

The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence can be used to measure T₂ relaxation times and field gradients (Meiboom and Gill, 1958). The standard sequence, which normally is used to generate repeated echoes, may be truncated and used instead to produce a free-induction-decay (FID) at the nᵗʰ echo:

\[ 90°_0 - (τ - 180°_0 - τ)_n - FID \]

After the FID has been Fourier-transformed, an echo amplitude can be measured at each peak. For unrestricted spin diffusion on a linear magnetic field gradient, when τ is too long to cause spin-lock (Santyr et al., 1988), and when chemical exchange may be ignored (Hills et al., 1990), the amplitude of the nᵗʰ echo is given by Woessner (1961):

\[ A(n, τ) = A_0 \exp\left(-2nγ^2DG^2τ^3/3 - 2nτ/T_2\right) \]  

(1)

where A₀ is the echo amplitude at n = 0, τ is the interval between a 180° pulse and the following echo, γ is the gyromagnetic ratio of the spins, D is a diffusion coefficient, and G is the gradient strength. For the truncated CPMG sequence, we may substitute \( t = 2nτ \), and Eq. 1 reduces to the following (Yu, 1993):

\[ A(t, τ) = A_0(t) \exp\left(-κτ^2t\right) \]  

(2)

where \( A_0(t) = A_0 \exp(-t/T_2) \) and \( κ = γ^2DG^2/3 \). Eq. 2 applies also to a nonlinear field gradient, in which case \( κ = Δω^2/3τ_c \), where Δω specifies the range of accessible resonant frequencies (assumed to be a Gaussian distribution), and τ_c is a correlation time for diffusion through the gradients (Majumdar and Gore, 1988; Callaghan, 1991). The equations become much more complex when diffusion is restricted; in that case, echo amplitudes depend on the size and geometry of the restrictions and on the probability that relaxation occurs upon encounter with a barrier (Robertson, 1966; Snaar and van As, 1993; Yu, 1993; Araujo et al., 1993).

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This paper reports measurements of $T_2$ relaxation times and magnetic field gradient strengths in the chloroplast and nonchloroplast compartments of maple leaves. Experimental results are compared with gradients calculated from a model leaf structure. The major objective is to introduce NMR techniques that generate new kinds of structural information about plant leaves.

**MATERIALS AND METHODS**

Shade leaves were harvested from Norway maple trees (A. platanoides, L.) growing in the university arboretum at Wageningen. Data from three different cultivars ("Crimson King," "Faassen's Black," and "Goldsworthy Purple") were averaged together because the NMR results were indistinguishable. Discs (4 mm diameter) were cut from fresh, fully hydrated leaves and placed in a sample holder that orient to the leaf surface perpendicular to the applied field (McCain et al., 1984). All measurements were made at 293 K.

**NMR measurements**

Experiments were performed on Bruker AMX spectrometers (Bruker Instruments, Rheinstetten, Germany) at 300 and 500 MHz using the truncated CPMG sequence. Typically, 16 FIDs were summed, 180° pulse widths were about 12 μs, and the recycle time was 1.5 s. Before Fourier transformation, the FIDs were multiplied by an exponential apodization function that reduced decay time to ensure constant peak width. Peak heights were measured; they were effectively equivalent to integrals because the widths were constant.

$T_2$ was measured by fixing $\tau$ and changing different values of $n$ to vary $t$, the total elapsed time between the initial 90° pulse and the FID (i.e., $t = 2n\tau$, where $\tau$ intervals are regarded as beginning or ending at pulse midpoints). From Eq. 2, it is clear that the CPMG method does not measure pure $T_2$ relaxation times, when spins diffuse through magnetic field gradients, instead one finds a hybrid relaxation time ($T_2^*$) that depends also on $\tau$ and on $\kappa$, a parameter that is proportional to the square of the gradient strength:

$$T_2^* = T_2/(1 + \kappa \tau^2 T_2)$$  \hspace{1cm} (3)

Raw $T_2$ data (peak heights, $A(t)$, with $i$ in ms) were fitted to a multieponential function that featured variable amplitude coefficients, $a_i$, (restricted to positive values), and a set of relaxation times fixed at integral powers of 2 ms.

$$A(t) = \sum a_i \exp(-t/2^i)$$  \hspace{1cm} (4)

This function was chosen because its $a_i$ values provide a $T_2^*$ distribution curve (Kroeker and Henkelman, 1986). Multieponential fits that varied both the amplitudes and relaxation times gave standard deviations similar to those obtained by fitting to Eq. 4 (when comparing functions that used the same number of variables), but were less convenient to compare with other data.

Field gradients were measured by fixing $\tau$ while simultaneously varying $\tau$ and $n$. Peak heights, $A(\tau, i)$, were fitted to a function with the same form as Eq. 2:

$$A(\tau, i) = \sum a_i \exp(-\kappa_i \tau^2)$$  \hspace{1cm} (5)

with variable amplitude coefficients, $a_i$ (restricted to positive values), and with a set of fixed $\kappa_i$ values spaced at uniform intervals on a logarithmic scale. The $a_i$ values provide a distribution function for $\kappa$.

**Model calculations**

A model was devised to simulate the magnetic environment within a leaf (Fig. 1). The thickness of each cell layer, the distribution and dimensions of cells and air spaces, the fraction of water space at each depth, and total thickness (100 μm) in the model were chosen to match average experimental values from A. platanoides shade leaves (McCain et al., 1988, 1993). Chloroplasts were supposed to cover the inner surfaces of cell walls in the palisade and spongy layers (but not the epidermal layers) and to extend inward from the walls a sufficient distance to include the same fraction of total water space as measured at the equivalent depth in real leaves (McCain et al., 1988, 1993). To eliminate edge effects, the model was assumed to extend a large (effectively infinite) distance in all directions parallel to the leaf surface beyond a central reference section in which gradients were calculated. A three-dimensional coordinate system was superimposed on the model structure, and each grid point, $(x, y, z)$, was identified as belonging to either water space or air space; grid points were 2 μm apart.

The gradient calculation was based on the assumption that the magnetic field would be uniform inside a leaf if there were no air space (i.e., that air spaces perturb what would be otherwise a homogeneous field, and that other sources of magnetic heterogeneity are insignificant compared with air). The local field in water-filled compartments was modeled by regarding air spaces to be occupied by spherical dipolar magnets that were centered on the grid points and had volumes equal to that of a lattice cube. The field, $B_i$, at any grid point $(x, y, z)$, in the reference water space includes a sum of dipolar fields from magnets at all grid points, $(x, y, z)_k$, in the air space:

$$B_i = B_0 + (3/4\pi)\Delta\chi B_0 \sum_k (3 \cos^2 \theta_k - 1) r^3_k$$  \hspace{1cm} (6)

$BLe$ is the strength of the uniform field that would exist throughout the water space if there were no air space, $\Delta\chi$ is the volume susceptibility difference between air and water ($\Delta\chi = 0.0 - 0.3$ ppm), $r_k$ is the length (in units of the lattice spacing) of a vector that connects position $k$ in the air space with position $i$ in the water space, and $\theta_k$ is the angle between that vector and the applied field. There is no need to calculate field strengths in the air space because no NMR signals come from that region. For comparison with experimental data, the diffusion coefficient was assumed to be $D = 2 \times 10^{-5}$ cm² s⁻¹, and gradient-strength parameters (Eqs. 2 and 5) were computed at grid points $(x, y, z)$ by using:

$$\kappa_j = (\chi D / 3) \left( (\partial B_j / \partial x)^2 + (\partial B_j / \partial y)^2 + (\partial B_j / \partial z)^2 \right)$$  \hspace{1cm} (7)

**RESULTS AND DISCUSSION**

Fig. 2 presents a series of NMR spectra from one A. platanoides shade leaf, providing an example of a raw data from a truncated CPMG experiment. Two peaks are typical in spectra from this species (McCain et al., 1984), although leaves from most other species (and from some A. platanoides cultivars) provide only one, broad peak. The peak on the right has been assigned to water in the chloroplasts, while the peak on the left is the signal from water in all other leaf compartments (McCain et al., 1988; McCain and Markley, 1986, 1992). When thylakoid membranes inside the chloroplasts form an ordered array as they do in these leaves, chloroplast water diffuses through dipolar magnetic fields from paramagnetic ions bound to the membranes, and the chloroplast signal can be displaced by as much as 3 ppm from that of water in the other leaf compartments. In effect, the chloroplasts contain a built-in "NMR shift reagent."
Spin relaxation

Fig. 2 was obtained with fixed $\tau$ and variable $t$. The slower decay rate in the chloroplast peak demonstrates that chloroplast water has the longer $T_2^*$. Peak heights from this and other experiments were measured and fitted to Eq. 4 to construct the curves shown in Fig. 3, which represent $T_2^*$ distribution functions. Each curve is an average of data from 16 different leaves. Chloroplast and nonchloroplast data were obtained simultaneously from each sample, but different samples were used at 300 and 500 MHz. Plotted points ($a_i$ values from Eq. 4, normalized so that $\sum a_i = 1$) indicate the fractions of nuclei that exhibit relaxation times near each integral power of 2 ms (from $2^n = 2$ ms to 128 ms). Experimental error and biological variation caused the $a_i$ values to scatter with a standard deviation of about 20%; with 16 samples, the standard deviation of the mean should be about 5%.

Only $^1$H nuclei in freely diffusing molecules have relaxation times that fall in the experimental range from 2 to 128 ms; these include most but not all of the $^1$H nuclei in the sample. Nuclei in rigid environments have relaxation times in the microsecond range and therefore produce no signals in the truncated CPMG experiment. Hydrogens in highly mobile regions of membranes, hydrogens that exchange slowly with water, as well as those in water molecules confined to small pores and in “bound” water, have relaxation times that range from microseconds to a few milliseconds and might be barely detectable (Araujo et al., 1993); perhaps these contribute to the elevated point at 2 ms in the non-chloroplast $T_2^*$ distribution (Fig. 3). There is no corresponding rise at 2 ms in the chloroplast signal because a nucleus must diffuse freely before its resonance can be shifted to the position of the chloroplast peak.

Both the chloroplast and the non-chloroplast $T_2^*$ distributions (Fig. 3) extend over a limited range and peak at a most probable value. The range is narrower, and the most probable relaxation times are longer in chloroplast water. Measurements at 500 MHz give distributions that are consistently shifted toward shorter $T_2^*$ than at 300 MHz. Interpolating between plotted points, nonchloroplast peaks occur at about 12 ms at 500 MHz or about 20 ms at 300 MHz, and the chloroplast peaks are at 22 and 32 ms (at 500 and 300 MHz, respectively). These numbers may be extrapolated to zero-field by using Eq. 3 where $k$ is $300^2/500^2 = 0.36$ times as large at 300 MHz as at 500 MHz. The extrapolated zero-field $T_2$ value for nonchloroplast water is $\approx 32$ ms, and for chloroplast water it is 43 ms.

When water is confined to small compartments, and when each encounter with the wall is a relaxation event, diffusion to the wall becomes the dominant relaxation mechanism. For example, walls appear to be responsible for most $T_2$ relaxation in water-saturated wood (Araujo et al., 1993). Maple leaf cells exhibit a variety of sizes and shapes, but typically their diameters (along the shortest axis) range from 10 to 20 $\mu$m (McCain et al., 1988). Theory predicts that water-filled compartments with these dimensions and with efficient wall relaxation should exhibit $T_2$ values in the range from 20 to 40 ms (Araujo et al., 1993). Experimental results are in good agreement with theory; a maximum occurs 32 ms in the $T_2$ distribution (extrapolated to zero field) of nonchloroplast water. Although $T_2$ data do not reveal a detailed mechanism for the interaction between
walls and water, they are at least consistent with the idea that wall relaxation is efficient in the nonchloroplast compartments.

In strong contrast to the nonchloroplast results, \( T_2 \) data demonstrate that wall relaxation must be relatively ineffective in chloroplasts. A maple chloroplast is only 2–3 \( \mu \)m in diameter (McCain et al., 1988); if it contained only water, efficient wall relaxation would require a \( T_2 \) of 4–6 ms (Araujo et al., 1993). But extrapolated data show a peak in the \( T_2 \) distribution at 43 ms. Furthermore, chloroplasts are packed with thylakoid membranes; if these were effective for wall relaxation, \( T_2 \) would be reduced to even shorter values. The long experimental relaxation times can be reconciled with theory only by concluding that wall relaxation is inefficient in chloroplasts.

Inefficient wall relaxation in chloroplasts may be related to the unusual lipids that are major components of chloroplast membranes. Thylakoids and the inner part of the chloroplast envelope are made from galactolipids that are different chemically from the phospholipid membranes found in other cellular compartments (Gounaris and Barber, 1983).

Inefficient wall relaxation in chloroplasts is an important phenomenon for leaf NMR because the chloroplast and nonchloroplast peaks could not be resolved without it. The mechanism for chloroplast peak displacement requires water molecules to diffuse throughout the chloroplast without relaxation within a period of a few milliseconds (McCain and Markley, 1986).

Several \( T_2 \) relaxation mechanisms can be shown to be relatively unimportant in leaves. Intercompartment exchange rates, e.g., are slower than spin relaxation rates. Saturation-transfer NMR experiments have been used to measure an 88 ms exchange lifetime in chloroplasts from another plant species (McCain and Markley, 1985); the same measurement is impractical in maple because \( T_1 \) (~300 ms) is not sufficiently longer than the exchange lifetime, but preliminary experiments demonstrate that water molecules remain inside maple chloroplasts for longer than 100 ms. Paramagnetic relaxation rates also are relatively slow, even though dipolar fields from Mn\(^{2+}\) ions shift the chloroplast water signal (McCain and Markley, 1986). Efficient paramagnetic relaxation requires a rapid exchange of water molecules in the first Mn\(^{2+}\) coordination sphere, but the ions are embedded in the thylakoid, and membrane-bound ligands may occupy their first coordination spheres.

**Magnetic field gradients**

Fig. 4 displays sample data that were acquired from a single leaf using the CPMG experiment with variable \( \tau \) and \( t = 20 \) ms. Echo amplitudes were measured from spectra that resembled those in Fig. 2. By fixing \( t \), all dependence on \( T_2 \) has been eliminated in this experiment. The decay in echo amplitude with increasing \( \tau \) provides evidence that water molecules move between different microenvironments, probably by diffusion along magnetic field gradients.

Note that chloroplast echo amplitudes approach a non-zero asymptote as \( \tau \) increases (Fig. 4); this indicates that some water molecules experience a constant average magnetic field as they diffuse through chloroplasts. One possibility is that some chloroplasts are located where the gradient is small; however, results from the calculation show that there are very few locations in a model leaf where the gradient remains sufficiently small over a volume as large as that of a chloroplast. A better explanation is that water molecules, confined within chloroplasts where there is in-
efficient wall relaxation, are able to diffuse throughout the volume during the time interval between echoes (i.e., 2τ); therefore, the molecules experience the same average environment between successive echoes, and the echoes refocus accurately (Robertson, 1966). Data in Fig. 4 suggest that water molecules begin to experience a constant environment after ~2τ = 2.5 ms, during which time they diffuse a distance of ~3.2 μm (if D = 2 × 10^{-5} cm^2 s^{-1}); the diameter of a maple chloroplast is 2–3 μm (McCain et al., 1988).

Fig. 5 displays a set of normalized q_i values (Σ_i q_i = 1) obtained by fitting 500 MHz data such as those in Fig. 4 to the function in Eq. 5; plotted points are averages from 16 different leaves (σ ≈ 20%). The non-zero asymptote (Fig. 4) causes some chloroplast water to apparently experience κ = 0; these molecules (~9% of the total) represent a fraction that is off-scale in Fig. 5. Note that the distribution of chloroplast gradients is displaced slightly to higher κ, and a minor peak near κ = 10^{10.5} s^{-3} is more pronounced in the chloroplast than in the non-chloroplast gradients; but even so, distributions are similar in the two compartments.

Fig. 5 shows that the most probable value of the gradient strength parameter in either compartment is near κ = 10^9 s^{-3}, but in chloroplasts another moderately probable value is near κ = 10^{10.5} s^{-3}. For linear gradients, these experimental results correspond to gradient strengths of 458 and 814 Gauss/cm, respectively (with κ = γ^2 DG^2/3 and D = 2 × 10^{-5} cm^2 s^{-1}). Or if gradients are nonlinear (a more realistic assumption), we may substitute τ_c = d^2/2D, where d is the distance that spins must travel to span Δω, into κ = Δω^2/3τ_c to estimate: κ = 2γD(Δω/d)^2 = 2γDγ^2(G^2) (Callaghan, 1991). The experimental κ values correspond to nonlinear r.m.s. gradients of 323 and 576 Gauss/cm, respectively.

Gradient strength in a leaf is determined by the size range of the heterogeneity (i.e., the sizes of cells and air spaces) and by magnetic volume susceptibility differences (e.g., the 0.7 ppm difference between air and water). In a 500 MHz NMR magnet, e.g., a difference of 0.7 ppm in total field strength across a distance of 8 μm corresponds to a gradient of 1.0 T/m or 100 Gauss/cm. Actual gradients in maple leaves may be larger than 1.0 T/m because most of the water is <8 μm from an air space and because the field is shifted in both positive and negative directions.

Fig. 6 shows the distribution of gradient strength parameters calculated from a model leaf. For direct comparison with experimental distributions, they are plotted in the same format as Fig. 5. Although experimental results show similar chloroplast and nonchloroplast gradient distributions, calculated chloroplast and nonchloroplast distributions are quite different from each other; the calculated chloroplast distribution peaks near κ = 10^{10.5} s^{-3}, while the nonchloroplast distribution peaks near 10^9 s^{-3}.

The calculated and experimental average gradient distributions (Figs. 5 and 6, solid lines) are similar; this suggests that the cellular architecture of the model leaf was a reasonable approximation to a real leaf. However, the calculated chloroplast and nonchloroplast distributions (Figs. 5 and 6, dashed lines) are quite different from experimental distributions, indicating that the model was unrealistic in its placement of the chloroplast and nonchloroplast compartments.

The model invariably places chloroplasts in contact with cell walls and restricts nonchloroplast water in the palisade.
and spongy mesophyl layers exclusively to interior regions of the cells; the model was designed this way because it is well known that chloroplasts in higher plants tend to be located preferentially near cell walls. Therefore, model chloroplasts are exposed to larger gradients because they always are adjacent to air spaces. If, instead, chloroplasts occasionally resided in the interior, and if nonchloroplast water sometimes reached the walls, then the actual gradient distributions might be weighted averages consisting of appropriate proportions of the interior and wall distributions. For example, the experimental chloroplast distribution (Fig. 5) can be reproduced approximately by adding 60% of the calculated chloroplast distribution (Fig. 6) to 40% of the calculated nonchloroplast distribution, and the experimental nonchloroplast distribution can be approximated by a sum made with the opposite proportions (40 and 60%, respectively). The significance of these quantitative results is uncertain because the literature supplies no quantitative measurements with which they can be compared; the fraction of chloroplasts in contact with the walls has not been reported.

CONCLUSIONS

1) The wall relaxation mechanism is inefficient in chloroplasts. 2) NMR relaxation data suggest that chloroplast water is confined to small compartments a few μm in diameter (consistent with the actual size of chloroplasts). 3) The combined effect of restricted diffusion and inefficient wall relaxation allows many chloroplast water molecules to experience a constant average magnetic field, even though the chloroplasts are located on a magnetic field gradient. 4) Average magnetic field gradients in real leaves resemble average gradients in the model leaf. 5) Experimental results suggest that chloroplasts are somewhat more likely to reside near cell walls, but they can be found also in the interiors of cells.

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