Water permeability of chloroplast envelope membranes

In vivo measurement by saturation-transfer NMR

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In tulip tree (*Liriodendron tulipifera*) leaves, the proton NMR signal from chloroplast water is resolved from that of water in other leaf compartments. We used the saturation-transfer NMR method to measure the mean water molecule residence time within a chloroplast, (88 ± 17) ms at 20°C. From the measured chloroplast dimensions, we calculate an effective permeability coefficient of (9 ± 2) × 10^{-4} cm/s for the chloroplast envelope membrane. This is the first in vivo measurement of chloroplast water permeability.

Chloroplast *Water exchange* *Membrane permeability* *Water compartment*

1. INTRODUCTION

The proton nuclear magnetic resonance (NMR) spectrum of a plant leaf is predominantly the spectrum of water, its major mobile constituent. Therefore, we may expect the NMR spectrum of a leaf to be a single peak. Leaves from most plant species do show a single NMR peak. However, the leaves of some deciduous trees display complex, orientation-dependent spectral patterns that change as the angle between the leaf surface and the applied magnetic field is varied [1]. Different components of the patterns have different proton spin-lattice relaxation times, a fact that demonstrates the presence of discrete water compartments with different magnetic environments. We have proposed [1] that the anisotropic components are signals from water in chloroplasts that have their thylakoid membranes aligned with respect to the leaf surface, and we have developed a theoretical model that explains the NMR results [2]. Water in other leaf structures contributes to the isotropic spectral component.

Proton spin relaxation has been used to detect water compartments in wheat leaves [3] and crowns [4], maize roots [5] and leaves [6], dogwood stems [7], ivy bark [8], *Elodea* [9], and *Nitella* [10]. Water is generally present in plant tissue in two or more compartments that are isolated (on an NMR time scale) by slow exchange rates. The compartments in bark and stems have been identified [7,8] as extracellular and intracellular water. Different relaxation times from vacuolar and cytoplasmic water can be distinguished in maize roots [5].

NMR has been used to measure water permeation rates through plant membranes. Stout et al. [11] measured water proton relaxation rates in *Chlorella* cells suspended in media containing Mn^{2+}; they found a mean residence time of 25 ms for water molecules inside the cells and a diffusional water permeability coefficient (P_d) of 2.1 × 10^{-3} cm/s for the cell walls. P_d values for other plant membranes [5,8,9,12] are in the range from about 1 × 10^{-4} to 5 × 10^{-2} cm/s. Water exchange through the thylakoid membrane inside chloroplasts is fast; Wydrzynsky et al. [13], using isolated, broken chloroplasts, measured exchange lifetimes of less than 20 ms.

Techniques used previously to measure plant membrane permeabilities are not applicable to the study of chloroplast envelope membranes in vivo;

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however, our ability to resolve an NMR signal from chloroplast water, as distinct from water in other compartments, enabled us to use the method of saturation transfer [14]. The chloroplast signal can be saturated differentially, effectively 'labeling' water protons in one compartment differentially. As labeled water molecules diffuse to other compartments they carry the saturation to other spectral components. Water exchange rates may be measured by following the time evolution of the NMR spectrum after a saturation event.

2. EXPERIMENTAL

Leaf tissue from *Liriodendron tulipifera* (tulip tree) was chosen because the spectra are reasonably well resolved and highly reproducible. *L. tulipifera* leaves were field collected from three different trees and studied immediately; 4 mm leaf disks were excised and oriented perpendicular to the magnetic field using the NMR-tube inserts described previously [1]. Spectra were recorded at 20°C on a Nicolet NT-470 spectrometer operating at 470 MHz. Fig.1 shows the spectrum of a typical leaf; all *L. tulipifera* specimens gave almost identical spectra. We have assigned the high field shoulder (peak C) to chloroplast water [1] based on its orientation dependence. The major peak (B) is an isotropic signal from water in the cytoplasm and vacuole as well as from extracellular water. A low field shoulder, often seen in spectra of other species and labeled 'peak A' in these [1] is weak or absent in *L. tulipifera*.

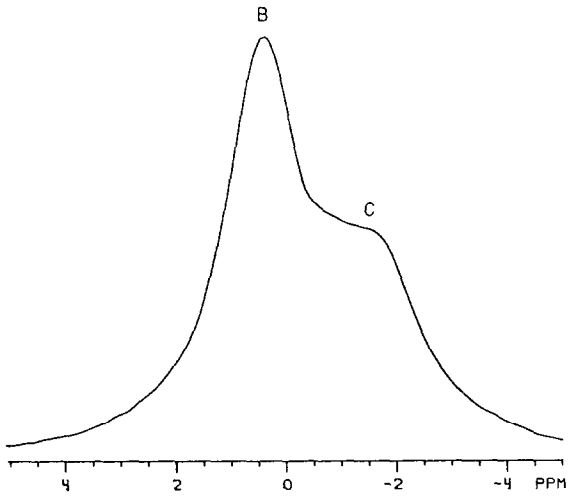

Fig.1. Proton NMR spectrum (470 MHz) of a section of *L. tulipifera* leaf oriented with its surface perpendicular to the applied magnetic field. Field intensity increases to the right; the chemical shift reference point is arbitrary. Peak B (the major peak) is from isotropic water in the vacuole, cytoplasm, and extracellular space. The chemical shift of peak C (a high-field shoulder) depended on the orientation of the leaf in the static magnetic field; it is assigned to water in chloroplasts.

For saturation transfer experiments the pulse sequence used was SP-*t*-OP-AT-PD, where SP is a 25 ms partial saturation pulse applied using the proton decoupler and t is a variable delay time. Typically, a set of 32 different t intervals, ranging from 5 ms to 3.5 s, were employed in each experiment. After a high power 90° observation pulse (OP), the free induction decay was recorded during an 85 ms acquisition time (AT). A 3.5 s pulse delay (PD) was inserted to allow the system to return to equilibrium, and the pulse sequence was repeated 8 times at each t value. A complete experiment required 20 min; during this time interval the leaf spectrum and its proton relaxation times do not change perceptibly.

3. RESULTS AND DISCUSSION

Table 1 presents representative results for the spin-lattice relaxation times of peaks B (T_{1B}) and C (T_{1C}) as measured by a standard inversion-recovery pulse sequence. Data from more than 30 *L. tulipifera* specimens show that T_{1B} is consistently 10–15% smaller than T_{1C}. In other plant species, T_{1B} is less than T_{1C} by amounts ranging from 10 to 40% [1]. The experimental T_1 data for peaks B and C could be fit adequately to single-exponential recovery functions. This suggests that the T_1 values of all major components of peak B are similar.

Campbell et al. [14] derived equations for all types of saturation transfer in a two-compartment model. In their notation, our measurements correspond to 'Experiment II' with a 'selective' presaturation pulse and no dipolar interaction. The presaturation pulse need not be absolutely selective; the only requirement is that at $t = 0$ the saturation intensity is greater in one compartment than in the other.

Assuming that rapid diffusion homogenizes the water protons within each compartment, then saturation intensity introduced into peak C at time

Table 1

Results of relaxation time and saturation transfer measurements of 8 *L. tulipifera* samples at 20°C. Values at the bottom are means (and SD)[a]

Spin-lattice relaxation times (ms)		Saturation-transfer relaxation times (ms)		Exchange lifetimes (ms)
T_{1B}	T_{1C}	τ_s	τ_f	τ_e
745	821	915	40.2	74
757	912	936	28.9	103
738	760	853	35.0	67
644	643	751	33.9	89
586	781	934	30.0	107
689	735	928	30.5	103
768	847	703	31.8	97
642	802	839	31.5	62
696 (± 62)	788 (± 75)	857 (± 84)	32.7 (± 3.4)	88 (± 17)

[a] The ^{1}H NMR longitudinal relaxation times of water in chloroplasts (T_{1C}) and in 'other' (T_{1B}) compartments (vacuole, cytoplasm, extracellular space) were measured by an inversion-recovery experiment. The slow (τ_s) and fast (τ_f) time constants for the decay of saturation intensity were obtained by fitting the data of fig.3 to equation (1). The mean residence time for water within chloroplasts (τ_e) was calculated from eqn (2)

$t = 0$ decays as a double exponential function of time:

$$S(t) = S_f e^{-t/\tau_f} + S_s e^{-t/\tau_s} \quad (1)$$

where $S(t)$ is the saturation intensity in peak C, τ_f and τ_s are fast and slow time constants, respectively, and S_f and S_s are intensity coefficients. The more rapid exponential decay (time constant τ_f) is caused by the flux of water from one compartment to another. The slower decay constant (τ_s) is approximately equal to the spin-lattice relaxation time. The assumption of rapid diffusion is supported by the contrast between the experimental mean lifetime of water in the chloroplast (88 ms) and the calculated time required for water to diffuse a distance equal to the average radius of a chloroplast (3 ms) [1]. Saturation intensity in peak B also follows eqn (1) with the same τ_f and τ_s values but with different intensity coefficients.

The mean residence time for water protons inside the chloroplasts (τ_e) is given by the following expression:

$$\tau_e = \frac{\tau_s \tau_f (S_s^* S_f - S_f^* S_s)}{S_s^* S_f (\tau_s - \tau_f)} \quad (2)$$

where the starred symbols (S_f^* and S_s^*) are saturation intensity coefficients measured from peak B. Eqns 1 and 2 can be derived easily from those in [14], assuming that exchange is fast compared to spin-lattice relaxation.

A set of 32 spectra (fig.2A) were obtained by saturating the high field shoulder (peak C). Each trace was recorded using a different delay interval (t) following the selective saturation pulse. The notch visible in the lower traces corresponds to the signal removed by saturation. By $t = 3500$ ms (the top trace) the spectrum has recovered completely and resembles fig.1.

Fig.2B displays a set of traces that represent the difference between the final (top) spectrum and each of the first 31 spectra of fig.2A. Negative deviations in the difference spectra are a measure of saturation intensity. Fig.2B shows that immediately after the presaturation pulse, saturation intensity grows rapidly in peak B and decays rapidly in peak C. At long delay times the saturation intensities in peaks B and C become approximately equal as they slowly decay together, showing that chloroplast water exchanges with approximately an equal quantity of cytoplasmic water.

Fig.3 presents a plot of saturation intensities measured from the difference spectra. The difference peaks (in fig.2B) are not fully resolved; therefore, saturation intensities were measured by curve-fitting, assuming the two peaks have equal width. The solid lines represent least-squares fits to double exponential functions with $S_s = 741$ (arbitrary units), $S_f = 162$, $S_s^* = 530$, $S_f^* = -88$, $\tau_f = 40.2$ ms, and $\tau_s = 915$ ms. The negative value of S_f^* indicates that saturation intensity initially increases with time in peak B. From these data we calculate $\tau_e = 74$ ms; this value is entered on the top line of table 1 where it is compared with data from 7 other leaf samples.

Saturation-transfer experiments in which the major peak (peak B rather than C) was saturated yielded τ_f values similar to those reported in table 1. However, saturation transfer from peak B to C

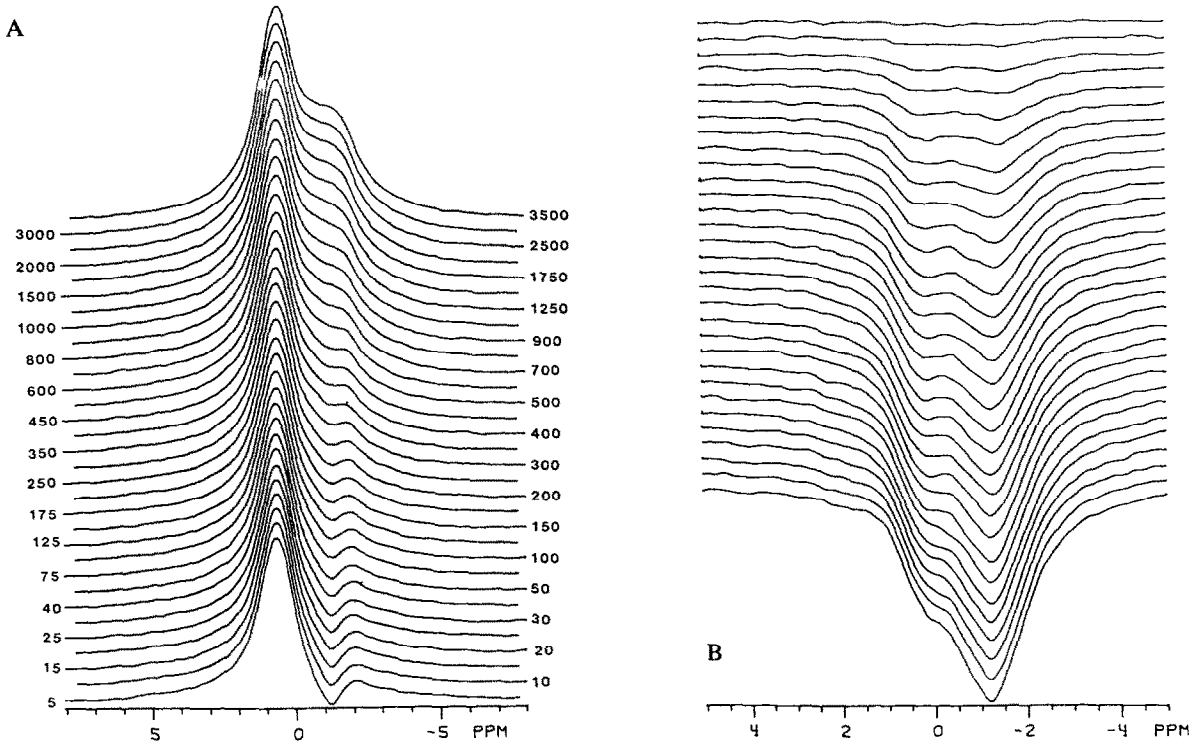

Fig.2. Saturation transfer ^{1}H NMR spectra of a *L. tulipifera* leaf section oriented with its surface perpendicular to the applied magnetic field. (A) A set of 32 traces showing recovery of the spectrum following saturation of the peak assigned to chloroplast water. Saturation power was applied for 25 ms at a frequency corresponding to −1.2 ppm on the arbitrary chemical shift scale. Each trace was obtained after waiting a variable delay time (t) following the saturation pulse. Traces are labeled with t values which ranged from 5 to 3500 ms. (B) A set of 31 ^{1}H NMR saturation-transfer difference spectra for delay times t = 5 to 3000 ms. Each trace represents the difference between the corresponding trace in (A) and the top trace (3500 ms) in (A). Negative deviations are a measure of saturation intensity.

is less efficient than the reverse process; a smaller fraction of the total saturation was transferred. Saturation intensities in the major peak always remained much larger than those in the high field shoulder, even at long delay times. From this we conclude that a large fraction of the saturation introduced into peak B resides in compartments that do not exchange water protons rapidly with the chloroplasts. For this reason, data obtained by saturating peak B cannot be analyzed using eqn 2; it would require a 3-compartmental model. The result that T_{1B} is less than T_{1C}, despite rather rapid chloroplast-cytoplasm exchange, suggests that protons in compartments unconnected with the chloroplasts have shorter relaxation times than protons in the chloroplasts and in the surrounding cytoplasm. It appears that the lifetime for water exchange between non-chloroplast compartments is comparable to T_1 based on the observation (in many plant species) that T_{1B} is only slightly shorter than T_{1C}. This conclusion is consistent with the findings of Bacic and Ratkovic [5] who found an exchange lifetime of 155 ms across the cell membranes in maize roots.

Under the light microscope, *L. tulipifera* chloroplasts appear ellipsoidal, with a long axis of 5.7 μm (SD + 0.7 μm). The short axis measures 3.8 ± 0.6 μm. Neglecting any effects the thylakoid membrane may have on water flux, the water permeability coefficient (P_d) can be calculated [9] from τ_e (average value 88 ± 17 ms) and the chloroplast dimensions using $P_d^{-1} = (A/V)\tau_e$,

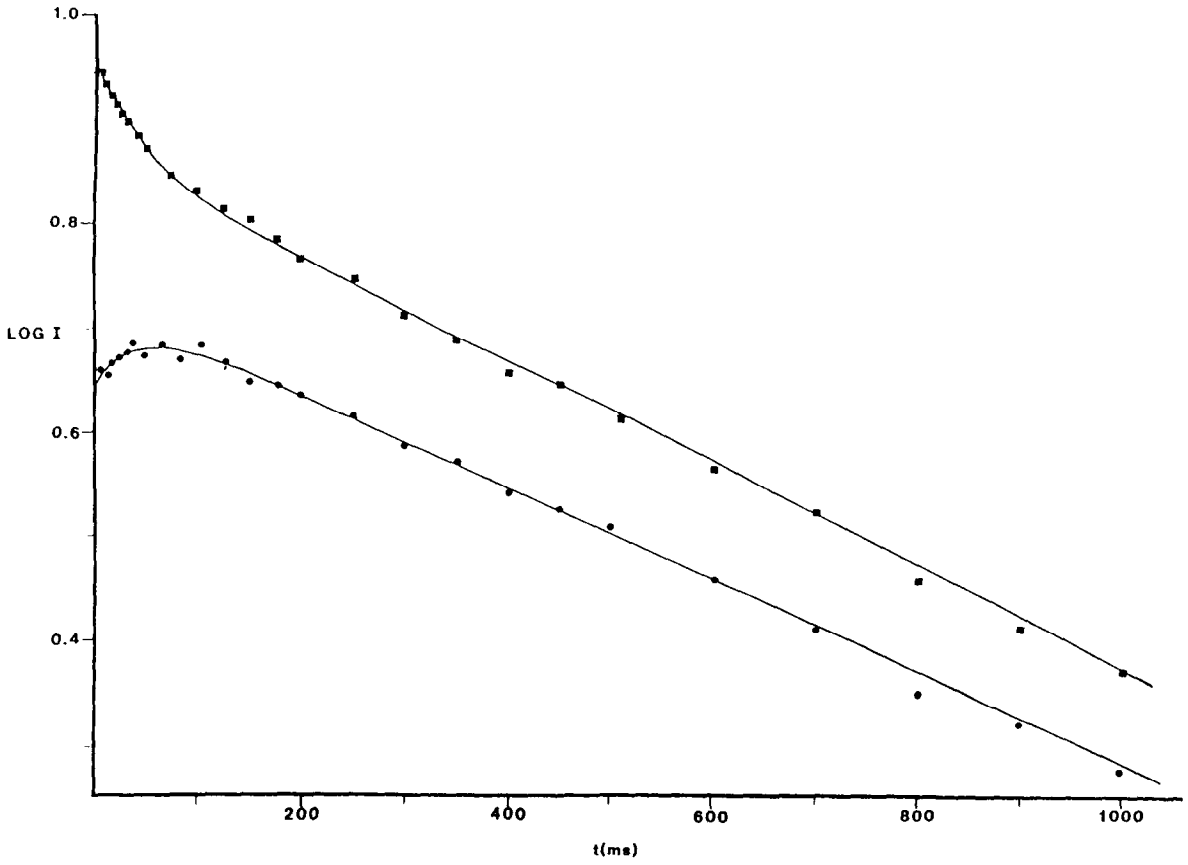

Fig.3. Time course of saturation intensity following irradiation of the ^1H NMR peak of chloroplast water in a *L. tulipifera* leaf [13]. The saturation intensity (*I*) was measured from the difference spectra in fig.2B. Upper curve: chloroplast water signal (−1.2 ppm). Lower curve: isotropic signal from water in the cytoplasm, vacuole, and extracellular space (0.2 ppm). Solid lines represent least-squares double-exponential fits of the data to eqn 1. The initial, non-linear part of each curve indicates exchange control, whereas the later, linear part represents relaxation control of the magnetization. Extrapolation of the linear component of each curve to time $t = 0$ shows that the saturation intensity lost from the chloroplast is approximately equal to that gained by the cytoplasm.

where A/V is the surface to volume ratio. We found $P_d = (9 \pm 2) \times 10^{-4}$ cm/s for the chloroplast envelope membrane.

Chloroplasts are bounded by a permeable, double membrane that readily passes small neutral molecules such as CO_2 or H_2O but not larger ones such as sucrose [15]. If chloroplast envelopes were to present no barrier to diffusion, then the mean residence time for water within a chloroplast would be approx. 3 ms, i.e., the time required for a water molecule to diffuse a distance half the mean diameter of a chloroplast [1]. The much longer experimental residence time (88 ms) suggests that a considerable barrier to diffusion exists, while the experimental value of the permeability coefficient is in the normal range observed for plant membranes [13].

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