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Some plant leaves have orientation-dependent EPR and NMR spectra

(water compartmentalization/chloroplasts/thylakoid membrane/manganous ion)

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ABSTRACT **Proton nuclear magnetic resonance (^{1}H NMR) spectra of leaves from 50 plant species were obtained at a spectrometer frequency of 470 MHz. Water present in leaf samples gives rise to characteristic spectral patterns. Most species show only one broad ^{1}H NMR peak; however, the leaves of some plants display complex, orientation-dependent spectra in which a common three-line pattern is discerned. The pattern varies with the angle between the leaf surface and the external magnetic field. Proton relaxation measurements show the presence of at least two water compartments in the leaves. The compartments are responsible for different components of the spectral pattern. EPR spectra, obtained at 35 GHz and at a temperature of −180°C, of plant leaf sections are dominated by the strong signals of manganous ions. We find that most plant leaves have isotropic Mn^{2+} EPR spectra. However, in some species (including ones that exhibit orientation-dependent ^{1}H NMR spectra) we detect orientation-dependent intensities in the forbidden lines; the spectra indicate that Mn^{2+} ions occupy binding sites with axial or lower symmetry on nonrandomly oriented membranes. Both the NMR and the EPR results suggest that the chloroplasts of some plants are preferentially aligned with respect to the leaf surface.**

Photosynthesis takes place in a highly ordered membrane system (1). The membranes (thylakoids) occur in parallel stacks (grana) inside the chloroplasts, and most chloroplasts are found within layers of specialized cells. The existence of such a highly structured system suggests the possibility that photosynthetic membranes may be preferentially aligned with respect to the leaf surface. Indeed, some plant cells actively control the orientation of their chloroplasts in response to light stimuli (2).

It is difficult to measure the net alignment of thylakoids in a leaf. Grana are easily viewed by electron microscopy, but to do so it is necessary to focus on individual chloroplasts or on very small regions of the leaf. Net thylakoid alignment (as a statistical property) might be more readily measured if the membranes provided an orientation-dependent spectroscopic signal. We have found such signals in both NMR and EPR spectra.

Plant leaves are mostly water. Therefore, one might expect the ^{1}H NMR spectrum of a leaf to be essentially the spectrum of water—a single line. In previous ^{1}H NMR studies of plant material, a single broad line usually has been observed. One well documented exception is in the spectrum of dogwood stems, which have orientation-dependent patterns caused by geometrical effects of the sample shape (3). Previous ^{1}H NMR studies of plant tissue have concentrated on the effects of freezing (4). Spin–lattice (t_1) and spin–spin (t_2) relaxation times have also been measured; for example, exchange times between water compartments were determined in ivy bark (5). Leaf samples seldom have been studied.

The EPR spectrum of a plant leaf always shows the presence of manganous ions (6, 7); most leaf manganese is concentrated in the chloroplasts. The characteristic Mn^{2+} EPR spectrum consists of six strong lines (from hyperfine coupling to ^{55}Mn, $I = 5/2$) and ten weaker lines (the so-called "forbidden" lines resulting from the simultaneous change of electron and nuclear spin quantum numbers). Forbidden lines are absent when Mn^{2+} experiences an octahedral crystal field; for axially distorted fields, forbidden-line intensities depend on the angle between the distortion axis and the applied magnetic field (8).

We have discovered orientation-dependent ^{1}H NMR and Mn^{2+} EPR spectra in plant leaves. Only a few species show the effects, but in these we find evidence of water compartmentalization, including evidence that one of the compartments is anisotropic and preferentially aligned with respect to the leaf surface. These observations provide a new approach for studying the structure and organization of the photosynthetic apparatus and for investigating the distribution and chemical environment of water and manganous ion in plant leaves.

MATERIALS AND METHODS

NMR Measurements. A Nicolet Magnetics NT-470 spectrometer was used for ^{1}H NMR measurements at 470 MHz. Free induction decays were collected using 1,000 data points in a ±5 kHz window following a 90° (7 μsec) pulse; 32 averaged transients collected with a 2-sec recycle time provided excellent signal-to-noise ratios. Additional ^{1}H NMR spectra were obtained at 200 MHz with a Nicolet NT-200. Sample temperatures were 18–20°C, except as noted. The results were unaffected by the sample spinning rate. Water was used as an external chemical shift reference. A standard inversion–recovery experiment was used to measure t_1; t_2 was measured with a Carr–Purcell sequence (9).

Young, but fully grown, leaves were collected in the field and used immediately. In some cases, we recorded an NMR spectrum <3 min after a leaf was severed from the living plant. By using a sharp cork borer, 4-mm-diameter discs were excised from leaf areas without large veins. Samples were assembled in standard 5-mm NMR tubes. To study leaves oriented perpendicular to the magnetic field—i.e., perpendicular to the NMR sample tube axis—we first inserted a 1.5-cm cylinder cut from Pyrex rod (nominally 4-mm diameter, selected for close fit to the inner diameter of the NMR tube), followed by the leaf disc, and finally a longer section of rod (≈16 cm). Such an assembly has very little air space; the leaf disc is enclosed in Pyrex.

To study orientation effects, four additional sample tube

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inserts were constructed. Three Pyrex rods were each cut at a point 1.5 cm from one end; cuts were made at angles of 30°, 45°, and 60° to the rod axis. Cut rods were assembled with leaf discs as described above. Finally, to study samples with surfaces parallel to the field, assembly began with a 1.5-cm section of rod, and then the leaf disc was positioned between the two halves of a 4-mm length of rod that had been split down its axis; assembly was completed with a 16-cm length of rod. Altogether, the five insert assemblies allowed the leaf disc to be oriented so that a vector normal to its surface made angles of 0°, 30°, 45°, 60°, and 90° to the applied field.

Inserts were tested using a control sample of moist filter paper. At every angle, the filter paper gave a single line with constant width (≈1 ppm at half height) and no evidence of unresolved structure. As the filter paper disc was rotated in the magnetic field, the line center shifted progressively; from 0° to 90°, it moved downfield by about 1 ppm. The angular dependence of the shift is easily explained because the magnetic field strength, H, inside a plane surface with finite thickness is given by (3, 10)

$$H = H_0 (1 - \delta \cos^2\theta), \qquad [1]$$

where H_0 is the applied magnetic field, θ is the angle between H_0 and a vector normal to the surface, and δ is the difference between the volume susceptibility of the region inside the surface and that of the surrounding medium. The shift observed in filter paper fits this equation with $\delta = 1$ ppm. We conclude that the inserts are magnetically homogeneous; they do not distort the spectra or contribute spurious signals.

EPR Measurements. We used a Varian E-109Q (Q-band, 35 GHz) EPR spectrometer equipped with a rotatable magnet. This instrument is well suited for these studies because Mn^{2+} EPR spectra are better resolved at Q-band than on a conventional X-band (9 GHz) spectrometer and because the rotatable magnet allows us to vary the magnetic field orientation without adjusting either sample position or cavity tuning. Operating conditions were 100-kHz modulation at 5 G amplitude; microwave power levels were <1 mW.

Fully grown leaves were collected in the field and examined within 1 hr of harvesting. Samples were prepared by cutting leaves into 2 × 6 mm sections, avoiding veins; three to five such sections were stacked into a Teflon jig, inserted into a 3-mm quartz EPR sample tube, and plunged into liquid nitrogen. Sample temperatures were maintained at *ca.* −180°C in the spectrometer cavity.

In describing the experimental geometry, we shall refer to a laboratory-fixed axis system. The sample and the EPR cavity are fixed, while the magnet may be rotated about the y-axis. Samples are inserted in the cavity with leaf surfaces parallel to the xy plane. Initially, the magnetic field is parallel to the z-axis (the rf field is parallel to y). We define this orientation (static field normal to the leaf surface) as the 0° angle. Successive spectra were obtained while rotating the magnet through 7.5° or 15° increments. Control spectra were obtained from samples with leaf surfaces parallel to the xz plane; in these samples the magnetic field vector remains parallel to the leaf surface regardless of magnet rotation. Another set of control spectra were obtained from macerated leaves packed into EPR sample tubes.

RESULTS AND DISCUSSION

NMR Survey Spectra. Leaves from 50 different plant species were examined. Most species showed one broad ^1H NMR line, often with a hint of structure in poorly resolved shoulders. Oaks, grasses, and thick-leafed plants with a high water content (such as spinach, Fig. 1, curve e) were among this group. The remaining species, mostly deciduous trees, displayed complex spectra. Some especially well resolved examples are shown in Fig. 1 (curves a–d); these spectra illustrate the range of species variation. The spectra in Fig. 1 were obtained at 470 MHz, but we observed almost identical spectra at 200 MHz. Therefore, the linewidths and peak separations are proportional to the magnetic field strength, and we may conclude that dipolar coupling effects, which are independent of field strength, make no significant contributions to the line shapes.

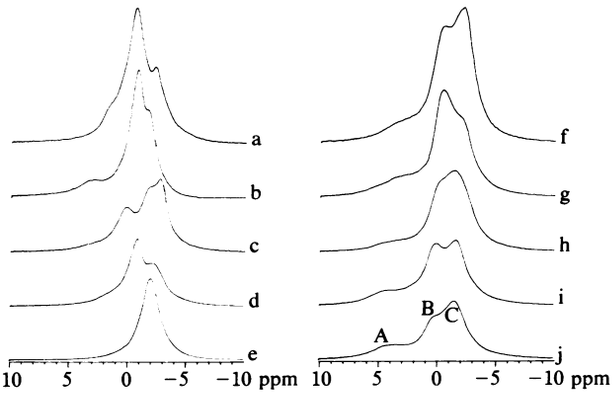

FIG. 1. ^1H NMR spectra, obtained at a spectrometer frequency of 470 MHz, of leaves from different species of plants: a, pawpaw (*Asimina triloba*); b, boxelder (*Acer negundo*); c, redbud (*Cercis canadensis*); d, tulip poplar (*Liriodendron tulipifera*); e, spinach (*Spinacia oleracea*); f–j, hawthorn (*Crataegus* sp.). A disc of leaf material 4 mm in diameter was cut from a freshly picked leaf and oriented in the NMR probe so that the leaf surface was perpendicular to the applied magnetic field. On the chemical shift scale, 0 ppm is approximately the position of the bulk water signal.

Although line positions and relative intensities do vary, all spectra in Fig. 1 (except spinach) appear to consist of three lines. To facilitate discussion, these are labeled A, B, and C, starting from the low field (left-hand) side. A is a weak line or shoulder (sometimes very weak as in curve d); B and C are stronger, with C usually from half to twice the size of B. All species with resolved spectra fit the general ABC pattern, but the spectrum of each species is characteristic. From species to species, the patterns differ in resolution, line position, and relative line intensities. Among specimens within a species, the major differences are in the relative line intensities. Curves f and g show the degree of variation within a single species of hawthorn; these spectra were selected as representative of results from 17 different leaf discs taken from four individual trees. All hawthorn spectra show the ABC pattern more or less well resolved, and all have about the same line positions.

NMR Orientation Dependence. Plant leaves showing well resolved ABC patterns were studied as a function of orientation. The spectrum of an individual leaf disc does not change noticeably in the 10 min required to examine all five angles. Two typical sets of orientation-dependent spectra are shown in Fig. 2. The 0° results may be compared with Fig. 1. At 30°, line spacings in the ABC pattern contract, and at 45° or 60° only a single line usually can be discerned. Structure reappears at 90° in the form of a low-field line or shoulder, sometimes accompanied by a weak line on the high-field side. The 90° spectrum resembles a poorly resolved mirror image of the 0° pattern. We find also a progressive angle-dependent offset similar to that described in *Materials and Methods* for moist filter paper.

NMR Spin–Lattice Relaxation. We measured spin–lattice relaxation times in many leaves and found t_1 to be quite species dependent; it varied from 70 msec in the single line of white oak (*Quercus alba*) to 1.2 sec in line C of Jerusalem artichoke (*Helianthus tuberosus*). However, it is not very specimen dependent. Repeated measurements on different leaves of the same species gave t_1 values that agree to within

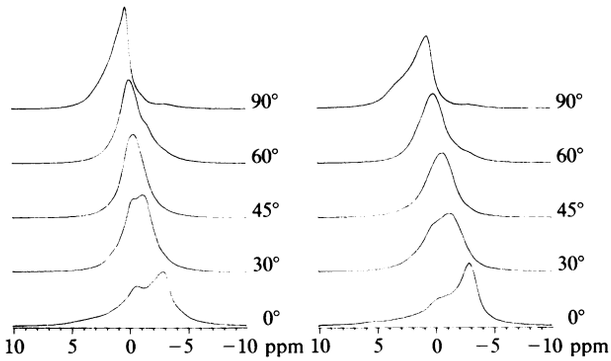

FIG. 2. ^{1}H NMR spectra, obtained at a spectrometer frequency of 470 MHz, of plant leaf discs (4 mm diameter) oriented at different angles in the magnetic field. Two species of trees are represented. (*Right*) Buckeye (*Aesculus glabra*). (*Left*) Cottonwood (*Populus deltoides*). The angle is defined as that between the applied magnetic field and a vector normal to the leaf surface.

±20%. Within our experimental accuracy of ±10%, t_1 was found to be independent of magnetic-field strength (at 200 and 470 MHz), and for periods up to 1 hr, t_1 does not depend on the age of the sample. With few exceptions, the t_1 data obtained conformed to single exponential recovery functions.

If individual lines in the ABC pattern could be labeled, it would be easier to follow their positions as the leaf angle is changed. t_1 relaxation provides an appropriate label. In each spectrum with an ABC pattern, lines B and C have significantly different t_1 values. (Line A is weak and too close to line B to be measured accurately.) For example, lines B and C in a hawthorn specimen at a 0° angle had t_1 values of 0.38 and 0.53 sec, while in a persimmon (*Diospyros virginiana*) leaf they were 0.21 and 0.28 sec, respectively. The existence of two distinct t_1 values means that at least two water compartments must be present in the leaf and that diffusion across whatever barrier separates the compartments does not lead to complete proton exchange in times less than t_1.

t_1 was measured as a function of orientation. In the hawthorn spectrum at 30°, the partially resolved lines B and C gave t_1 values of 0.36 and 0.50 sec. At 45° and 60°, the single line had t_1 values of 0.41 and 0.40 sec, respectively. At 90°, t_1 values of the low-field component and the major line were 0.50 and 0.42 sec, respectively. As signals overlap, the measured t_1 becomes a weighted average of t_1 from each component; this explains the intermediate values at 45° and 60°. It is clear that the spectrum inverts as the sample is rotated from 0° to 90°. Line C (the long t_1 component in hawthorn) appears as a low-field shoulder at 90°. Similar observations were made with a number of different species. In each case we find that line C crosses through the center of the spectrum as the leaf is rotated.

Inversion of a spectrum caused by sample rotation normally indicates the presence of magnetic anisotropies aligned along a sample-fixed axis system, as in a crystal. The water protons detected are certainly not crystalline; they must be located in a fluid medium because linewidths and relaxation times are inconsistent with a solid phase. We conclude that the leaves contain at least one intrinsically anisotropic water compartment. The internal axis system of this compartment is aligned with respect to the leaf surface.

These observations suggest that chloroplasts are the anisotropic compartments responsible for line C. Chloroplasts are lens-shaped organelles 2–5 μm in size that often comprise 20–30% of the total volume in a leaf cell; they are surrounded by a semipermeable membrane and contain lamellar membrane structures (grana) that establish an internal axis system.

NMR Spin–Spin Relaxation. The t_2 measurements yielded nonexponential decay curves. To a fair approximation, our data could be fitted to the sum of two single-exponential functions, and can be interpreted by postulating the presence of two (or more) water populations, each with a characteristic t_2 value. Biphasic t_2 relaxation often has been observed for water in plant tissue (3–5); it has been explained (3) as the result of partitioning water into "bound" (i.e., motionally restricted) and "bulk" environments. Provided that exchange between compartments is rapid enough, a biphasic t_2 is compatible with a single-exponential t_1.

Typically, within a given sample, line C exhibits a larger fraction of the long t_2 component than does line B, but t_2 values were found to vary with the age of a specimen or from one specimen to another. The range of variation depends on the magnetic field strength: the short t_2 components clustered between 3 and 6 msec (with some values well outside that range) at 470 MHz; they ranged between 10 and 20 msec at 200 MHz. The long t_2 components clustered between 15 and 30 msec at 470 MHz; they ranged between 30 and 40 msec at 200 MHz.

The observed linewidths (*ca.* 500 Hz at 470 MHz) are larger than would be predicted from the t_2 values (10–100 Hz). We attempted to use selective proton decoupling in a "hole-burning" experiment to determine the intrinsic linewidths, but we found evidence instead for saturation transfer resulting from proton exchange. An analysis of these data will be published elsewhere.

NMR Effects of Drying and Freezing. Leaf discs from a number of species were prepared, and their ^{1}H NMR spectra were recorded every 15 min. In the interval between runs, leaf discs were removed from the sample holder and exposed to dry air. Over a period of time, the intensity of the signal faded as the leaf disc dried. However, line B always decreased faster than line C, especially during the first hour. Some spectra in which line C was initially smaller than B eventually developed the opposite intensity ratio. A typical series is shown in Fig. 3 (curves g–m). In other experiments, some of the drying leaf discs were immersed in water, then blotted dry, and reexamined; the results showed restoration of approximately the original signal pattern when dehydration lasted for only a short time (less than about 30 min). Leaf discs that were retained in the sample holder and not removed to dry showed very little change in a period of about 1 hr. These observations provide additional informa-

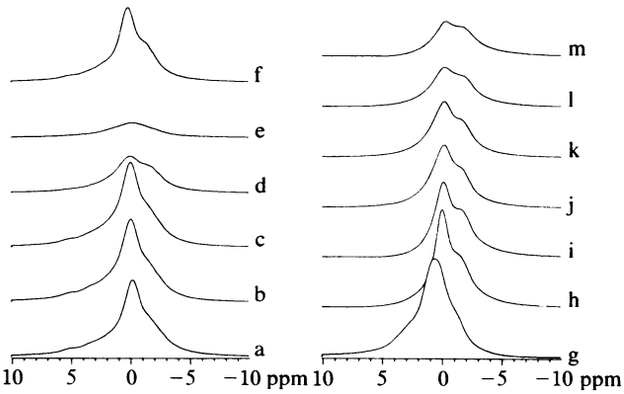

FIG. 3. ^{1}H NMR spectra at 470 MHz of leaf discs (4 mm diameter) from a tulip poplar (*L. tulipifera*) obtained with the leaf surface held perpendicular to the applied magnetic field. Curves a–f, a series of spectra of a fresh leaf specimen obtained at different temperatures. a, +5°C; b, 0°C; c, −1°C; d, −3°C; e, −5°C; and f, warmed to +5°C after −5°C. Curves g–m, a series of spectra obtained from a single specimen beginning 5 min after the leaf was picked from the tree (curve g) and continuing through successive 15-min intervals of air drying until 95 min after picking (curve m).

tion that helps in assigning ^1H NMR signals to specific water compartments. Drying rates suggest that lines C and B are derived from different compartments and that the compartment responsible for line C dries more slowly than that responsible for line B. Vacuoles often contain more water than any other plant cell compartment, and they are the first to lose water under conditions of water stress. Perhaps line B may be assigned, at least in part, to vacuoles.

Curves a–f show spectra recorded from a single leaf disc of tulip poplar subjected to a series of progressively lower temperatures before being rewarmed. As the leaf freezes, its ^1H NMR signal decreases substantially but does not disappear. The residual signal (curve c) represents unfrozen water associated with substances that depress the freezing point. The spectrum of the thawed leaf (curve f) does not reproduce exactly the initial spectrum (curve a). Our results are quite similar to those of published ^1H NMR studies of cold acclimation—for example, in wheat leaves (11)—except that the previous studies did not resolve signals from different cell compartments.

EPR Results. Leaves of 28 plant species were studied. The Mn^{2+} EPR spectrum is easily seen in every specimen, but it varies considerably in intensity and resolution. In some species (oaks) the Mn^{2+} signal is strong but poorly resolved. In others (spinach), it is well resolved but weak, and it is superimposed on the spectra of organic radicals (narrow signals at $g = 2$) often detected in chloroplasts (12). Our best results came from *Photinia glabra* (redtop, an ornamental shrub). We examined about 20 leaves from several individual *Photinia* plants and found strong well-resolved Mn^{2+} signals. The Mn^{2+} spectrum is much stronger than that of spinach—about 100 times as strong for equal sample volumes. Signals are so strong that they mask completely the spectra of organic radicals in the chloroplasts. *P. glabra* leaves also give a typical three-line ^1H NMR spectrum.

The Mn^{2+} ions we detect in these leaves are not those at the active site for photosynthetic O_2 release. Active-site Mn^{2+} is EPR silent under normal conditions (12–14). Nor is our signal due to aquo ions, $Mn(H_2O)_6^{2+}$, which have broader signals and no forbidden lines. Our spectrum appears to be that of Mn^{2+} ions in a storage pool (13, 14). In some species, storage pool Mn^{2+} is far more abundant than either active-site Mn^{2+} or aquo ions. The function of storage-pool ions has not been established.

Fig. 4*a* shows a typical *P. glabra* spectrum. All leaves of this species give very similar results; the resolution varies slightly from leaf to leaf, and line shapes are a subtle function of orientation, but these effects would not be readily apparent in spectra reduced to the size of Fig. 4*a*. The 6 major lines are the allowed ones; their intensities are independent of orientation (except for small line-shape effects). Between each adjacent pair of allowed lines are 2 forbidden lines (10 in all). The low-field line of each forbidden pair is nearly obscured by overlapping wings from the allowed lines. Fig. 4*b* shows the 2 forbidden lines at the very center of the spectrum (on a much expanded scale). A trace was recorded every 7.5° from angles of 0° to 90°. Nearly all *Photonia* leaves studied showed orientation-dependent forbidden-line intensity variations of >10%. In the best examples, the intensity varied by a factor of two over the range 0°–90°. Control spectra from macerated leaves and leaves with surfaces perpendicular to the axis of magnet rotation showed angle-dependent variations of <5%.

Spectra resembling Fig. 4 have been observed before in manganous–protein complexes. The spectrum of Mn^{2+} in concanavalin is one example (15, 16). Such spectra have been successfully simulated (16) using a model that assumes a quadratic crystal field for the metal ion. Theory requires that the forbidden-line intensities be orientation dependent (8). For ions in a site with axial symmetry, the forbidden-line

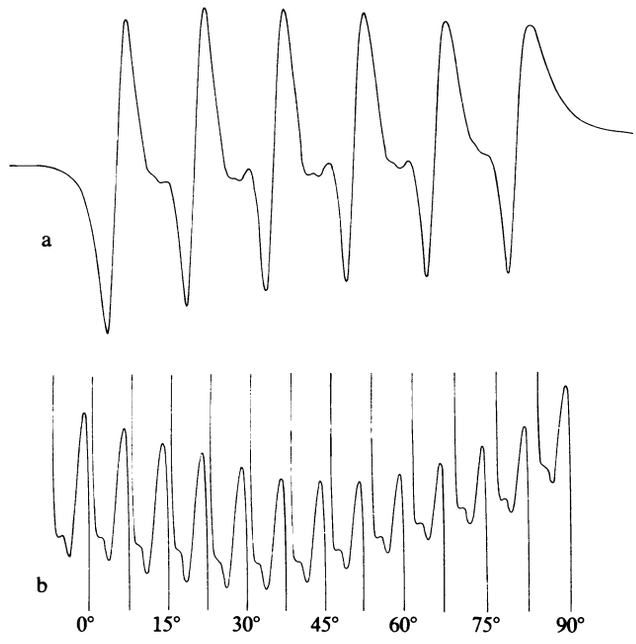

FIG. 4. EPR spectra at 35 GHz of leaf sections from redtop (*Photinia glabra*). (*a*) A typical spectrum. (*b*) The region at the center of the same spectrum on a much-expanded scale showing the innermost forbidden lines as a function of sample orientation in the magnetic field. The sample orientation was changed from 0° to 90° in increments of 7.5°.

intensity is proportional to $\sin^2 2\theta$, where θ is the angle between the magnetic field and the unique axis. Our observation of intensity variations is direct evidence of nonrandom alignment. The manganous ions in *P. glabra* must be bound to membranes that are at least partially oriented with respect to the leaf surface.

We have devised a computer model that simulates NMR and EPR spectra closely resembling those we report here. The model fits both types of spectra; it also reproduces the pattern of orientation dependence we observe. Our model assumes that Mn^{2+} ions are bound to specific binding sites on oriented planar surfaces. Details will be published elsewhere.

Conclusion. Both the NMR and the EPR experiments show that the leaves of some plants contain partially aligned, magnetically anisotropic structures; the results strongly suggest that these structures are thylakoid membranes. This conclusion is hardly surprising, because photosynthetic systems are known to be highly ordered. More important, these observations introduce a new method for studying the organization and structure of leaves and thylakoids. Using ^1H NMR or EPR one could compare the degree of chloroplast orientation with environmental factors such as the direction and intensity of light to which a leaf had been exposed. Also, it would be interesting to compare the species differences in ^1H NMR or EPR spectra with structural differences observed using microscopy. Because signals from different water compartments can be resolved, ^1H NMR might be used to measure rates of transmembrane water exchange *in vivo*. ^1H NMR also could be used to study water stress or cold acclimation, allowing quantitative measurements on organelles in intact leaves.

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