JOURNAL OF MAGNETIC RESONANCE **127,** 17–25 (1997)
ARTICLE NO. MN971185

Using Bulk Magnetic Susceptibility to Resolve Internal and External Signals in the NMR Spectra of Plant Tissues

Yair Shachar-Hill,*,† Douglas E. Befroy,‡ Philip E. Pfeffer,* and R. George Ratcliffe‡

**U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, Pennsylvania 19038; and ‡Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom*

Received January 14, 1997

Internal and external NMR signals from a variety of plant cells and plant tissues can be resolved by changing the bulk magnetic susceptibility (BMS) of the perfusing medium with [Gd(EDTA)]$^-$ or Dy(DTPA-BMA). This separation is observed in samples consisting of cylindrical cells oriented along the B_0 field, and is consistent with established theoretical predictions about BMS effects. Evidence is presented that the shifted signals represent material outside the tissue as well as some contribution from intercellular spaces and cell walls, while intracellular signals are unshifted. The paramagnetic complexes used to separate the signals are shown to be nontoxic and to have no effect on a number of transport processes. The method has been applied to roots, shoots, and giant algal cells, facilitating the interpretation of the *in vivo* spectra from a range of biologically important magnetic isotopes. The potential of the method for studies of transport is illustrated with experiments showing: (i) $^{14}N/^{15}N$ isotopic exchange of nitrate in roots; (ii) the influx of HDO into root and shoot segments; and (iii) the use of saturation transfer to follow water movement into and out of plant cells.

INTRODUCTION

Resolving the contributions from internal and external pools is an important objective in NMR studies of transmembrane transport, and clear discrimination can only be expected when a property of the observed signal depends on the location of the detected species (*1*). In general, differences in chemical shift provide the most unambiguous discrimination and such differences may arise in several ways. First, there may be intrinsic chemical shift differences between the signals from different compartments, usually due to the effects of pH, ionic environment, or BMS. Second, external paramagnetic shift reagents may be used to induce chemical shifts in the signals from cations (reviewed in (*2*)) and some anions (*3, 4*). Third, external paramagnetic and ferromagnetic reagents can be used to alter the magnetic susceptibility and to generate differences in chemical shift between internal and external compartments (*5–7*). The first two of these mechanisms lack generality with regard to the observed species, for example, paramagnetic shift reagents are rarely useful for the *in vivo* discrimination of the signals from neutral molecules, and it is perhaps only the third that offers the opportunity to resolve internal and external signals from the full range of detectable ions and metabolites. Unfortunately susceptibility effects are critically dependent on sample geometry (*6, 8, 9*, and references therein) and this imposes severe limitations on their exploitation. Indeed susceptibility effects are more usually a complication in NMR, and it has only been in rather particular circumstances that such effects have been exploited (*9–11*). However for many plant tissues, the regular cylindrical geometry and the permeability of the extracellular space suggests that susceptibility reagents could provide an effective method for separating internal and external signals. Here we show that complexes of gadolinium and dysprosium may be successfully used with perfused root and shoot segments to resolve intracellular and extracellular signals from a range of small molecules. This resolution allows transport and exchange to be monitored in a variety of situations of biological interest.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays*, L. cultivars W 7551, FRB 73, and LG20.80) seeds were germinated in the dark for 2–3 days at 25–30°C on absorbent paper soaked in 0.1 m*M* $CaSO_4$. For experiments involving nitrate and ammonium uptake, the nitrate transport system was induced by incubating 2-day-old seedlings for 12–18 h in an aerated medium containing 10 m*M* KNO_3/0.1 m*M* $CaSO_4$. For experiments on intact seedling, 2-day-old seedlings were placed on a perforated sheet of Styrofoam over buckets of continuously aerated 0.1 m*M* $CaSO_4$ solution so that their roots grew straight down. These seedlings were grown hydroponically for 2–4 days under continuous illumination; 10 m*M* NaCl was added

1090-7807/97 $25.00

to the growth medium for the last 24 h. Experiments were conducted on five different sample types: (i) 2.5-cm-long root segments taken from 5 mm behind the root tip of straight primary roots; (ii) 2.5-cm shoot segments cut from straight shoots between the seed and the first node; (iii) 15-mm root tips; (iv) 5-mm root tips; and (v) intact seedlings. *Chara corallina* cells, the gift of Dr. R. M. Spanswick (Cornell University), were grown in artificial pond water (*12*). Intact cells, 2–5 cm long and approximately 1 mm in diameter, were excised. Leeks (*Allium porrum*, L. cv Musselburgh) were grown for several months as described previously (*13*), and straight sections, 4–6 cm long, were cut from the middle region of the root. Cells, tissues, and seedlings were placed in 10-mm NMR tubes and carefully oriented along the axis of the tube. *Chara* cells and leek root segments were aligned by placing them in a 4-cm-long section of a 9-mm od. glass tube which was then inserted into the NMR tube. Samples of long root tips were arranged so that the tips were in the sensitive volume by placing half the tissue at the bottom of the tube with the tips facing up and half above with the tips pointing down. Cells and tissues were oxygenated in buffer solutions using either an airlift system (*14*) or continuous perfusion at flow rates of at least 6 ml/min.

NMR Spectroscopy

^{1}H, ^{14}N, ^{15}N, ^{31}P, ^{35}Cl, and ^{37}Cl spectra were recorded at 7.05 T on a Bruker CXP 300 spectrometer, and ^{1}H, ^{2}H, ^{13}C, ^{23}Na, and ^{31}P spectra were recorded at 9.4 T on a Varian Unity*plus* 400 spectrometer using 10-mm probeheads. Proton spectra and spectra of quadrupolar nuclei were recorded under fully relaxed conditions (i.e., pulse angles of between 60° and 90°, recycle times of greater than 3 T_1). Spin–lattice relaxation times were measured by inversion recovery. Saturation of water signals during magnetization transfer experiments was achieved by selective irradiation for 5 s with the minimum power required to suppress the signal. Proton-decoupled ^{13}C NMR spectra were obtained using the GARP sequence throughout, and proton decoupled ^{14}N ammonium signals were obtained by gated broadband decoupling. Susceptibility shifts in simple solutions were characterized using coaxial NMR tubes; paramagnetic complexes were added to the solution in the outer of the two tubes. Bulk susceptibility shifts were measured by recording the ^{1}H spectrum of the coaxial sample.

Optical Microscopy

Short segments, around 2 mm long, were excised from 5 mm behind the tip of primary roots of maize seedlings with a stainless steel razor blade and immersed in a solution of 1% glutaraldehyde buffered with 0.1 *M* imidazole HCl at pH 6.8 for 2 h. The segments were subsequently embedded in epoxy resin, sectioned, stained with methylene blue, and mounted for optical microscopy according to methods described by Richardson and co-workers (*15*). Images were acquired with a Star 1 cooled CCD camera (Photometrics, Ltd., Tucson, AZ) on a Nikon Diaphot inverted microscope. Digital images were processed and displayed using IPLab software (Signal Analytics Corp., Vienna, VA).

Reagents

Analytical grade gadolinium (III) salts were obtained from Aldrich Chemical Co. and sprodiamide (the bis-*N*-methylamide of Dy.diethylenetriaminepentaacetate, Dy (DTPA-BMA)) was generously supplied by Nycomed (Oslo, Norway).

RESULTS AND DISCUSSION

Extensive analysis of the susceptibility effects to be expected in the spectra of samples of various geometries suggests that cells of right cylindrical shape aligned along the B_0 magnetic field should provide readily interpretable spectra in the presence of an external susceptibility reagent (*6, 8*). Many plant roots and shoots are rather regular in anatomy, being cylindrical in shape and consisting largely of cells that are themselves cylindrical with their long axes parallel to the tissue axis. Maize roots are one such tissue, and Figs. 1A and 1B show that the shape of most of the cells is rather close to a right cylinder with an aspect ratio (i.e., axial length divided by the radius of the cross section) of over 6. The principal axes of the cells are oriented parallel to the tissue axis. These considerations suggest that the use of media of different bulk magnetic susceptibility should allow the separation of internal and external signals.

Figure 1C is a ^{1}H spectrum of a sample of maize root segments oriented parallel to the axis of the NMR tube and perfused with oxygenated buffer containing 10 m*M* $[Gd(EDTA)]^{-}$. The separation of the two water signals in this spectrum, which was only observed when the root tissue was aligned along the tube axis, was very close to that obtained in a phantom sample of two concentric coaxial tubes in which the outer compartment contained 10 m*M* $[Gd(EDTA)]^{-}$ (data not shown). Using Dy(DTPA-BMA) instead of $[Gd(EDTA)]^{-}$ increased the separation in spectra of root segments and a comparison of the two contrast reagents in phantoms at different concentrations showed that Dy (DTPA-BMA) gave shifts that were larger by a factor of 1.5–1.6 than $[Gd(EDTA)]^{-}$. This value is intermediate between the 1.34 predicted from the relative paramagnetic moments of the two rare earth cations (*9*) and the 1.8 reported by Fossheim and co-workers (*16*) for the ratio of bulk magnetic susceptibilities of Dy(III) versus Gd(III) complexes. Springer and co-workers (*6, 8*) have shown that the following holds for right cylinders in a medium to which a para-

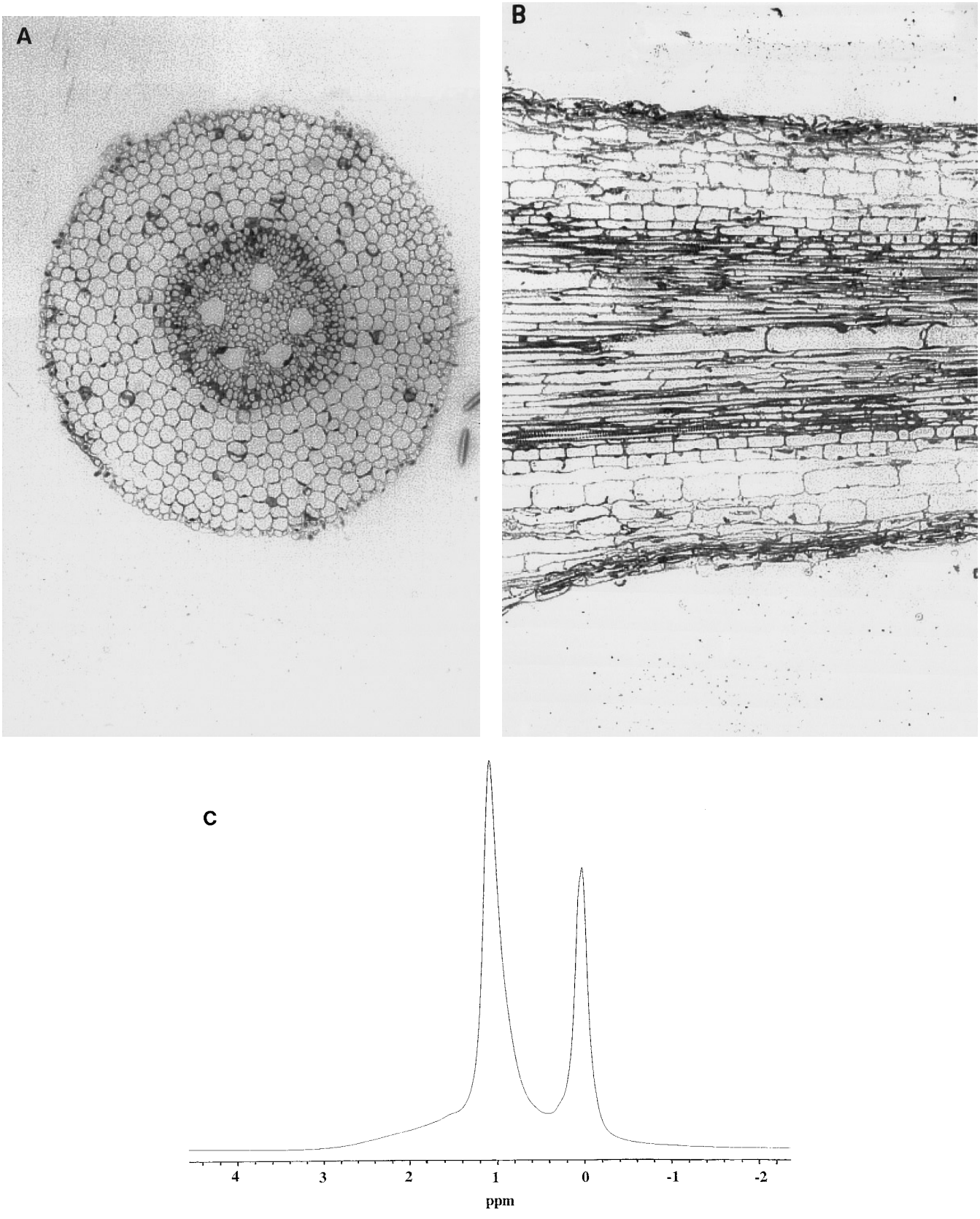

FIG. 1. The separation of internal and external water signals from perfused maize roots by the addition of a paramagnetic reagent and the regular anatomy that makes this possible. (A) Optical micrograph showing transverse section through a maize root segment. (B) As (A) but longitudinal section. The diameter of the cross section (A) is close to 2 mm, and the micrograph in (B) is shown at the same magnification as (A). (C) The 400-MHz ^{1}H spectrum of a sample of maize root segments perfused with oxygenated buffer containing 50 m*M* glucose, 1 m*M* $CaCl_2$, 10 m*M* HEPES, pH 7, and 10 m*M* $[Gd(EDTA)]^{-}$.

magnetic reagent has been added: (i) the separation between internal and external signals is caused by a shift of the external signal to higher frequency (lower field); (ii) there is rather little dependence of the predicted shifts on length for right cylinders with an aspect ratio of above 6, so that the separation is of the same magnitude as for infinite cylinders; and (iii) there is a strong dependence of shift on the orientation of the long axis of such cylindrical shapes relative to the field axis. The observations on root segments in the presence of gadolinium or dysprosium complexes were entirely consistent with these predictions and indicate that the separation of the signals was due to a BMS effect.

In order to explore the usefulness of this approach we examined a number of different plant cells and tissues and acquired spectra of several nuclei that are commonly used for *in vivo* NMR spectroscopic studies of transport and metabolism in plants (*17, 18*). Figure 2 shows representative results from giant algal cells (Fig. 2A), shoot segments (Figs. 2B and 2C), and excised root tissues (Figs. 2D, 2E, and 2F). Leek root segments also yielded spectra with resolved internal and external signals (not shown). The requirement to align the tissue along the B_0 axis was easily satisfied, but in the case of root tips it was necessary to use a minimum length that exceeded the diameter of the 10-mm tube rather than the 5-mm root tips commonly used in *in vivo* NMR studies of plants (*17, 18*). As would be expected for shifts due mainly to susceptibility effects, the separation between the internal and external signals, measured in ppm, was largely independent of the observed nucleus so that the successful resolution of the signals only depended on their linewidths. Signals from cations (Figs. 2D and 2F), anions (Figs. 2D and 2E), and neutral molecules (Figs. 2A, 2B, and 2C) were separated to a similar extent for the same concentration of a given susceptibility reagent. Paramagnetic shift reagents, for example, Co^{2+} in the case of chloride (*3*) and $[Dy(TTHA)]^{2-}$ in the case of sodium and ammonium (*2*), can be used to resolve signals of the ions observed in Fig. 2, and in general such contact and pseudocontact shifts are larger than the shifts induced by similar concentrations of susceptibility reagents. However, paramagnetic shift reagents are not generally applicable to neutral molecules, because of the weak binding interaction, and in these cases the susceptibility effects illustrated in Figs. 2A, 2B, and 2C are likely to be particularly useful. Moreover paramagnetic shift reagents are not available for all biologically important ions nor can they be used to follow both cations and anions in the same sample (Fig. 2D), and in such cases susceptibility effects provide an attractive alternative. We have previously shown (*4*) that Gd^{3+} may be used as a shift reagent for nitrate, but since Gd^{3+} is toxic it cannot be used for studies of nitrate in roots. In contrast Gd(DTPA-BMA) was used to resolve internal and external nitrate signals from maize root segments and was found to be nontoxic. In analyzing the origin of the separation in that case it is now apparent that it is due predominantly to susceptibility effects. This is consistent with the analysis by Chu *et al.* (8, and references therein) who have convincingly argued that the shifts induced by $[Gd(EDTA)]^-$ and by Dy(DTPA-BMA) in ^{23}Na spectra of phantoms are dominated by BMS effects.

BMS effects can also be exploited in the spectra of whole seedlings, and Fig. 3 shows the resolution of internal from external water, chloride, and sodium signals in the roots of intact maize seedlings. Differences in the shift induced in the signals of the three nuclei (also seen in phantoms) are due to small contact or dipolar shifts induced by the Dy (DTPA-BMA). In a previous study (*3*), it was shown that narrow ^{35}Cl signals of chloride can be seen in the *in vivo* NMR spectra of algal and higher plant cells. This is in marked contrast to the rather broad signals recorded from mammalian cells and tissues, and it was suggested that this signal arises from chloride in the vacuole where there is little broadening of the signals by binding to macromolecules. In Figs. 2E and 3 it may be seen that such narrow signals are also observable from maize root tissues. To explore further the usefulness of this approach for salt stress studies we followed the course of: (i) osmotic shrinkage in maize root tissue exposed to 300 m*M* sucrose; and (ii) the uptake of chloride and sodium into maize root segments exposed to 100 m*M* salt for over 12 h (data not shown). Previous NMR studies of salt stress in plants have allowed the monitoring of sodium uptake in cells and excised tissues (reviewed in *17*), and the ability to follow water as well as both anions and cations in the roots of intact plants extends the usefulness of *in vivo* NMR in this field.

Several observations indicated that the susceptibility reagents used here had no effect on the viability or physiological status of the plant material. Thus Dy(DTPA-BMA), like its gadolinium analogue (*4*), had no effect on the growth of maize root tips over 24 h and no effect on the uptake of nitrate or chloride by maize root segments over the same period. Moreover *in vivo* ^{31}P NMR spectra of maize root segments showed no changes in vacuolar or cytoplasmic pH or of NTP or phosphomonoester levels for several hours following the addition of $[Gd(EDTA)]^-$. It was also observed that the separation between the internal and external signals in spectra such as those of Figs. 1–3 was constant for at least 24 h, showing that there was little, if any, entry of the reagents used into the cells. An exception to these findings was a substantial loss of resolution in spectra of *Chara* cells that began several hours after exposure to $[Gd(EDTA)]^-$. Since *Chara* grows in very dilute solutions, exposure to the relatively high concentrations of ions when $[Gd(EDTA)]^-$ is added may cause membrane leakiness. The nontoxicity of these compounds to higher plant tissues is unsurprising, since the EDTA and DTPA-BMA complexes of Gd have been widely used as contrast reagents

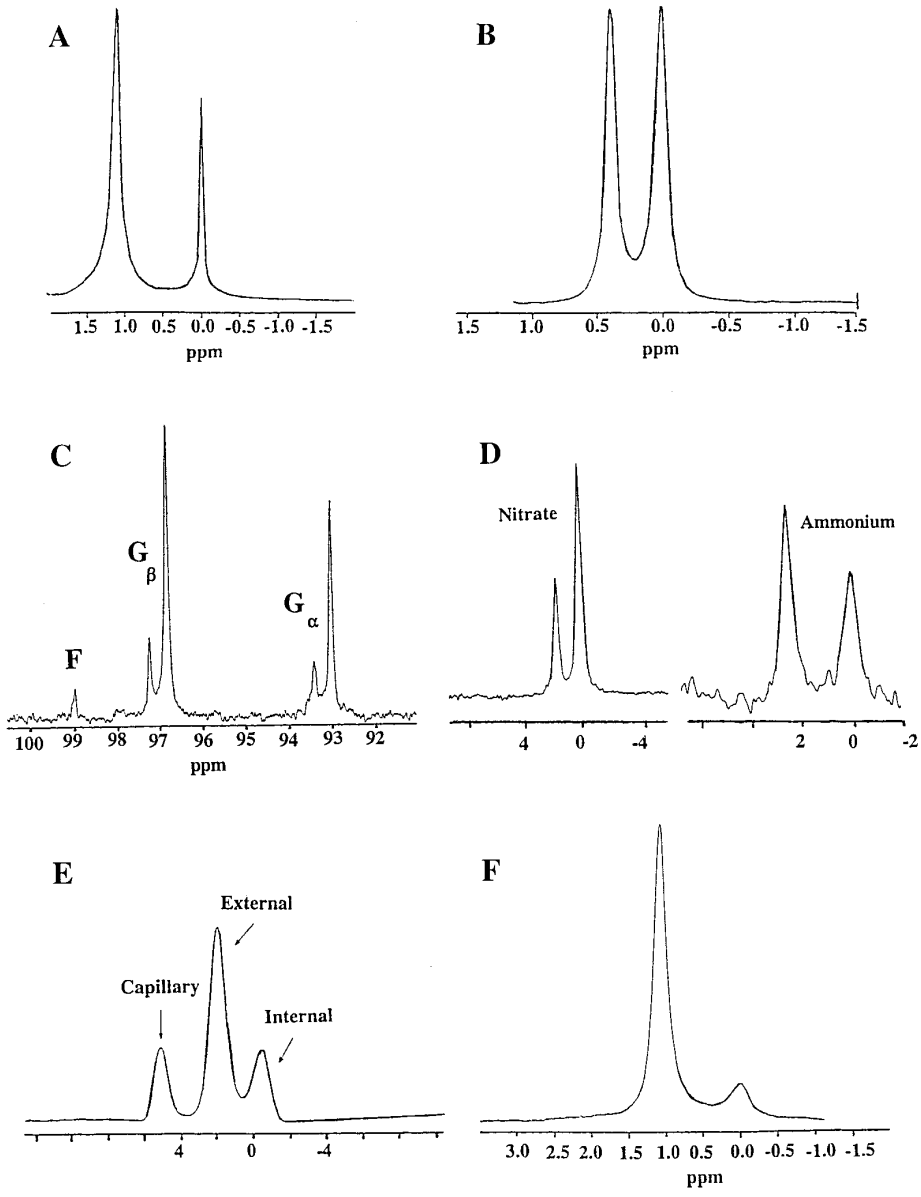

FIG. 2. Susceptibility allows the separation of internal and external signals in a range of plant materials. (A) A 400-MHz ^{1}H water spectrum (one scan) from about 15 *Chara* cells, each being rod shaped, close to 1 mm in diameter, and 2–5 cm long. (B) A 61.4-MHz ^{2}H spectrum from maize shoot segments after exposure to 3% D_2O for 3 h. (C) A 100.6-MHz ^{13}C subspectrum of the glucose in maize shoot segments in the presence of 0.5 m*M* $^{13}C_1$ glucose, showing the anomeric carbon resonances of glucose and fructose. G and F refer to signals from glucose and fructose C1 carbons respectively; the subscripts α and β refer to the two anomeric forms of glucose. While the external glucose signals are from highly labeled glucose (>99%), the internal glucose has natural abundance levels of ^{13}C but is present at much higher levels. Fructose is not present in the perfusate. (D) 21.68-MHz ^{14}N nitrate and ammonium subspectra from oriented maize root tips in medium containing 20 m*M* NH_4NO_3. The tissue had previously been exposed to 10 m*M* $K^{14}NO_3$ during the last 12–18 h of seedling growth. Thus the internal nitrate had mostly accumulated during seedling growth, whereas the internal ammonium accumulated during the NMR experiment (spectrum acquired 3.5–4 h after the tissue was first exposed to ammonium). (E) A 29.4-MHz ^{35}Cl spectrum of oriented maize root tips in the presence of 20 m*M* chloride. This sample contained a capillary tube containing a 2 *M* aqueous solution of CsCl, giving rise to a downfield reference signal. (F) A 105.8-MHz ^{23}Na spectrum of root segments in medium containing 50 m*M* NaCl 3 h after the NaCl was added to the perfusate. The samples from which spectra (A) and (F) were taken were perfused with solutions containing 10 m*M* $[Gd(EDTA)]^-$, 10 m*M* HEPES, and 0.5–1 m*M* $CaSO_4$ at pH 7. The samples of (B) and (C) were perfused with solutions containing 5 m*M* $[Gd(EDTA)]^-$. The samples which yielded spectra (D) and (E) were oxygenated with an airlift in medium containing 20 m*M* Dy(DTPA-BMA).

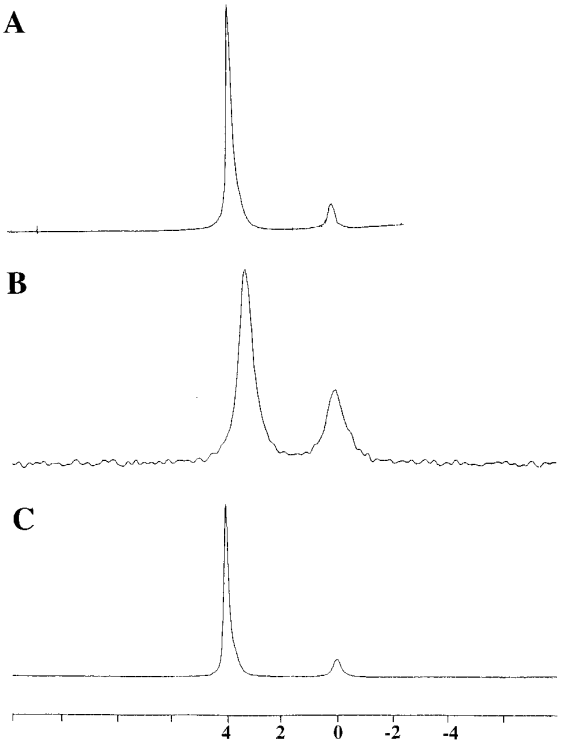

FIG. 3. (A) ^{1}H, (B) ^{35}Cl, and (C) ^{23}Na spectra at 7.05 T of the roots of intact maize seedlings. Several intact seedlings were placed in a 10-mm tube in medium containing 10 m*M* NaCl and 20 m*M* Dy(DTPA-BMA) so that the straight primary roots were aligned along the axis of the tube with the shoots protruding from the top. Spectra of this kind showed no loss of resolution even after 24 h of exposure to the susceptibility reagent.

in imaging of mammals without adverse effects (*9*), and it opens up the possibility of making novel observations on the transport properties of functioning plant tissues. Several examples are given in the following paragraphs.

The separation of internal and external HDO signals in the deuterium spectrum in Fig. 2B allows the detection of the bulk movement of water into the cells of plant tissues. Using 3% D_2O as a label yields very strong spectroscopic signals so that the flux of water can be followed accurately with high time resolution. Figure 4 is a series of 10-s ^{2}H spectra, showing only the internal resonance, following the addition of 3% D_2O to the perfusate reservoir. The first spectrum shows the small natural abundance signal before the label had reached the sample; subsequently the influx of label into the cells was monotonic with a rapid initial phase. The high signal-to-noise and time resolution, as well as the possibility of following both influx and efflux of the label in such experiments, make it a potentially useful tool for examining water relations in plant tissues. This type of experiment is related to imaging experiments in which the influx of D_2O can be followed either directly or through the depletion of the ^{1}H signal (*19*). While the imaging experiments yield information at high spatial resolution, the time resolution in the experiment in Fig. 4 is far better and it requires a nontoxic level of D_2O.

The potential for monitoring ion transport was further explored by following the ^{14}N signals of nitrate in maize root segments during isotope exchange of [^{15}N]nitrate for [^{14}N]nitrate. Figure 5 shows the intensity of the internal nitrate signal over 6 h in the presence of either [^{14}N] or [^{15}N]nitrate at 10 m*M*. When exposed to [^{14}N]nitrate the internal [^{14}N]nitrate levels rise, whereas in [^{15}N] nitrate the internal [^{14}N]nitrate level falls due to the replacement of internal [^{14}N] by [^{15}N]nitrate. Since the tissues were maintained using an airlift system (see Materials and Methods) with a small total volume of suspending medium (several milliliters as compared to several hundred milliliters used in the perfusion system), the intensity of the external [^{14}N]-nitrate signal in the same spectra from which Fig. 5 is derived (data not shown) represents the efflux of nitrate from the root segments. This allows one to deduce that the majority of the loss of the [^{14}N]nitrate signal is due to the efflux of nitrate rather than to its metabolism (it has been previously shown that nitrate in plant roots is fully NMR-visible (*20*)). This experiment demonstrates the potential of the susceptibility method for following net transport and for using NMR to measure isotopic exchange rates of ions and metabolites in plant tissues. Clarkson and co-workers have demonstrated the usefulness of ^{15}N labeling followed by mass spectrometric detection for following transport of ammonium and nitrate in plant tissues (*21*). While the present method using ^{14}N and ^{15}N NMR is less sensitive for following short term fluxes, it is complementary in allowing the direct and noninvasive measurement of nitrate and ammonium levels and turnover in tissues over longer times. In an analogous experiment, we were able to follow the isotopic exchange of chloride in maize root segments by monitoring internal and external ^{35}Cl signals after substituting $Na^{37}Cl$ for the unlabeled NaCl in the medium (data not shown).

Discrimination between internal and external signals using susceptibility reagents also allows faster transport processes than those illustrated in Figs. 4 and 5 to be investigated by magnetization transfer. Saturation transfer between internal and external ^{1}H water signals was readily observed in maize root segments (Fig. 6) and *Chara* cells. In the case of maize root segments, the extent of saturation transfer, together with the measured T_1 of the internal water, indicates that the average residence time for intracellular water was about 400 ms. This value may be compared with the residence time of 150 ms observed for maize seedling roots by Bačić and Ratković (*22*) using the relaxation enhancement method of Conlon and Outhred (*23*), and with the value of 900 ms calculated by Snaar and Van As (*24*) from NMR diffusion measurements of apple fruit. The present method may be

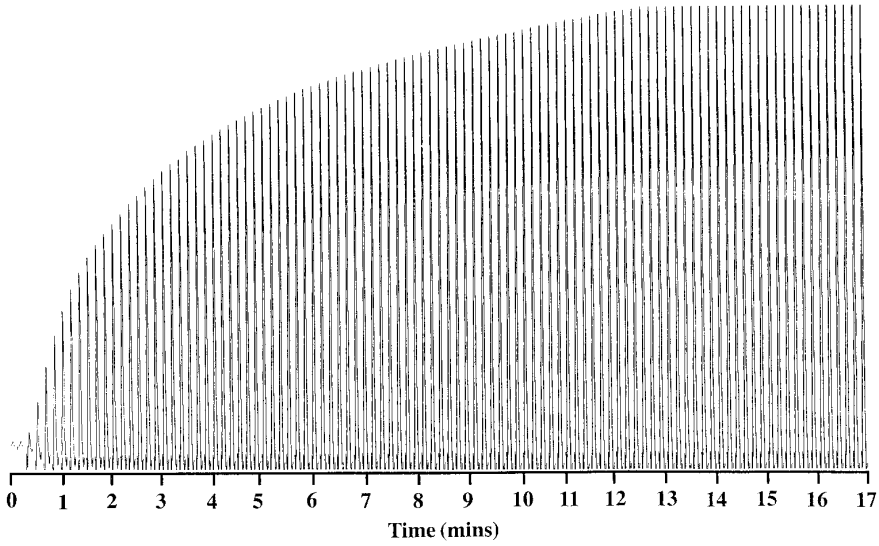

FIG. 4. Stacked plot of the internal ^2H signal as a function of time to follow D_2O influx into maize root segments after adding 3% D_2O to the perfusing medium. Root segments were perfused with oxygenated buffer containing 10 m*M* [Gd(EDTA)]$^-$, 10 m*M* HEPES, 50 m*M* glucose, and 0.5 m*M* $CaSO_4$ at pH 7, and 10-s spectra were recorded consecutively over a period of 17 min. The external signal (not shown) increased from the natural abundance level to a maximum value in spectra 2–4. Gaussian multiplication was used for resolution enhancement.

argued to have advantages since it is direct and involves fewer assumptions.

Several observations suggest that the unshifted resonance in the spectra in Figs. 1–6 is dominated by intracellular material and that the shifted signal is extracellular. First, chemical shifts in *in vivo* spectra of oriented plant tissue samples are similar to those in phantom samples of concentric tubes where an external compartment contains a susceptibility reagent. Second, the relative sizes of internal and external signals depend on the fraction of the sample volume occupied by the tissue and on the relative concentrations of the species being observed. Third, when 10 m*M* rhamnose was added to the perfusate of a sample of maize root segments (which was being perfused with medium containing 10 m*M* [Gd(EDTA)]$^-$), a single, shifted, rhamnose methyl

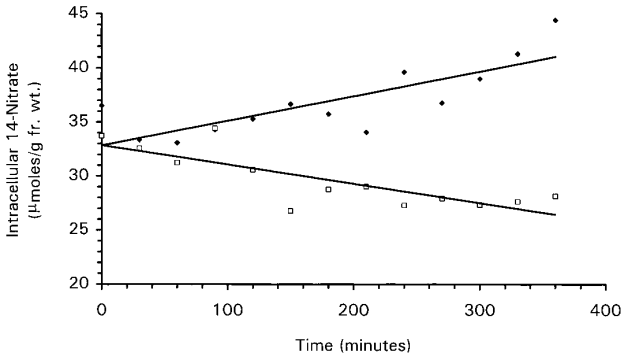

FIG. 5. Nitrate exchange in maize root segments. The internal ^{14}N nitrate signal was followed in the presence of either 10 m*M* [^{14}N]nitrate (♦) or 10 m*M* [^{15}N]nitrate (□). The tissue in each experiment had previously been exposed to 10 m*M* $K^{14}NO_3$ during the last 12–18 h of seedling growth before the root segments were excised and the time courses begun. The suspending medium (volume about 10 ml in the airlift system, see Materials and Methods) contained 20 m*M* Dy(DTPA-BMA) at pH 7. The lines are least-squares fits to each of the two time courses.

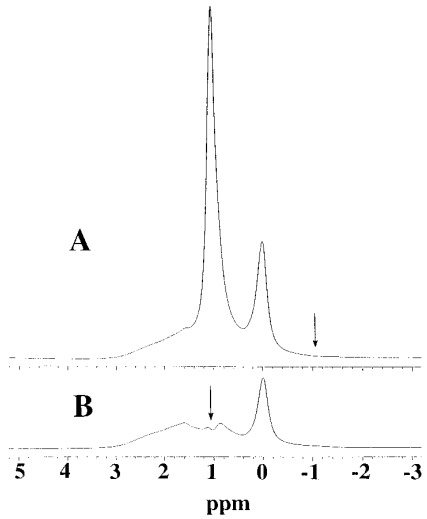

FIG. 6. ^1H water spectra showing saturation transfer in maize root segments. (A) Saturation at a control frequency upfield of the internal peak (to control for any reduction in the internal signal due to direct saturation as distinct from magnetization transfer), and (B) saturation at the frequency of the external water signal. The maize segments were prepared and perfused as described in the legends for Figs. 2D, 2F, and 4.

resonance was observed (data not shown). Rhamnose is not expected to enter the cells significantly. If there were a significant fraction of the sample, such as intercellular spaces, where rhamnose but not paramagnetic complexes could permeate, one would see an unshifted rhamnose signal. Fourth, when 3% D_2O was added to the perfusing medium the shifted peak of the ^2H signal increased as soon as the addition was made, and the unshifted peak increased steadily over time consistent with entry of the D_2O, but not of the paramagnetic complex, into the cells. Fitting of the spectrum in Fig. 1C (as previously described, *4*) shows that the two peaks have purely Lorentzian lineshapes with a ratio of integrated areas for the shifted and unshifted signals of 2:1. There is an additional component downfield which extends significantly under the shifted peak but contributes little to the unshifted peak. We attribute this additional component to external material since it relaxes with a T_1 of approximately 10 ms, like the shifted Lorentzian external peak. The additional spectroscopic feature may arise from inhomogeneous regions of the primary field, especially at the ends of tissue segments, since it is absent in whole root spectra (Fig. 3) and since some of it is further upfield than the maximum predicted for BMS effects (*6–8*). In any case, neither quantification of the internal signals nor magnetization transfer measurements (Fig. 6) are significantly affected because the internal signal is resolved from the other components.

The remaining question is whether signals from outside the cells but inside root and shoot tissue (i.e., in the intercellular spaces, cell walls, and the lumen of xylem tubes) are shifted. Using a fluorescent dye that does not enter cells it has been shown that in young maize roots the apoplasm of the cortex is permeated within 80 min, although there was variable penetration beyond the endodermis (*25*). Bačić and Ratković measured the T_1 relaxation of water in roots of intact maize seedlings that had been incubated in solutions of paramagnetics and concluded that Mn(EDTA) permeates the extracellular spaces of this tissue (*22*). Two observations suggest that a substantial fraction of the apoplastic signal is shifted in the presence of the reagents used here. First, the size of the shifted peak grows and the unshifted decreases by about 10–15% over the first 60–90 min of exposure to $[Gd(EDTA)]^-$ and then stabilizes; this is consistent with permeation of the complex into apoplastic spaces over the timescale reported elsewhere for maize roots (*22, 25*). Second, magnetization transfer from the shifted to the unshifted signal was observed in maize root segments when the external signal was saturated. If the signal from apoplastic water is not shifted, then saturation of the external signal could only be transferred by exchange between the perfusing medium and the total root water. Figure 4 shows that this rate is much slower than the T_1 of water and could not therefore allow for significant magnetization transfer. Thus the observed saturation transfer suggests that a substantial fraction of the signal from apoplastic water is shifted since apoplastic water can exchange across the cell membranes with intracellular water on a shorter timescale (*22, 24*). Note that the above observations do not prove that *all* the extracellular spaces are permeated by the paramagnetics.

Bulk magnetic susceptibility effects have been used to distinguish internal from external signals in cell suspensions of a bacterium (*7*) by addition of paramagnetic reagents, and this approach was applied to measuring movement of sugars and alcohol into these cells (*7, 26*). However, the work of Springer and co-workers shows that this approach is unlikely to allow quantitative separation of signals for cell suspensions because cells adopt a wide spread of orientations relative to the primary magnetic field so that there is a distribution of local field strengths resulting in non-Lorentzian lineshapes and substantial overlap between the internal and external signals. Indeed inspection of published spectra shows that this appears to be the case for bacterial cells suspensions as well as the example of red blood cells treated by Springer and co-workers (*6, 7, 9, 26*). Thus it appears that BMS effects can be considerably more useful for *in vivo* spectroscopy of plants tissues whose cells are cylindrical and oriented along the tissue axis than for most animal tissues or cell suspensions. Interestingly, intrinsic susceptibility effects associated with manganese in highly oriented chloroplast membranes have been observed in the ^1H spectra of the leaves of certain plants (*10*), and these have been exploited to follow the movement of water across the chloroplast membrane (*27, 28*).

In conclusion we have shown that BMS effects induced by adding paramagnetics to the suspending medium allow the separation of internal and external signals of many important ions and small molecules in the *in vivo* spectra of plant cells and tissues. The use of this method to follow transport, isotopic exchange, and magnetization transfer due to transmembrane water exchange further illustrates the wide applicability of the approach for studies of plant transport. The method is limited to cells and tissues of appropriate geometry and orientation, and it is not expected to be useful in suspensions of spherical or irregularly shaped cells.

ACKNOWLEDGMENTS

We thank the microscopic imaging group at ERRC for Figs. 1A and 1B. We are grateful to Professor C. S. Springer for valuable comments and analysis during the preparation of this manuscript. The authors thank Mr. A. Hene for financial assistance and are grateful for the support of Nycomed Imaging AS, Oslo.

REFERENCES

1. P. S. Belton and R. G. Ratcliffe, *Prog. NMR Spectrosc.* **17,** 241 (1985).
2. C. S. Springer, *Ann. Rev. Biophys. Chem.* **16,** 375 (1987).

3. Y. Shachar-Hill and R. G. Shulman, *Biochemistry* **31,** 6272 (1992).
4. Y. Shachar-Hill, P. E. Pfeffer, and R. G. Ratcliffe, *J. Magn. Reson. B* **111,** 9 (1996).
5. M. S. Albert, W. Huang, J. H. Lee, J. A. Balschi, and C. S. Springer, *NMR Biomed.* **6,** 7 (1993).
6. C. S. Springer, *NMR Biomed.* **7,** 182 (1994).
7. S. M. Schoberth and A. A. De Graaf, *Anal. Biochem.* **210,** 123 (1993).
8. S. C. K. Chu, Y. Xu, J. A. Balschi, and C. S. Springer, *Magn. Reson. Med.* **13,** 239 (1990).
9. C. S. Springer, *in* ''NMR in Physiology and Biomedicine'' (R. J. Gillies, Ed.), pp. 75–99, Academic Press, New York (1994).
10. D. C. McCain, T. C. Selig, Govindjee, and J. L. Markley, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 748 (1984).
11. Y. Xu, J. A. Balschi, and C. S. Springer, *Magn. Reson. Med.* **1,** 80 (1990).
12. D. W. Keifer and R. M. Spanswick, *Plant Physiol.* **62,** 653 (1978).
13. Y. Shachar-Hill, P. E. Pfeffer, D. Douds, S. F. Osman, L. W. Doner, and R. G. Ratcliffe, *Plant Physiol.* **108,** 7 (1995).
14. G. G. Fox, R. G. Ratcliffe, and T. E. Southon. *J. Magn. Reson.* **82,** 360 (1989).
15. K. Richardson, L. Jarett, and E. Finke, *Stain Technol.* **35,** 313 (1960).
16. S. Fossheim, C. Johansson, A. K. Fahlvik, D. Grace, and J. Klaveness, *Magn. Reson. Med.* **35,** 201 (1996).
17. R. G. Ratcliffe, *Adv. Bot. Res.* **20,** 43 (1994).
18. Y. Shachar-Hill, and P. E. Pfeffer (Eds.), ''Nuclear Magnetic Resonance in Plant Biology,'' American Society of Plant Physiologists, Rockville, MD (1996).
19. A. Connelly, J. A. B. Lohman, B. C. Loughman, H. Quiquampoix, and R. G. Ratcliffe, *J. Exp. Bot.* **38,** 1713 (1987).
20. P. S. Belton, R. B. Lee, and R. G. Ratcliffe, *J. Exp. Bot.* **36,** 190 (1985).
21. D. T. Clarkson, A. Gojon, L. R. Saker, P. K. Wiersema, J. V. Purves, P. Tillard, G. M. Arnold, A. J. M. Paans, W. Vaalburg, and I. Stulen, *Plant Cell Environ.* **19,** 859 (1996).
22. G. Bačić and S. Ratković, *Biophys. J.* **45,** 767 (1984).
23. T. Conlon and R. Outhred, *Biochim. Biophys. Acta* **288,** 354 (1972).
24. J. E. M. Snaar and H. Van As, *Biophys. J.* **63,** 1654 (1992).
25. M. J. Canny and C. X. Huang, *New Phytol.* **126,** 11 (1994).
26. S. M. Schoberth, B. E. Chapman, P. W. Kuchel, R. M. Wittig, J. Grotendorst, P. Jansen, and A. A. De Graaf, *J. Bacteriol.* **178,** 1756 (1996).
27. D. C. McCain and J. L. Markley, *FEBS Lett.* **183,** 353 (1985).
28. D. C. McCain, *Biophys. J.* **69,** 1105 (1995).