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Application of ^{39}K NMR Spectroscopy to Potassium Transport in Mung Bean Root Tips

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The intracellular K^+ concentration and its change in mung bean [*Vigna mungo* (L.) Hepper] root tips were investigated non-invasively with ^{39}K nuclear magnetic resonance spectroscopy using a membrane impermeable shift reagent, dysprosium (III) tripolyphosphate [$Dy(PPP_i)_2^{7-}$]. The K^+ resonance was shifted to higher magnetic field in proportion to the concentration of the shift reagent. In addition to a reference capillary peak for measuring the K^+ concentration, two well-resolved peaks (intra- and extracellular K^+ resonances) were observed in the ^{39}K NMR spectra of mung bean root tips. The intracellular K^+ concentration was determined to be 41 mM, which was similar to the value obtained by flame photometry. When 20 mM KCl was added to the external medium, the intensity of the intracellular K^+ resonance gradually increased and the net K^+ uptake rate was calculated to be 4.1 micromoles per gram fresh weight per hour. After removal of KCl from the perfusion medium, the intracellular K^+ concentration considerably decreased. With ^{31}P NMR method, 2.5 mM $Dy(PPP_i)_2^{7-}$ and 20 mM KCl had little effect on the ATP level in the cells. We have indicated that the ^{39}K NMR method can be used to determine the K^+ levels and net fluxes of the K^+ transport in perfused root tips successively.

Key words: Intracellular K^+ — ^{39}K NMR — Mung bean (*Vigna mungo*) — Potassium transport — Shift reagent.

Potassium transport and its regulation are fundamental processes in plant cells (Glass 1983), and changes in K^+ transport can affect cellular functions. Therefore, it is of interest to study intracellular K^+ concentrations and K^+ fluxes across the plasma membrane of plant cells. In order to determine the intracellular ion concentrations, the frame photometry (Silk et al. 1986) and ion selective microelectrode methods (Beilby 1986) have been applied. The flame photometry analysis requires destruction of the tissue, and the continuous measurement of the same tissue is impossible. In higher plants, which have relatively small cells, the puncture by the microelectrode substantially damages the cells.

NMR spectroscopy is a non-invasive and non-destructive method which allows us to study the cellular phenomena in the same sample successively (Gadian 1982). Although the ^{31}P NMR spectroscopy has been successfully applied to studies on many plant tissues and cells (Roberts 1984 and references therein), there were few NMR investigations on other nuclei such as metal cations. Since the natural abundances of ^{23}Na and ^{39}K, that have the nuclear spin, are 100 and 93.1% respectively, these ions are also observable with NMR without adding any isotopes. But the lack of separation of intra- and extracellular resonances had limited the application of the NMR technique to these cations.

In recent years, Gupta and Gupta (1982) demonstrated that $Dy(PPP_i)_2^{7-}$ could provide large shifts enough to separate intra- and extracellular Na^+ resonances in ^{23}Na NMR spectra. Their approach opened the possibility of applying the ^{23}Na NMR technique to studies of the ion transport in intact cells. In plant cells, the ^{23}Na NMR has been applied to millet suspension cells (Sillerud and Heyser 1984) and corn roots (Gerasimowicz et al. 1986). But no ^{39}K NMR studies on plant cells have been reported, since the NMR sensitivity for ^{39}K was lower than that for ^{23}Na.

Abbreviations: $Dy(PPP_i)_2^{7-}$, dysprosium (III) tripolyphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; K_{in}^+, intracellular K^+; K_{out}^+, extracellular K^+; K_{ref}^+, K^+ in the reference capillary; MDP, methylene diphosphate; NMR, nuclear magnetic resonance; UDPG, uridine 5′-diphosphate glucose.

In this paper, we applied the ^{39}K NMR spectroscopy to investigation of the intracellular K^+ concentration and its change in mung bean root tips in vivo. In addition, effects of the shift reagent and external KCl on energy metabolism were also studied with the ^{31}P NMR method.

Materials and Methods

Plant material—Mung bean seedlings [*Vigna mungo* (L.) Hepper] were grown in a growth medium (0.1 mM KCl and 0.05 mM $CaCl_2$) as described previously (Yazaki et al. 1988). Root tips, 1.5 mm long, were excised from the two-day-old seedlings. The root tips were rinsed and stored in a medium of 100 mM glucose and 0.05 mM $CaCl_2$ for 2 h.

Perfusion—The incubated root tips, approximately 2.4 g fresh weight, were transferred to a 10-mm NMR tube and then perfused with an oxygenated medium at a rate of 6 ml/min as described previously (Yazaki et al. 1988). In all experiments, the perfusion medium contained 100 mM glucose, 0.05 mM $CaCl_2$, and 15 mM MES-Tris (pH 6.5). The temperature was kept at 22±1°C. The volume of the perfusate in a reservoir was 80 ml and unless otherwise indicated, the perfusate was exchanged to a fresh one at intervals of 30 min to maintain the composition of the perfusate. During the initial perfusion for tuning the probe and shim coils in NMR magnet, 1.0 mM KCl was added to the perfusate to avoid K^+ loss from the cells.

NMR spectroscopy—The ^{39}K NMR spectra were obtained at 23.3 MHz using a JEOL GX500 NMR spectrometer. The spectra were accumulated with a repetition time of 0.5 s and a 90° pulse (67.5 μs) in 10 min. To separate intra- and extracellular K^+ resonances, a membrane impermeable shift reagent, dysprosium (III) tripolyphosphate [$Dy(PPP_i)_2^{7-}$], was used. The shift reagent was prepared by adding a stoichiometric amount of $DyCl_3$ to a solution of sodium tripolyphosphate ($Na_5P_3O_5$). In most experiments, the perfusion medium contained 2.5 mM $Dy(PPP_i)_2^{7-}$. In order to measure the K^+ concentration, a sealed coaxial capillary tube containing 12 mM $Dy(PPP_i)_2^{7-}$ and 30 mM KCl (210 μl) was inserted to an NMR tube with the sample. The K^+ concentration in the root tip cells was determined by comparing the area of the intracellular K^+ peak with that of the reference capillary peak, and taking the cellular volume into account. The cellular volume was determined by subtracting the volume of the outer and free spaces of the root tips from the effective volume in the NMR tube. Since $Dy(PPP_i)_2^{7-}$ can not enter the plant cells (Sillerud and Heyser 1984), K^+ ions in the cells are not affected by the shift reagent. On the other hand, K^+ ions in both the outer space and free space of the root tips are accessible to the shift reagent and they should contribute to the extracellular K^+ resonance. Accordingly the summed volume of the outer and free spaces was calculated from such a spectrum as shown in Fig. 4 by comparing the area of the extracellular K^+ peak (20 mM) with that of the reference capillary peak (30 mM, 210 μl).

The ^{31}P NMR experiments were performed at 161.8 MHz using a JEOL GX400 NMR spectrometer. Each spectrum required 2,000 transients with a repetition time of 0.2 s and 45° pulse (19.5 μs). Methylene diphosphate (MDP) in the capillary, which was inserted to an NMR tube with the sample, was used as an external reference. The intracellular pH was estimated by use of the standard reference curve of chemical shift versus pH for P_i, which was obtained according to the method of Pfeffer et al. (1986).

Flame photometry—The incubated root tips (0.02 g fresh weight) were frozen and then thawed in distilled water. The K^+ concentration in the extracts was measured using a Hitachi 180-50 atomic absorption spectrophotometer. The K^+ concentration in the cells was estimated from the water and K^+ content of the sample. The water content was calculated from the difference between the fresh weight and the dry weight of the sample.

Results and Discussion

Effects of shift reagent on ^{39}K and ^{31}P NMR resonances—Fig. 1 shows the effect of $Dy(PPP_i)_2^{7-}$ on the K^+ resonance in ^{39}K NMR spectra. Since a solution of 2.5 mM $Dy(PPP_i)_2^{7-}$ contained 25 mM Na^+ due to sodium tripolyphosphate, both K^+ and Na^+ ions are accessible to the shift reagent. Taking the Na^+ ions into consideration, we showed that the K^+ resonance was shifted to higher magnetic field in proportion to the concentration of $Dy(PPP_i)_2^{7-}$. Therefore, it is possible to shift the ^{39}K

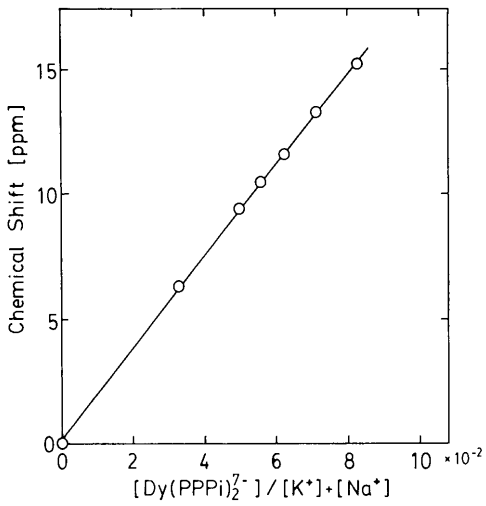

Fig. 1 Effect of $Dy(PPP_i)_2^{7-}$ on ^{39}K NMR chemical shift of the K^+ resonance. The chemical shift of K^+ resonance shifted to higher magnetic field was measured relative to the chemical shift of the KCl solution as an external reference (0 ppm).

resonance peak to appropriate positions by the shift reagent.

For biological application, it is important to investigate whether the shift reagent affects the metabolic state of the tissue. Then the effect of $Dy(PPP_i)_2^{7-}$ on the ^{31}P NMR resonances was checked, because the level of high-energy phosphate such as ATP reflects the viability of the tissue. The ^{31}P NMR spectrum in the presence of $Dy(PPP_i)_2^{7-}$ showed slight broadening, but the ATP level was maintained (Fig. 2). The broadening was probably due to the increased susceptibility difference between the intra- and extracellular compartments produced by the paramagnetic shift reagent. Besides the ATP level, the oxygen uptake rate measured with an O_2 electrode was almost unchanged in the presence of the shift reagent (data not shown). Moreover, the effect of the shift reagent on membrane permeability was also checked by measuring the K^+ content of the root tips using flame photometry. The K^+ content in the presence of the shift reagent was 90% of the control level. From these results, $Dy(PPP_i)_2^{7-}$ had little influence on the energy metabolism and the physiological functions of the tissue. Ogino et al. (1985) reported that $Dy(PPP_i)_2^{7-}$ had no deleterious effects on energy metabolism in human erythrocytes and Civan et al. (1983) also reported that the electrophysiological responses of frog skin were not impaired by the shift reagent.

Quantitation of K^+ levels by ^{39}K NMR—Fig. 3 shows the relationship between the K^+ concentration and integrated peak intensity in ^{39}K NMR spectra. The peak intensity was proportional to the K^+ concentration. Accordingly, we can quantitate the intracellular K^+ levels of the root tips from ^{39}K NMR spectra.

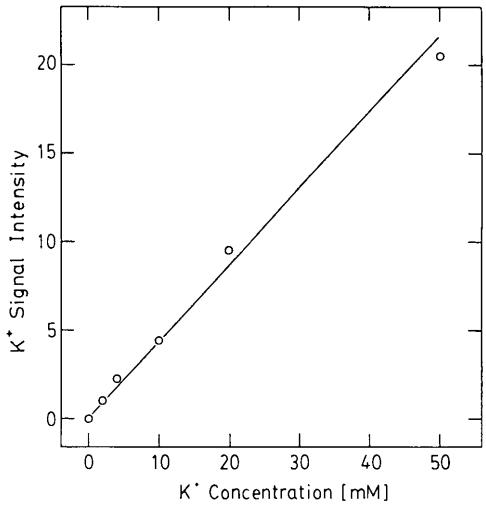

Fig. 3 Relationship between K^+ concentration and integrated K^+ signal intensity in ^{39}K NMR spectra. The K^+ signal intensity represents relative intensity of K^+ resonance in the standard KCl solution.

Ogino et al. (1983) observed intra- and extracellular K^+ resonances in ^{39}K NMR spectra of yeast cells by using $Dy(PPP_i)_2^{7-}$. For our experiments, the modification related to the reference capillary was introduced for measuring the K^+ concentration precisely. Fig. 4 shows the representative ^{39}K NMR spectrum of aerobic mung bean root tips. Three well-resolved peaks were observed in the

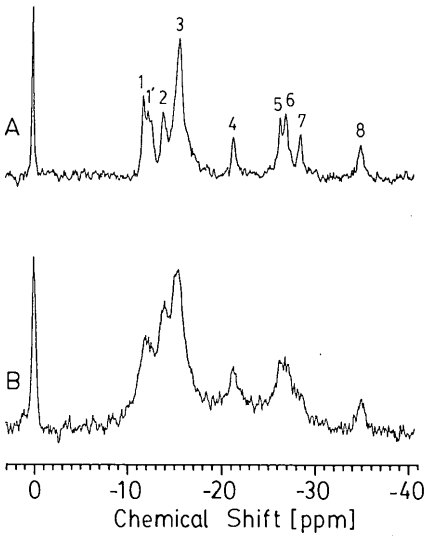

Fig. 2 Effect of $Dy(PPP_i)_2^{7-}$ on ^{31}P NMR spectra of mung bean root tips. A: The spectrum before addition of $Dy(PPP_i)_2^{7-}$. B: The spectrum in 30 min after addition of 2.5 mM $Dy(PPP_i)_2^{7-}$. Peak assignments: 1, G6P; 2, cytoplasmic P_i; 3, vacuolar P_i; 4, γ-ATP; 5, α-ATP; 6, UDPG and nicotinamide adenine nucleotides; 7, UDPG; 8, β-ATP. Peak 1′ seemed to be F6P.

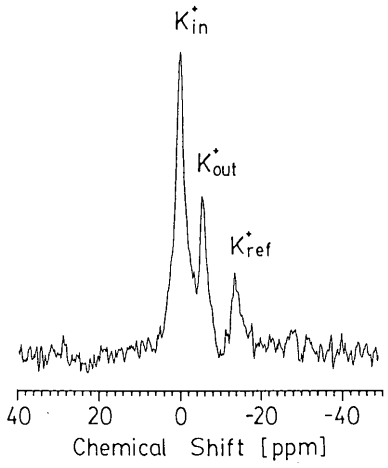

Fig. 4 Representative 23.3 MHz ^{39}K NMR spectrum of mung bean root tips in the presence of 20 mM KCl and 2.5 mM $Dy(PPP_i)_2^{7-}$. Peak assignments: K_{in}^+, intracellular K^+; K_{out}^+, extracellular K^+; K_{ref}^+, K^+ in the reference capillary.

spectrum. The unshifted peak (0 ppm) corresponds to K_{in}^+, and the peak shifted upfield (−8 ppm) corresponds to K_{out}^+. Moreover, the peak shifted upfield about −14 ppm corresponds to the reference capillary peak (K_{ref}^+). The peak assignments were based on the relation as shown in Fig. 1 and the observation that the chemical shift of K_{in}^+ was virtually unaffected by the presence of the shift reagent. The intracellular K^+ concentration was determined to be 41 mM for aerobic root tip cells. This concentration was similar to the value of about 45 mM using flame photometry. Brophy et al. (1983) also reported that the intracellular K^+ concentration in erythrocytes determined by ^{39}K NMR agreed with that by flame photometry.

Detect of K^+ uptake by NMR—The changes in ^{39}K NMR spectra of mung bean root tips by addition of 20 mM KCl are shown in Fig. 5. The intensity of the low field signal (K_{in}^+) increased. The chemical shift difference between the intra- and extracellular K^+ resonances remained constant throughout the experiments. This supported that the shift reagent could not enter the intracellular space. The observed increase in K_{in}^+ represents the influx of K^+ into the cells. Fig. 6 shows the time course of the intensity of the intracellular K^+ resonance in ^{39}K NMR spectra. K_{in}^+ was gradually increased. From this figure, the net K^+ uptake rate was calculated to be 4.1 μmol·(g fresh weight)$^{-1}$·h^{-1}. It is well known that the dual pattern of K^+ uptake (high affinity mechanism I at low concentrations, and low affinity mechanism II at high concentrations) is observed over wide range of K^+ concentrations (Epstein 1966). Our experimental conditions of 20 mM KCl corresponds to the mechanism II, and the uptake occurs mainly by diffusion.

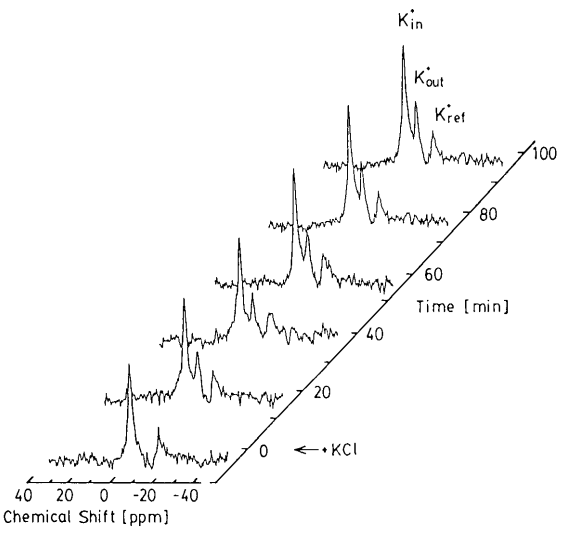

Fig. 5 Changes of ^{39}K NMR spectra by addition of 20 mM KCl. The root tips were firstly perfused with the solution containing 2.5 mM $Dy(PPP_i)_2^{7-}$. Then 20 mM KCl was added to external solution at time 0 min.

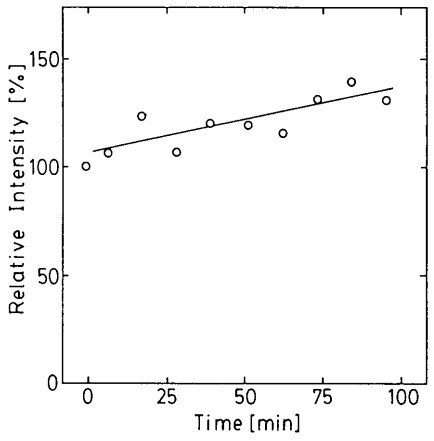

Fig. 6 Time course of relative intracellular K^+ intensity (the initial intensity is 100%) by addition of 20 mM KCl to the perfusion solution.

Glass (1978) reported that K^+ influx values for excised roots recovered to values comparable with those for intact plant in 2 h after the excision. Since we performed the NMR measurements more than 2 h later after the excision, it was thought that there was no significant wounding effect on the influx data obtained using the excised root tips.

Detect of K^+ loss by NMR—Fig. 7 shows changes in ^{39}K NMR spectra of mung bean root tips, after removal of KCl from the external medium. The root tips were preloaded with 20 mM KCl for 100 min. When KCl was removed from the perfusate, the signal of the intracellular K^+ decreased considerably. This indicates that the intracellular K^+ concentration decreased. From the time course of the peak intensity, the efflux rate was calculated to be 11 μmol·(g fresh weight)$^{-1}$·h^{-1}.

In plant cells, the major compartments are the cytoplasm and the vacuole. With the present ^{39}K NMR

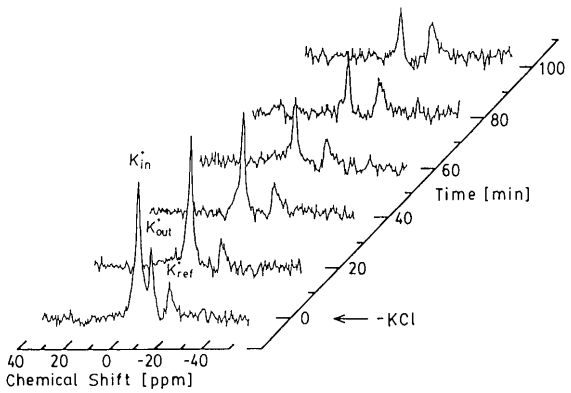

Fig. 7 Changes of ^{39}K NMR spectra of mung bean root tips after KCl was removed from the external medium. This experiment was subsequently performed after processes in Fig. 5.

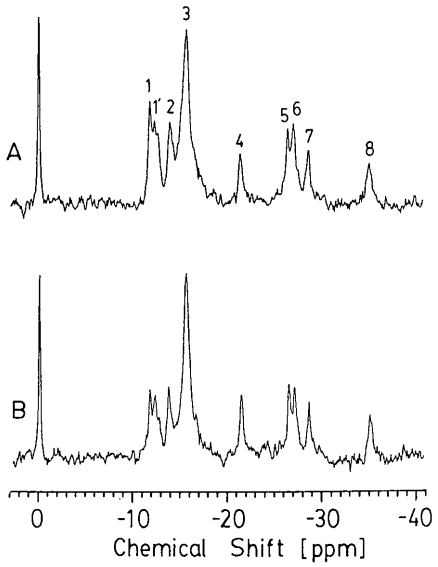

Fig. 8 Effect of external KCl on ^{31}P NMR spectra of mung bean root tips. A: The spectrum before addition of KCl. B: The spectrum in 30 min after addition of 20 mM KCl. Peak assignments are given in the legend to Fig. 2.

method, we could not determine the distribution of K^+ between the cytoplasm and the vacuole, but measured total amount of K^+ present in root tip cells. K^+ ions in the cell walls and intercellular spaces are accessible to the shift reagent and do not contribute to the intracellular K^+ resonance. If K^+ ions are bound tightly to the cell walls, these ions are invisible in NMR spectra. Using the radioactive isotope, the compartment analysis has been applied to estimation of the amounts of isotope in each compartment (Pitman and Saddler 1967, Macklon and Higinbotham 1970). This technique might be able to apply to the NMR method. In fact, Sillerud and Heyser (1984) demonstrated from the ^{23}Na NMR determination, that the efflux data could be fitted to the two compartment model.

Effect of external KCl on ^{31}P NMR spectra—The ^{31}P NMR spectra obtained before and after the addition of 20 mM KCl to the perfusate are shown in Fig. 8. The intensity of ATP resonances did not change and this indicates that 20 mM KCl has no remarkable effects on energy metabolism in root tips. In addition, the pH value of the vacuole was constant.. But the cytoplasmic pH increased from 7.3 to 7.6 in 30 min under 20 mM KCl. It has been generally accepted that K^+ uptake is coupled either directly or indirectly to H^+ efflux (Poole 1978). Therefore, this increase in the cytoplasmic pH seemed to result from the coupling of K^+ uptake and H^+ efflux. But the precise stoichiometry between H^+ efflux and K^+ uptake could not be determined, because the cytoplasmic buffer capacity and the proton production by metabolic processes were unknown.

In summary, we have indicated that the ^{39}K NMR technique provides an alternative non-invasive method for determination of the intracellular K^+ levels and net fluxes of the K^+ transport in mung bean root tip cells in vivo. With NMR methods, the intracellular K^+ levels could be monitored directly and successively. In particular, the combination of the ^{39}K NMR and ^{31}P NMR methods might permit us to investigate simultaneously the intracellular K^+ levels and energy metabolism and to relate the intracellular pH to the K^+ transport. Further investigations associated with electrophysiological properties and coupling to other ions are recommended for revealing the transport process and its mechanisms.

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