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Water transport in maize roots under the influence of mercuric chloride and water stress: a role of water channels

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Abstract

The influence of inhibitor of water channels, $HgCl_2$, on water diffusion in maize (*Zea mays* L.) seedling roots was investigated with the pulsed nuclear magnetic resonance (NMR) method. Blocking of water channels decreased the water permeability of cell membranes by 1.5 - 2 times. This effect of $HgCl_2$ was exhibited only in the roots of seedlings grown in a nutrient solution containing Ca^{2+} and was reversed with Hg-scavenging agent β-mercaptoethanol. Subsequent incubation of Ca^{2+}-deficient roots in the nutrient solution with Ca^{2+} recovered the sensitivity to $HgCl_2$. The water stress decreased water diffusion rates similarly to $HgCl_2$ and the effects of water stress and $HgCl_2$ were not additive. The obtained data demonstrate the possibilities of the pulsed NMR method for study of the transmembrane water exchange *in vivo* in connection with water channel functioning.

Additional key words: diffusion rate, nuclear magnetic resonance, transmembrane transfer, *Zea mays.*

Introduction

The important role of water channels (aquaporins) in the regulation of transmembrane water transfer in plant cells is generally accepted. Aquaporins function as narrow protein pores, which facilitate essentially passive movement of water molecules. It has been estimated that as much as 70 - 90 % of the water moving from cell to cell passes through these pores (Henzler and Steudle 1995, Tazawa *et al.* 1997, Zhang and Tyerman 1999). A large number of aquaporin genes are expressed in a wide variety of plants and plant parts. At least 31 aquaporin homologues are expressed in maize (Chaumont *et al.* 2001). Barrieu *et al.* (1998) have shown that tonoplast aquaporin (ZmTIPI) is highly expressed in the endodermis and xylem parenchyma of the maize root. Hukin *et al.* (2002) have demonstrated the expression of two plasmalemma aquaporin genes in growing maize roots. There are indications that control of water flux through aquaporins could be important during cell division and elongation (Barrieu *et al.* 1998, Chaumont *et al.* 1998, Kaldenhoff *et al.* 1998). Two functions of aquaporins are postulated: the maintenance of the intracellular osmotic equilibration, and the regulation of transcellular water transport. An optimal water balance is essential in the plant homeostasis and aquaporins may be one of the mechanisms involved under changing environmental and developmental conditions (Baiges *et al.* 2002).

To date there is evidence for the dependence of the water channel activity on external factors such us the water and salt stress (Guerrero *et al.* 1990, Azaizeh *et al.* 1992, Yamaguchi-Shinozaki *et al.* 1992, Carvajal *et al.* 1999, Steudle 2000), and nutrient deprivation (Carvajal *et al.* 1996, Clarkson *et al.* 2000). Some aquaporins were shown to be up-regulated (Guerrero *et al.* 1990) while others down-regulated (Yamada *et al.* 1995) by drought conditions. So far vagueness exists regarding whether and how aquaporins activity may be regulated. It has been shown that water transport activity of some aquaporins is controlled at the protein level by phosphorylation (Maurel *et al.* 1995, Johansson *et al.* 1998, Kjellbom *et al.* 1999). According to Johansson *et al.* (1996) the phosphorylation of plasma membrane aquaporin PM28A of spinach leaf is Ca^{2+} dependent.

A functional role of aquaporins at cellular level is being quite successfully studied. When transport processes are studied immediately in plant tissues,

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Abbreviations: D_{ef} - effective diffusion coefficient of water; DD - diffusional decay, g - gradient pulse amplitude; P_d - coefficient of diffusional water permeability of membranes; R - relative echo amplitude; t_d - diffusion time.

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researchers face the difficulties because of the absence of suitable equipment for water flow measurement (Steudle 1997). In this case, it is necessary to consider the different ways of water transport in the tissue (apoplast, symplast and transmembrane way) and their contribution to the overall water flow (Steudle 1997, 2000), which depends on the factors, such as the type of tissue, a stage of its development, a nature of driving force of the flow (osmotic or hydrostatic), and external conditions (Steudle 1997).

In this paper we propose to use the spin-echo NMR method for study of water transport through cell membranes in maize seedling roots. This method is the most adequate one for water transport studies at all levels and is widely used in the investigation of plant water transfer (Van As *et al.* 1980, Anisimov and Ratković 1992, Van Dusschoten *et al.* 1995, Van der Weerd *et al.* 2001, Krishnan *et al.* 2004). Earlier we used this method for the study of the water transport under the influence of membrane-active compounds and environmental conditions (Anisimov *et al.* 1983, Ionenko and Anisimov 2001).

The purpose of this paper was to study the water transmembrane transfer in maize root segments under the influence of water channel inhibitor by the spin-echo nuclear magnetic resonance (NMR) method. An indication of the water channel activity can be obtained by treatment with mercury, which acts as an efficient blocker of most aquaporins (Maggio and Joly 1995, Carvajal *et al.* 1996, Tazawa *et al.* 1997, Barrowclough *et al.* 2000, Javot and Maurel 2002). The objective of this study was also to investigate the influence of water deficiency and Ca^{2+} content in growth medium on the water diffusion rates in connection with the water transport activity of aquaporins.

Materials and methods

Growth conditions and sample preparations: The experiments were conducted with 7- to 9-d-old seedlings of maize (*Zea mays* L. cv. Donskaja 1) grown in hydroponic culture with continuous aeration, under the 12-h photoperiod and temperature of 20 - 23 °C. One part of seedlings was grown on ¼ Hoagland-Arnon solution, and the other part on the same solution but without Ca^{2+} ions (Ca^{2+} was replaced by Na^{+}). To study the influence of the water channel inhibitor on water diffusion, the roots of intact seedlings were incubated in the nutrient solution containing 0.1 mM of $HgCl_2$ for 15 min. Then a part of seedlings was incubated for 15 min in the solution containing 5 mM of β-mercapto-ethanol. The concentrations of these solutions and incubation times were chosen after Maggio and Joly (1995), Tazawa *et al.* (1997), Zhang and Tyerman (1999), which have shown that the reduction of the hydraulic conductivity of plant roots under the $HgCl_2$ treatment can be reversed by the subsequent incubation in β-mercapto-ethanol. It has been claimed that 0.1 mM $HgCl_2$ is non-toxic (Willmer *et al.* 1999) and do not induce cell membrane leakage (Wan and Zwiazek 1999).

To study the effect of polyethylene glycol (PEG)-induced water deficit, the intact seedlings were transferred into the nutrient solution containing PEG-6000 for 1 h. The solution osmotic potential was of -0.3 MPa. Root water content of control and water stressed seedlings was determined by weighing. Then a part of water stressed seedling roots was treated with $HgCl_2$ (0.1 mM, 15 min).

After the exposure to the above-mentioned solutions, roots were rinsed with distilled water and blotted by filter paper, and root segments 10 - 12 mm long (elongation zone) were excised. The apical zone (2 mm long) of the each root was removed. Root segments were placed in a 10 mm diameter tube with their axes aligned parallel to the tube axis and the end of the tube was sealed by a rubber stopper. Each sample tube containing 15 to 20 segments was placed into the probe of the NMR diffusion meter and thermostated at 20 °C. The water diffusion was measured in the radial direction of the root segments. The measuring time of one sample at one diffusion time was 15 min.

The NMR method of water diffusion coefficient measurement: NMR spin-echo with pulsed magnetic field gradient (Stejskal and Tanner 1965) was used to study water transport in maize seedling roots. General principles of NMR and spin-echo specificity are described in detail in (Abraham 1961, Farrar and Becker 1971). There are two approaches in the NMR spin-echo method to the study of water transfer: relaxational and diffusional. The diffusional method registers the translational displacement of water molecules over a limited time range. In a heterogeneous system (for example, plant tissue) containing different compartments and barriers (membranes), the measured displacement yields information about the water molecule mobility in each compartment, distances between membranes, and their water permeability (Snaar and Van As 1992, Van Dusschoten *et al.* 1995). It has sufficient accuracy and sensitivity and does not require the marker introduction: protons of biosystems, first of all water protons, make markers. The NMR method can be used for the selective estimation of different components of water transfer, namely apoplast, symplast and intercellular components (Anisimov and Ratković 1992, Anisimov *et al.* 1998, Ionenko and Anisimov 2001).

The determination of the molecule self-diffusion coefficient in the diffusional method is based on the registration of the loss of phase coherence of precession frequency in the spin ensemble due to their translational displacement in the magnetic field gradient. A magnetic field gradient is created in a sample volume by passing an

electric current through special coils which surround the tube containing the sample. Thus, the magnetic field gradient marks the volume of the sample, and the self-diffusion of protons in the field gradient then results in a decrease in the spin-echo signal amplitude. This decrease is proportional to the intensity of the magnetic field gradient and the shift of water molecules during the time of observation (so-called diffusion time t_d) from the moment of the start of the first pulse gradient (the beginning of the observation) to the moment of the start of the second pulse gradient (the end of the observation). The projection of the diffusional displacement of water molecules on the magnetic field gradient vector during the experiment is registered.

A three-pulse stimulated echo sequence (Tanner 1970) with a pulsed magnetic field gradient was used to measure the water diffusion. The characteristic feature of this method is that it gives an opportunity to increase the observation time for diffusing molecules, t_d. This method is based on the registration and analysis of diffusional decay (DD) of spin-echo signals depending on parameters of magnetic field gradients. DD of the echo is expressed as:

$$R = \exp [-\gamma^2 \delta^2 g^2 (t_d - 1/3\, \delta) D], \quad (1)$$

where γ is the proton magnetogyric ratio [constant equal to 2.67×10^8 $T^{-1}s^{-1}$; T (tesla) is unit of magnetic field], D is the diffusion coefficient, g and δ are the amplitude and duration of the pulses of the magnetic field gradient, t_d is the diffusion time (the interval between gradient pulses), and R is the relative echo amplitude equal to A(g)/A(0), where A(g) is the echo amplitude when the magnetic field gradient is switched on and A(0) is the echo amplitude when the magnetic field gradient is switched off. Diffusional decays were obtained while changing the values of g [32 steps; T^2 m^{-2}] with fixed values of δ [μs] and t_d [ms].

Physical meaning and value of measured diffusion coefficients are determined by chosen t_d values. In biological systems the increase in the diffusion time allows the successive observation of the mode of free diffusion with self-diffusion coefficient D_o, the mode of diffusion restricted by cell barrier structures - D_r, and the mode of retarded diffusion when cell walls are permeable for diffusing molecules with effective self-diffusion coefficient D_{ef} (Cooper *et al*. 1974) (Fig. 1). The term D_{ef} means the averaged diffusion coefficient for long diffusion times.

The experiments were carried out on the NMR spin-echo diffusion meter at a frequency of 16 MHz with a pulsed magnetic field gradient. The diffusion meter control, and DD treatment were carried out using a computer. The relative echo amplitude, R, versus the amplitude of the magnetic field gradient pulses, g, was recorded at variations of the pulse duration, δ, and intervals between them, t_d, as parameters. Diffusion coefficients were determined using relation (1) from the exponential DD ln R versus g^2 with successive subtraction of exponentials (beginning with the slowly decaying exponential) when DD was multiexponential. Water diffusion coefficients were calculated for each component.

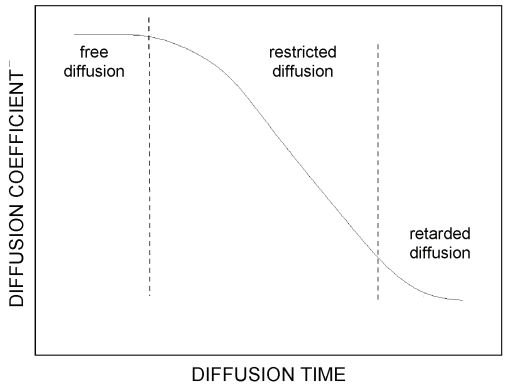

Fig. 1. The idealized dependence of the diffusion coefficient, D_{ef} , on diffusion time, t_d , in systems with permeable walls.

The coefficients of the diffusional water permeability of plasma membrane P_d for root cells were determined using the equation (Crick 1970):

$$1/D_{ef} = 1/D_0 + 1/(P_d \times a) \quad (2)$$

where D_{ef} is the effective coefficient of water diffusion measured in the region of retarded diffusion (at t_d = 500 ms); D_0 is the diffusion coefficient of water at $t_{d\ min}$ = 15 ms; a is the characteristic restricting dimension estimated from the equation of Einstein-Smolukhovsky ($a^2 = 6D_r \times t_d$). For maize roots, a is 21 ± 1.5 μm, corresponding to the average diameter of root cells (Anisimov *et al*. 1998).

Statistics: The experiments were repeated for 3 - 5 samples. Each diffusional decay is an average of 7 - 10 measurements (accumulations of the echo signal amplitude). The statistic analysis was carried out using the *Microsoft Origin* software. Differences between the control and variants with $HgCl_2$ treatment and water stress were statistically significant ($P < 0.05$).

Results and discussion

The dependences of diffusional decays of relative echo amplitudes R on gradient pulse amplitudes g^2 for maize seedling roots are non-exponential and could be decomposed into three components (Fig. 2). Decomposition of DD was carried out by the successive subtraction of exponentials in the dependence R (g^2) beginning with the slowly decaying exponential. The dynamics of the behavior of DD is characteristic of the processes with

restricted diffusion and exchange in biological systems, where the decrease of the decrement of decay with the increase of diffusion times results from the relaxational redistribution of contributions of the fractions of apoplast, cytoplasm and vacuolar symplast water to the echo signal. According to the conventional processing of diffusion measurements (Anisimov and Ratković 1992, Anisimov *et al.* 1998), the fast component, characterized by high diffusion coefficients (of the order of 10^{-9} m^2 s^{-1}), relates to the extracellular and vacuolar water, and the slow component (D_{ef} of the order of 10^{-10} - 10^{-11} m^2 s^{-1}) relates to the intracellular water which is motion-limited both by the membrane permeability and the interactions with cell components. Intermediate component of DD can be related to the distribution of diffusional displacements near restricting structures (first of all membranes) and attributed to less motion-limited water molecules. Van Dusschoten *et al.* (1995) also pointed out the multi-exponentiality of the diffusional decay in apple parenchyma tissue and correlated the decay components with different water compartments.

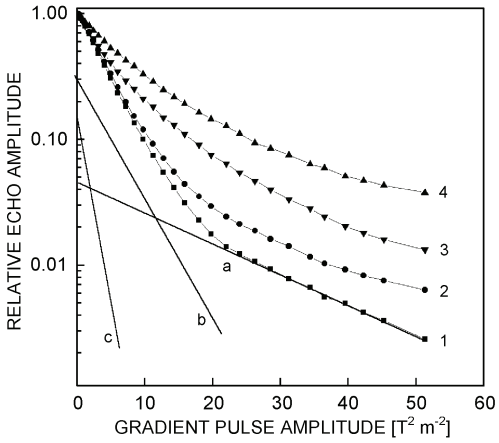

Fig. 2. Diffusional decays of relative echo signal amplitude, R, versus gradient pulse amplitude, g^2, for maize roots at diffusion times t_d = 15 ms (*1*, *2*) and t_d = 300 ms (*3*, *4*), for control (*1*, *3*) and sample treated by 0.1 mM $HgCl_2$ (*2*, *4*). *Straight lines* show separate exponentials received by decomposition of diffusional decay by the successive subtraction of exponentials: *a* - slow, *b* - intermediate, *c* - fast. Water diffusion coefficients (D_{ef}) for the slow component of diffusional decay at t_d = 300 ms equal to 4.0×10^{-10} m^2 s^{-1} for control and 2.5×10^{-10} m^2 s^{-1} for sample treated by $HgCl_2$. The curves are an average of 7 - 10 accumulations of the echo signal amplitude.

The 15-min treatment of roots with mercuric chloride slowed down the diffusional decay and decreased the diffusion coefficients of slow and intermediate components of DD (Figs. 2, 3). The dependence of D_{ef} of slow component of DD on t_d (Fig. 4) within the region t_d > 200 ms, the so-called region of retarded diffusion, characterizes mainly the intercellular water transport and depends on plasmalemma permeability. The decrease in water diffusion rates (calculated from slow and intermediate components of DD) in this region under the influence of $HgCl_2$ is probably connected to the block of plasmalemma and tonoplast water channels. A number of studies have shown that mercurials caused the reversible changes in the conformation of channel proteins by binding to SH-groups of proteins, that resulted in a complete block of the water path (Chrispeels and Maurel 1994, Maggio and Joly 1995, Tyerman *et al.* 1999, Murata *et al.* 2000).

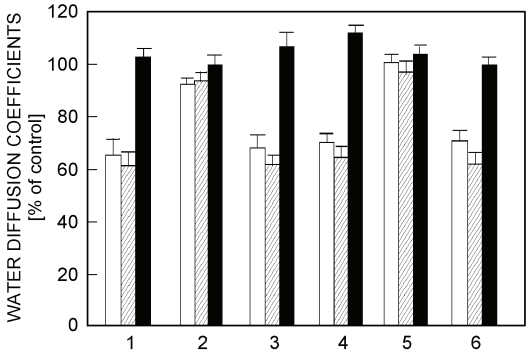

Fig. 3. Relative values of water diffusion coefficients for slow (*empty columns*), intermediate (*stripped columns*) and fast (*black columns*) components of diffusional decay under the influence of: *1* - 0.1 mM $HgCl_2$, *2* - consecutive treatments with 0.1 mM $HgCl_2$ and 5 mM mercaptoethanol, *3* - PEG-induced water stress (-0.3 MPa, 1 h), *4* - consecutive application of water stress and $HgCl_2$ treatment, *5* - $HgCl_2$ treatment of Ca^{2+}-deficient seedling roots, *6* - consecutive treatments of Ca^{2+}-deficient roots with 1.5 mM $Ca(NO_3)_2$ and 0.1 mM $HgCl_2$. *Bars* show SE (n = 5).

The calculations of the coefficients of the diffusional water permeability of plasma membrane P_d using the equation (2) shown P_d decrease in roots affected by $HgCl_2$ almost by a factor of two ($1.7 \pm 0.2 \times 10^{-5}$ m s^{-1}) as compared to the control ($3.2 \pm 0.15 \times 10^{-5}$ m s^{-1}).

It should be noted that the blocking effect of $HgCl_2$ was exhibited only when the nutrient solution for plant growth contained Ca^{2+} (Ca^{2+} ion concentration of 1.5 mM)

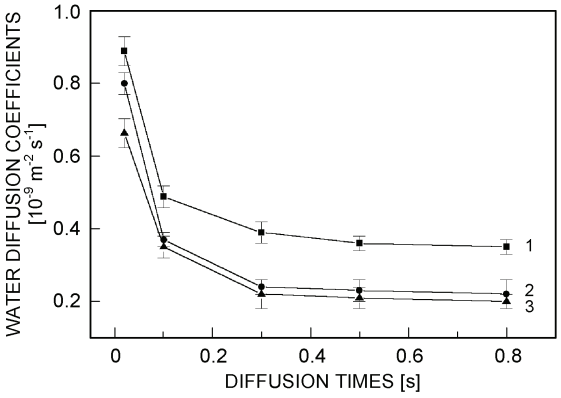

Fig. 4. Dependence of water diffusion coefficients, D_{ef}, of the slow component of diffusional decay on diffusion time, t_d, for maize roots: *1* - control, *2* - 0.1 mM $HgCl_2$ (15 min), *3* - PEG-induced water stress (-0.3 MPa, 1 h). *Bars* show SE (n = 5).

(Fig. 5). The absence of $HgCl_2$-sensitivity of roots was revealed in plants growing on the Ca^{2+}-deficient nutrient solution. Subsequent addition of Ca^{2+} ions to Ca^{2+}-deficient roots [1.5 h incubation in the nutrient solution with 1.5 mM $Ca(NO_3)_2$] restored Hg^{2+}-sensitivity (Fig. 3). These data confirm an important role of Ca^{2+} in the regulation of water channel activity and are consistent with the results of Johansson *et al.* (1996, 1998), who demonstrated the dependence of water-transport aquaporin activity (phosphorylation of plasma membrane protein PM28A of spinach leaf) on submicromolar concentrations of Ca^{2+}.

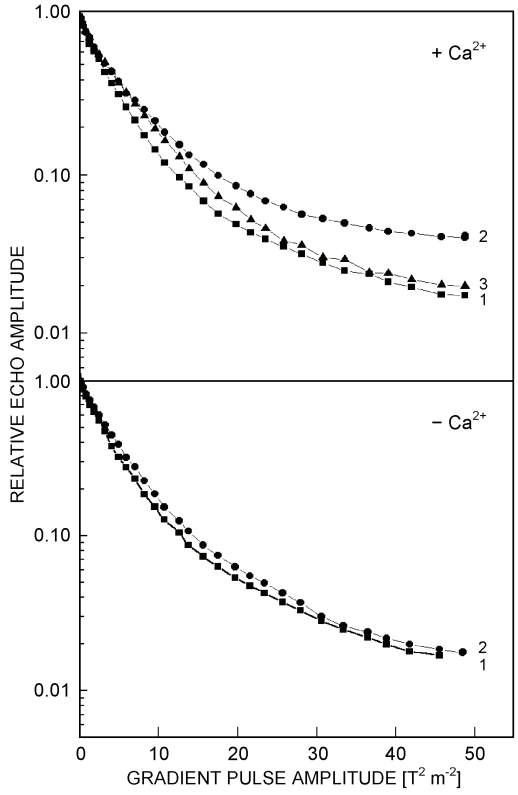

Fig. 5. Diffusional decays of the relative echo signal amplitude, *R*, versus gradient pulse amplitude, g^2, for maize seedling roots, grown on standard nutrient solution with Ca^{2+} of 1.5 mM (*upper part*) and on Ca^{2+}-deficient solution (*lower part*): *1* - control, *2* - 0.1 mM $HgCl_2$ (15 min), *3* - consecutive treatments with 0.1 mM $HgCl_2$ and 5 mM mercaptoethanol. Measurements were made at the diffusion time t_d = 300 ms. The curves are an average of 7 - 10 accumulations of the echo signal amplitude.

The inhibition of water diffusion was reversible with a 15 min exposure of $HgCl_2$-treated roots in 5 mM β-mercaptoethanol (Figs. 3, 5). The reduction of the flow rates under $HgCl_2$ treatment and their subsequent recovery with β-mercaptoethanol point to: *1*) the absence of any essential side effects caused by the $HgCl_2$ treatment and *2*) the presence of an essential protein-mediated way of water transport in maize roots (Maggio and Joly 1995).

Water stress (1 h incubation of intact seedling roots in PEG solution prior to NMR measurements) caused the changes in D_{ef} similar to those caused by mercuric chloride (Figs. 3, 4). The changes in root water content of PEG-pretreated roots were slight (95 ± 1.5 and 92 ± 1.3 % of fresh mass for control and water stress samples, respectively) and could not cause the observed decrease of D_{ef}. Moreover a number of studies have shown that replacing the nutrient solution with test solutions of higher osmotic pressure (mannitol, PEG 4000) caused a biphasic response in root cell: fast decline in pressure potential accompanied by growth cessation and subsequent recovery (within 10 - 30 min) of these parameters (Kuzmanoff and Evans 1981, Frensch and Hsiao 1994). The invariability (or even slight increase) of the fast component of DD under the influence of PEG-induced water stress (Fig. 3) can result from the recovery of pressure potential.

It should be noted that the roots subjected to water stress as well as the roots of seedlings grown in Ca^{2+}-deficient nutrient solution did not respond to the addition of $HgCl_2$ to the nutrient medium (Fig. 3). A number of studies have demonstrated the absence of $HgCl_2$-sensitivity of hydraulic conductivity of plant roots growing under non-standard conditions, for example, in nutrient deprived roots of wheat (Carvajal *et al.* 1996, Clarkson *et al.* 2000), and in hypoxia-treated cells (Zhang and Tyerman 1999). The decrease in D_{ef} of slow and intermediate components of DD under the influence of water deficiency was probably connected with the reduction of water transport activity of aquaporins. One of the mechanisms lying in the basis of reduction of aquaporin activity under water stress can be the repression of aquaporin phosphorylation (Johansson *et al.* 1996, 1998). Johansson *et al.* (1998) have demonstrated the decrease in the phosphorylation level of channel proteins in the spinach leaf plasma membrane in response to water deficiency, which resulted in water channel closing. Our results with PEG-induced water stress correlate with our previous data (Ionenko and Anisimov 2001) where the effect of water deficit on D_{ef} was explained in terms of re-distribution of water flows through different transport ways, *i.e.*, by the decrease of the rate of transmembrane water diffusion and the increase of the water transport rate in apoplast and, apparently, intercellular endoplast.

Thus, the similarity of changes in water transfer parameters (D_{ef}) in maize seedling roots in response to the effect of $HgCl_2$ and water deficiency, and also the absence of $HgCl_2$-sensitivity of roots subjected to water stress, point most likely to disturbed conductivity of water channels under the PEG-induced water stress. The possible closure of water channels could help to limit root water loss under adverse drying conditions. The dependence of $HgCl_2$-sensitivity on addition of Ca^{2+} to the growing medium indicates an important role of this ion in the regulation of water channel activity. The dependence of water flow inhibition (by mercury) on the Ca^{2+} supply together with the reversibility of mercury inhibition by mercaptoethanol point to the absence of

essential side (toxic) effects caused by a metabolic poison treatment.

The obtained results of diffusional measurements demonstrate the possibilities of the pulsed NMR method for study of the transmembrane water exchange in plant tissues in connection with functioning of water channels without interfering with the hydrodynamic system of plant. The sensitivity of the measured water diffusional parameters to the effect of water channel blocker allows one to look forward to applying this method to study the water transport activity of aquaporins and their role in water transfer switching to various paths along plant tissues under stress conditions.

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