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Regulation of Intercellular Water Exchange in Various Zones of Maize Root under Stresses

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Abstract—Apical root meristems and segments of root elongation zone were sampled from 4- to 5-day-old *Zea mays* L. seedlings. The vacuolar ATPase and pyrophosphatase, the tonoplast marker enzymes, and the tonoplast α-, δ-, and γ-aquaporins were visualized by means of indirect immunofluorescent microscopy with the use of the respective antibodies. Following cell plasmolysis (700 mM mannitol, 2.5 h), the vacuolar ATPase and pyrophosphatase were detected in cell wall pores where plasmodesmata remained detached from the plasmolyzed protoplasts. This finding provides further evidence for existence of the vacuolar symplast in the elongation zone of maize root, which may ensure intercellular continuity of plant tissues. The pulsed NMR method was used to study the self-diffusion of water molecules. The diffusive decay in the root elongation zone was nonexponential, and it was transformed to three exponential terms with characteristic coefficients of self-diffusion; two of these coefficients (D_2 and D_3) characterize the water self-diffusion in the cytoplasmic and vacuolar symplasts of root, respectively. The root apical meristem was also investigated with NMR technique by virtue of paramagnetic doping of the apoplast. This approach allowed selective studying of water diffusion within the symplast compartments. Partial dehydration with PEG-6000, 12 and 20%, for 2.5 h and chemical stressors (ABA and salicylic acid, 0.1 mM, 24 h) were applied to modify water permeability of plasmodesmata and tonoplast aquaporins. The transcellular water permeability increased in the root meristem under the action of all stress factors. In the root elongation zone exposed to partial dehydration, the water exchange in the apoplast became the dominant component. Other stress factors affected water relations in different manners. ABA elevated the water permeability of the vacuolar symplast, in contrast to salicylic acid that decreased water conductance of both the cytoplasmic and vacuolar symplasts.

Key words: Zea mays - plasmodesmata - aquaporins - water permeability - regulation - immunofluorescent microscopy - NMR

INTRODUCTION

Water movement in plant tissues occurs through three pathways: (1) the apoplast, i.e., tissue free space that comprises intercellular spaces, cell-wall channels between the cellulose microfibrils, and xylem vessels; (2) the symplast, i.e., protoplasts of all cells interconnected by plasmodesmata into a continuous system; and (3) the transmembrane route—from cell to cell with the intermediate efflux through the vacuoles and across the plasma membrane into the intercellular space.

The plasmodesmata, lined up with the plasma membrane, contain a hollow central element, a desmotubule formed by the membrane of the endoplasmic reticulum [1]. The desmotubular membrane is linked to other endomembranes, which provides a continuous membrane space of a plant tissue [2, 3]. It is thought that the permeability of plasmodesmata is actively regulated by the ATP-dependent sphincter, localized in the neck regions of the plasmodesmata [4–6] and/or by rapid reversible depositions of callose in the cell wall around the plasmodesma [4]. Using the NMR method, we obtained indirect evidence in favor of the Gamalei's hypothesis [3] on the existence of two transport pathways in the plasmodesmata [5, 7]. There is some evidence for the occurrence in plant tissues of two symplastic systems—the cytoplasmic and vacuolar symplasts.

Cell membranes represent the main barrier for the transcellular water movement. In the last few years it became clear that water crosses biological membranes not only through the lipid bilayer but also through the pores formed by aquaporins, specific membrane proteins intrinsic to animal and plant cells [8]. The plant cell aquaporins represent a family of integral mem-

Abbreviations: D—coefficient of self-diffusion; DMSO—dimethyl sulfoxide; FITC—fluorescein isothiocyanate; PBS—phosphate buffer solution; Pipes—piperazine N,N'-bis[2-ethanesulfonic acid]; PMD—paramagnetic doping; TIPS—tonoplast aquaporins (tonoplast intrinsic proteins).

brane proteins with mol wt 20–27 kD and are divided in two subfamilies: the plasma-membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs) [9]. The discovery of aquaporins raised the question of their significance for the radial water transport in the root. The proposal has been put forward that the bulk of radial water flow in roots can proceed via the transcellular route under some conditions [10, 11] and can be controlled by changes in aquaporin frequency in the membrane and by opening–closing of these water channels [10, 12].

The dominance of each route in water transport depends on the plant species, the stage of plant development, as well as on the driving force of water absorption, i.e., hydrostatic and osmotic gradients [13, 14]. At high-rate water flow in the case of open stomata at low relative humidity of air, water moves predominantly through the apoplast, since the apoplast hydraulic resistance is comparatively low. Conversely, the apoplastic movement is insignificant at slow rates of water flow [14, 15], and the major water flow moves along the transcellular pathway that includes the transmembrane water transfer and the transport through the plasmodesmata [16]. In the world literature, the transport routes via plasmodesmata and plasma membranes are usually not separated, and both components of this water transport are termed "cell-to-cell" movement. This is explained by technical difficulties of discriminating these two pathways [17].

The hydraulic root conductance is known to change under various abiotic stresses, such as high salinity, anoxia, and thermal stress. A considerable role in such regulation of water hydraulic conductance belongs to aquaporins [17–19].

In this study we attempted to analyze regulatory changes in water permeability of aquaporin pores and plasmodesmata in various zones of maize root under exposure to water deficit stress and some chemical stressors (ABA and salicylic acid). An additional goal was to obtain evidence in support of the idea of vacuolar symplast. Such a continuum, together with a cytoplasmic symplast, would ensure the intercellular continuity of plant tissues.

MATERIALS AND METHODS

We used the roots of 4- to 5-day-old etiolated maize (*Zea mays* L.) seedlings. Seeds were soaked for 8 h in a running tap water and then placed into filter paper rolls and germinated at 25°C in a dark thermostat. The water deficit stress was imposed by placing the intact seedling roots into PEG solutions (12 and 20%). These treatments provided, respectively, weak and moderate water losses of 5 and 10%. The plasmolysis was evoked with 700 mM mannitol (Sigma, United States). The treatments with chemical stressors—a phytohormone ABA (0.1 mM, Sigma) and salicylic acid (0.1 mM, Sigma)—lasted for 24 h. Two root zones were taken for measurements: the meristem occupying 1.5–2 mm-long root apex and the elongation zone positioned within 2–8 mm of the root apex [20].

Self-diffusion of water in root tissues was studied by means of NMR spin-echo technique with a pulsed magnetic field gradient [21]. We recorded the relative echo amplitude R as a function of squared magnetic field gradient (g^2) at fixed durations of gradient pulses (δ) and fixed periods for observation of diffusion ($t_d = 100$ ms). The coefficients of self-diffusion (D) were calculated from the formula $\ln R = -\gamma^2\delta^2 g^2 t_d D$, where γ is a constant (the proton gyromagnetic ratio) and D is the coefficient of self-diffusion of water molecules. The method of determining D values of water in roots was described previously [5, 21].

In our earlier study, we interpreted the origin of D values obtained by decomposing the diffusive decay in roots into three exponential terms with respective D [5]. The highest coefficient (D_3) characterizes the averaged self-diffusion of water in the vacuolar symplast and the apoplast, whereas the middle-valued coefficient (D_2) was assigned to water transport in the cytoplasmic symplast of roots. The slowly decaying portion of three-component diffusive decay provides information on self-diffusion coefficient D_1, which is independent of the diffusion observation time; in the context of our study this coefficient was not considered here. However, we applied a methodological approach that allowed us to eliminate the contribution of water transport through the apoplast and to observe water transport through the tissue symplast only (the so-called paramagnetic doping, PMD) [23, 24]. In this method, the diffusive displacement of water molecules over the sample volume is measured after preliminary infiltration of the extracellular space with paramagnetic ions that feature high relaxation efficiency and cannot permeate (or permeate slowly) into the cell interior. The diffusion distance is eventually confined to the symplast, because the water molecules coming out of the cell become invisible for NMR signal detection. This invisibility results from the fast relaxation in the presence of paramagnetic ions, while water molecules entering the cell have already lost their magnetization. We used manganese ions as paramagnetic agents. The root samples were exposed to 2.5 mM $MnCl_2$ for 15 min.

The roots were gently wiped with a filter paper, cut into segments measuring 2 mm from the root apex (meristem) and subsequent 5–6 mm from the apex (elongation zone). The samples from the elongation zone were placed into the detector unit of diffusometer in such a way that the magnetic filed gradient was directed parallel to the root radius; thus, the water self-diffusion in the radial direction could be determined. The meristem samples were randomly oriented in the detector unit because of their small size.

The tonoplast visualization was accomplished by means of indirect immunofluorescent microscopy and

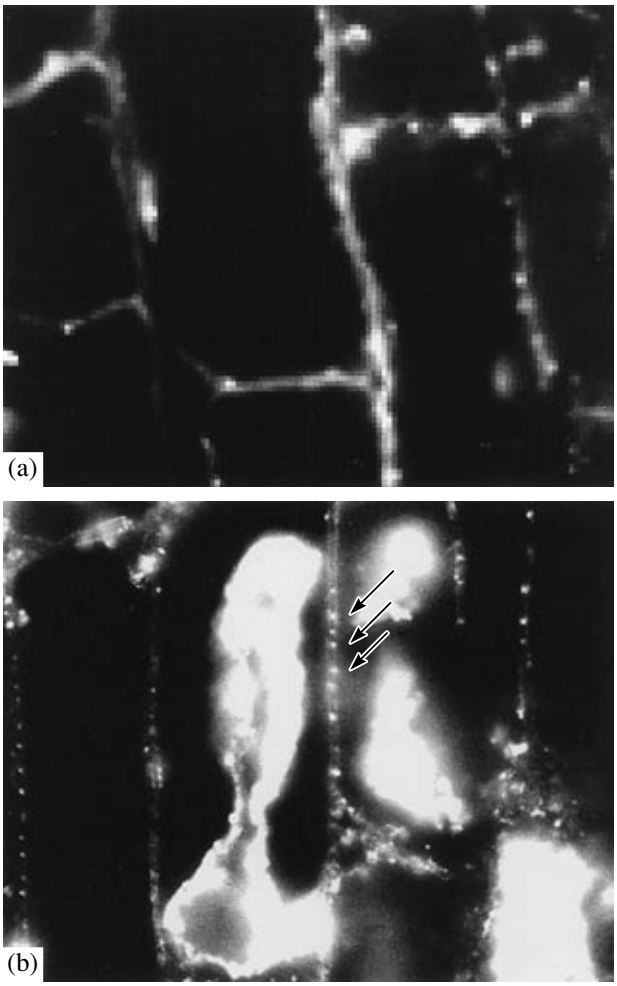

Fig. 1. Visualization of subcellular location of pyrophosphatase in the elongation zone of maize roots after plasmolysis.
(a) Control cells; (b) mannitol-treated (700 mM, 2.5 h) cells. Arrows point to the location of the protein in cell walls of plasmolyzed cells (in the vicinity of plasmodesmata). Magnification ×100.

the tonoplast marker proteins, i.e., vacuolar ATPase and pyrophosphatase, according to the method of Balušska *et al.* [25]. The apical root segments measuring 8 mm were vacuum-infiltrated with 4% formaldehyde dissolved in a stabilizing buffer with pH 6.9 (the buffer contained 50 mM Pipes, 50 mM $MgSO_4$, 50 mM EGTA, and 12% dimethyl sulfoxide (DMSO)). The segments were infiltrated for 2 min and incubated in the same buffer for another 60 min. The root segments were then transferred to a fresh stabilizing buffer for 30 min and then incubated in the phosphate buffer solution (PBS), pH 7.3 (0.14 M NaCl, 2.7 mM KCl, 6.5 mM KH_2PO_4) for 15 min. Next, the segments were dehydrated in a series of ethanol–PBS solutions (30, 50, 70, 90, and 97% ethanol); each treatment lasted for 30 min. An ethanol-soluble Steedman's wax with a low melting point was used as a fixative agent [26]. The Steedman's wax was prepared by mixing PEG-400 distearate melted at 65°C with hexadecanol at 9 : 1 ratio. The infiltration of tissues with the wax was performed at 37°C. First, the wax–ethanol mixture was infiltrated for 12 h, and then the pure wax was applied for 1–2 h. The root segments were placed into special cups and allowed to polymerize for 8 h. The 10 μm thick sections were prepared with a microtome. They were placed into a drop of distilled water, adhered to glass plates, and gently dried on object plates (Super-Frost*/plus glasses, no. 041300, Germany) covered with glycerol-albumine. After the removal of wax, the sections were rehydrated in a series of ethanol–PBS solutions (97, 90, and 50% ethanol; 10 min each treatment). Next, the glasses with samples were placed in PBS for 10 min to accomplish protein labeling. The anti-vacuolar H^+-ATPase and anti-pyrophosphatase [27] dissolved in PBS at 1 : 100 and 1 : 200 ratios were used as primary antibodies. In addition, we used anti-α-TIPs, anti-δ-TIPs, and anti-γ-TIPs [28] dissolved in PBS at 1 : 100, 1 : 30, and 1 : 50 ratios, respectively. The samples were incubated with primary antibodies for 1 h. Next, the sections were rinsed with PBS for 10 min and treated with secondary fluorescein-labeled antibodies. Fluorescein isothiocyanate (FITC) was obtained from Sigma. FITC-conjugated anti-rabbit IgG was dissolved in PBS at a ratio of 1 : 100. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, United States).

The samples prepared were examined with an Axiovert 405 M microscope (Zeiss, Germany) equipped with epifluorescent and conventional FITC filters (BP 450–490, LP 520). The photographs were taken with an AxioCam digital camera (Zeiss). The immunofluorescence experiments were conducted in the Institute of Botany, Bonn University (Germany).

Data in Figs. 4–6 represent mean values and their standard errors.

RESULTS AND DISCUSSION

By using indirect immunofluorescent microscopy and antibodies for two tonoplast-specific proteins (vacuolar ATPase and pyrophosphatase as tonoplast markers), we exposed root samples to mannitol-induced plasmolysis and found that the ATPase and pyrophosphatase were present in cell walls of plasmolyzed cells in the regions of plasmodesmata pit fields (Figs. 1 and 2). This observation provides direct evidence for the continuity of vacuolar compartment. This continuity can be realized through the transition of the tonoplast membrane in the form of tube through the plasmodesma (desmotubule), with the resulting formation of the second, vacuolar transport route within the plasmodesma. This membrane continuum would ensure the intercellular integrity of the plant tissue.

The meristematic cells differ in their structure from the cells of the elongation zone. The characteristic features of meristematic cells are small dimensions, dense

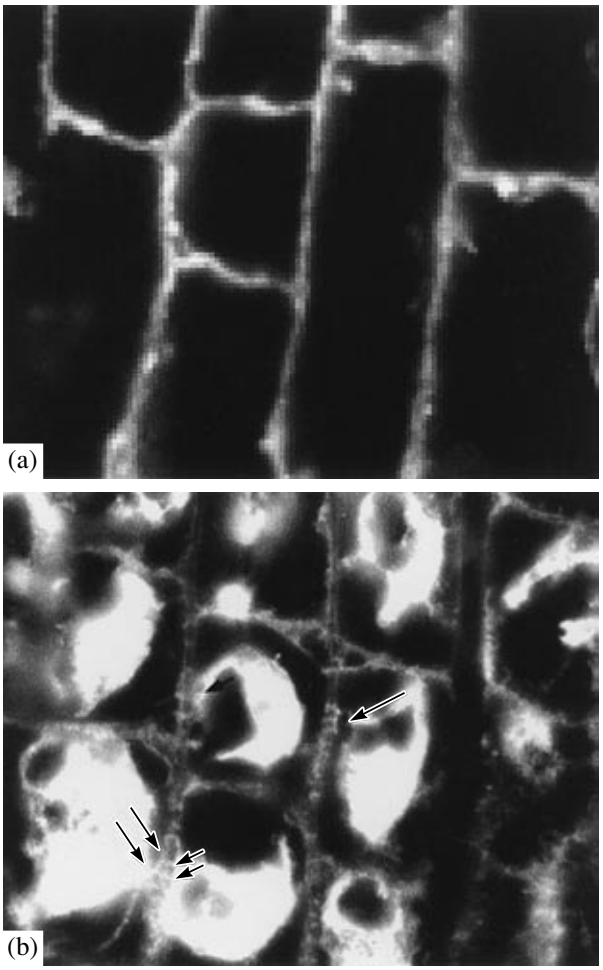

Fig. 2. Visualization of subcellular location of ATPase in the elongation zone of maize roots plasmolyzed with mannitol. (a) Control cells; (b) mannitol-treated (700 mM, 2.5 h) cells. Arrows point to the location of the protein in cell walls of plasmolyzed cells (in the vicinity of plasmodesmata). Magnification ×100.

cytoplasm, and abundant plasmodesmata [28, 29]. Small vacuoles in these cells are distributed around the nucleus and are not spatially interconnected. Apparently, the vacuolar symplast has not yet been formed in dividing cells; it develops only in the elongation zone.

The diffusive decay in the root meristem consisted of two components in contrast to three-component diffusive decay in the root elongation zone (Figs. 3a and 3b). When the PMD procedure was applied to the meristematic region, the signal of apoplastic water was eliminated and the diffusive decay became monoexponential (Fig. 3c) with a single self-diffusion coefficient D. This coefficient reflects the unobscured water mobility in the cytoplasmic symplast. Thus, the two components and the respective self-diffusion coefficients in these samples seem to characterize the water permeability of the cytoplasmic symplast (D_1) and the apoplast (D_2).

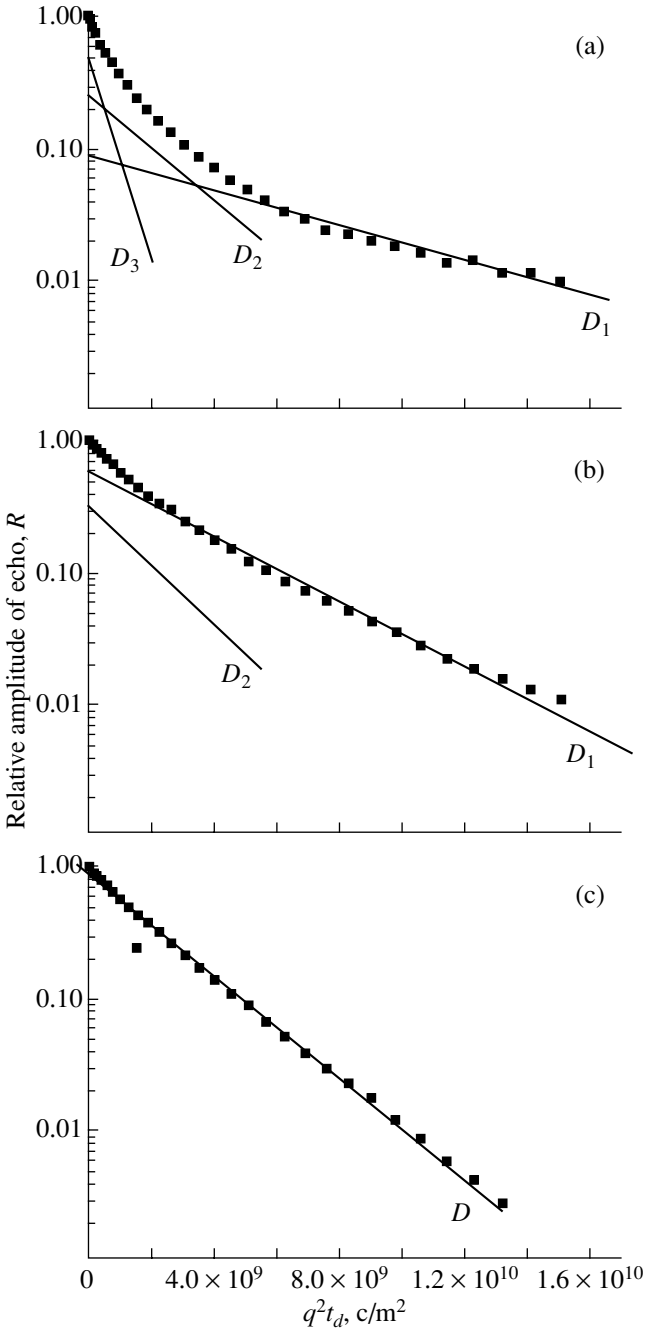

Fig. 3. Diffusive decay of stimulated echo in different zones of maize root.

(a) Three-component diffusive decay in the elongation zone; straight lines correspond to decomposed exponential terms with self-diffusion coefficients D_1, D_2, and D_3; (b) two-component diffusive decay in the meristem zone; straight lines correspond to decomposed exponential terms with self-diffusion coefficients D_1 and D_2; (c) monoexponential diffusive decay in the meristem zone after infiltrating the apoplast with paramagnetic ions (Mn^{2+}); a straight line represents a single exponential term with a self-diffusion coefficient D. The abscissa corresponds to the parameter $q = -\gamma g \delta$, where γ is a gyromagnetic ratio for protons (physical constant), g is the amplitude of the gradient pulse, δ is duration of the gradient pulse, and t_d is the observation time.

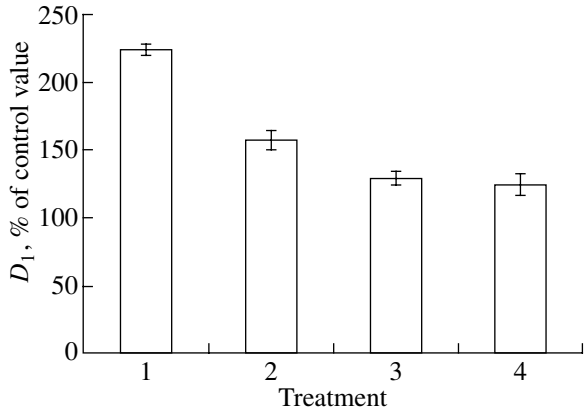

Fig. 4. Changes in self-diffusion coefficient D_1 in the root meristem.

The observation time was 200 ms. (1) Weak dehydration (12% PEG-6000, 2.5 h); (2) moderate dehydration (20% PEG-6000, 2.5 h); (3) ABA (0.1 mM, 24 h); (4) salicylic acid (0.1 mM, 24 h). The self-diffusion coefficient D_1 of unstressed sample was 1.44×10^{-10} m^2/s.

In root meristems exposed to weak dehydration, the coefficient D_1 increased more than twofold with respect to the control value, i.e., unstressed samples. A substantial increase in D_1 was also observed under moderate stress (Fig. 4), which implies stimulation of water transport through the cytoplasmic symplast. The application of PMD procedure to root meristems confirmed the increase in coefficient of water self-diffusion through the cytoplasmic symplast in stressed samples (Fig. 5). The literature contains evidence that water deficit is accompanied by the cessation of water transport through the apoplast; under these conditions the bulk of water flow driven by the osmotic force proceeds through the transcellular route [17]. The results of our pulsed NMR experiments suggest that partial dehydration increases the water conductivity of cytoplasmic symplast in the root meristem.

A principally different situation was observed in the root elongation zone: the D_2 coefficient reflecting the permeability of cytoplasmic symplast to water decreased under partial dehydration (Fig. 6a). At the same time, the increase in D_3 coefficient was observed. It is hardly possible that water conductivity of the vacuolar symplast increases under given conditions of cell plasmolysis, possible shrinkage of vacuoles, and the detachment of plasmodesmata. A most likely explanation is that slight dehydration of cells in the root elongation zone elevates water transport through the apoplast. This would raise the D_3 coefficient known to characterize the average coefficient of water self-diffusion in the vacuolar symplast and the root apoplast.

Thus, the dehydration of root tissues apparently increases the water permeability of the transcellular route in the meristem and of the apoplastic route in the elongation zone.

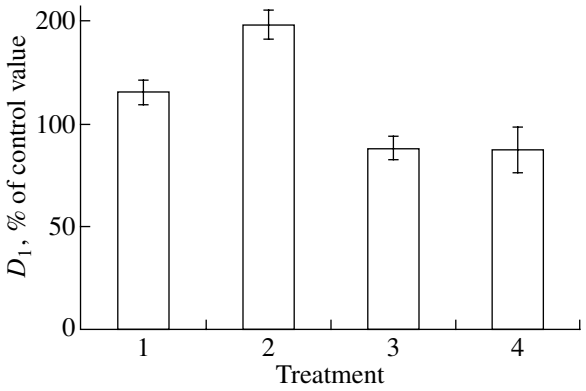

Fig. 5. Changes in self-diffusion coefficient D in the root meristem upon paramagnetic doping (2.5 mM $MnCl_2$, incubation time 15 min).

See Fig. 4 for designations of treatments.

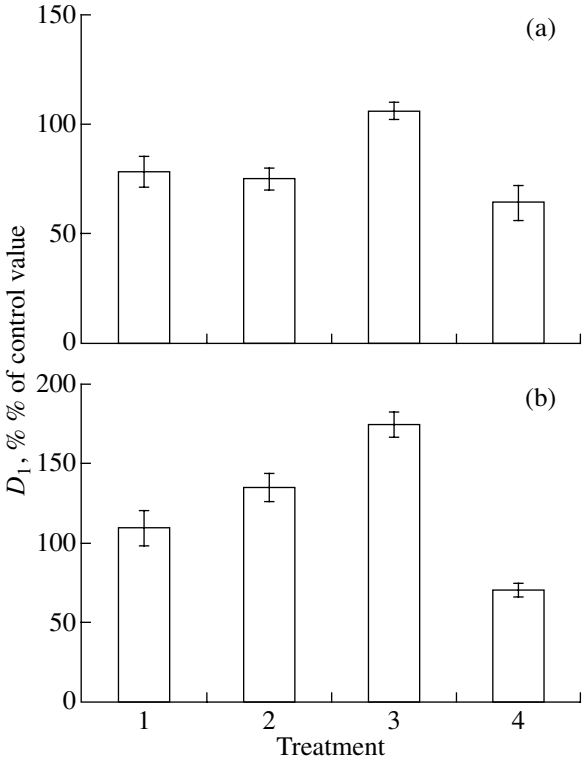

Fig. 6. Changes in self-diffusion coefficients (a) D_2 and (b) D_3 in the elongation zone of maize root.

The values D_2 and D_3 for the untreated sample were 3.44×10^{-10} and 1.02×10^{-10} m^2/s, respectively. Treatment designations are the same as in Fig. 4.

Effects of ABA on water relations in plant tissues are being actively investigated. It was found that this phytohormone, synthesized in leaves under stress conditions, is transferred to roots, thus increasing their absorptive and pumping capacity. The researchers exploring the origin of ABA-stimulated hydraulic conductivity tend to believe that such stimulation reflects the increase in transcellular water flow caused by virtue

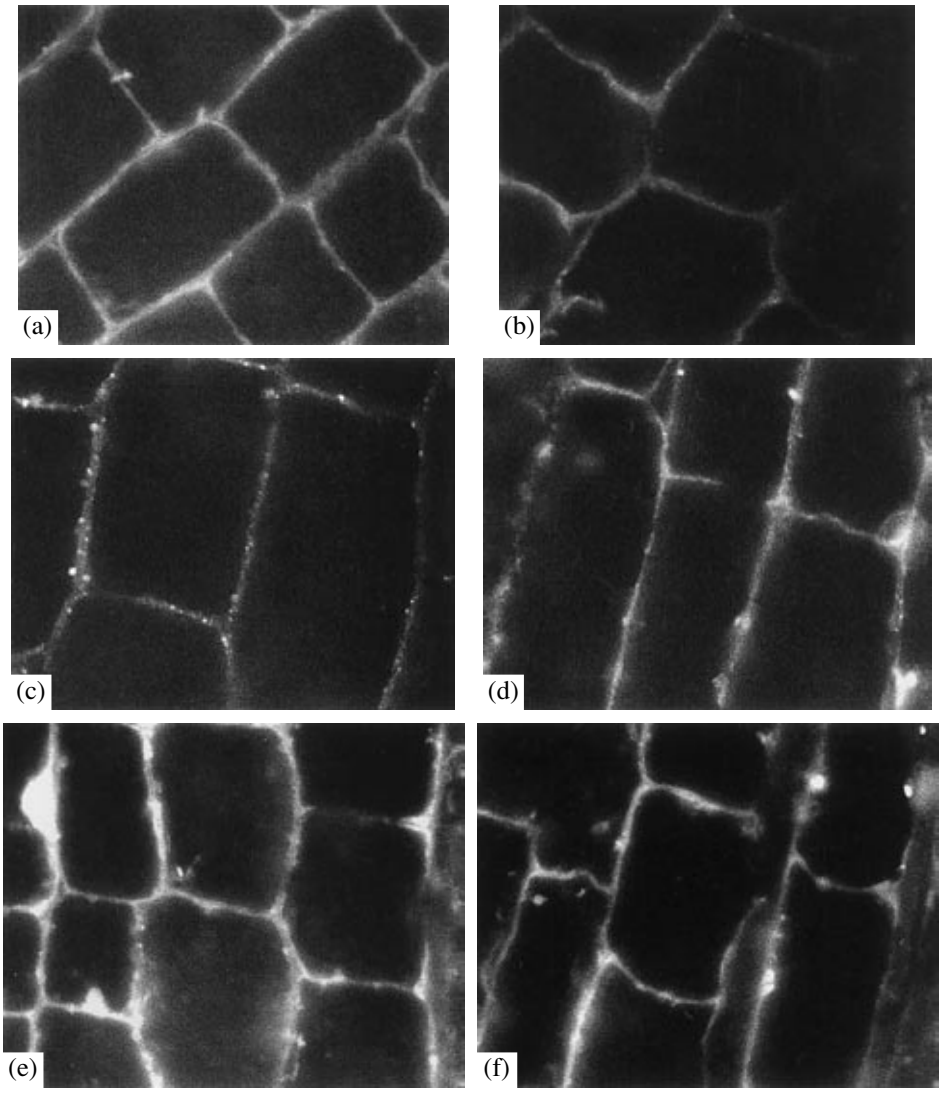

Fig. 7. Visualization of various types of the tonoplast aquaporins in the elongation zone of maize roots treated with ABA. (a, c, e) Untreated samples; (b, d, f) ABA treatment (0.1 mM, 24 h); (a, b) α isotype; (c, d) δ isotype; (e, f) γ isotype. Magnification ×100.

of aquaporin activity in the membranes [30]. Nevertheless, as noted above, discrimination between the transmembrane and symplastic pathways is technically impossible so far. Therefore, the role of symplast and, particularly, of plasmodesmal permeability in ABA-enhanced water transport in root tissues was not discussed up to date. Here we attempt to compensate for the lack of this discussion.

In the root meristem ABA caused a slight increase in D_1 coefficient (Fig. 4) that characterizes water conductivity of the cytoplasmic symplast. However, the application of PMD in order to exclude the contribution of transmembrane water flows and to monitor the plasmodesmal conductivity from changes in self-diffusion coefficient, revealed an insignificant decrease in the self-diffusion coefficient under the action of ABA (Fig. 5). These experimental data imply that ABA does not enhance the water conductivity of plasmodesmata in the root meristem and that the D_1 increase in conventional experiments without PMD resulted from stimulation of the overall transcellular water exchange due to higher water permeability of the plasma membrane. This may result from the increase in the amount of aquaporin isoforms in the membrane and/or from the increase in the functional activity of aquaporins.

In the elongation zone, i.e., the root region with two developed symplasts, we observed the ABA-induced increase in D_3 coefficient and a constant D_2 value (Fig. 6). These coefficients reflect changes in water permeability of the vacuolar (D_3) and cytoplasmic (D_2) symplasts [5]. Apparently, the change in D_3 coefficient can be a consequence of the increasing water permeability of the vacuolar symplast. What is a possible mechanism of such an increase?

Using indirect immunofluorescent microscopy, we established the presence of three main aquaporin isotypes—α, δ, and γ—in maize root tissues. However, we did not observe greater aquaporin labeling in the elongation zone under the action of ABA (Fig. 7). Since ABA treatment did not cause appreciable changes in water permeability of cytoplasmic symplast, the changes in water permeability of its membrane barriers, including the tonoplast, seem unlikely. As the water permeability of the vacuolar membrane remains constant and the composition of the tonoplast aquaporin isoforms remains invariable, the probability of ABA-induced changes in aquaporin functional activity seems very low. Apparently, the ABA-induced increase in coefficients of water self-diffusion through the vacuolar symplast can be interpreted as evidence that ABA treatment elevates water permeability of the vacuolar symplast in the root elongation zone due to the increase in water permeability of the respective channels in plasmodesmata.

By comparing ABA effects in two root zones, we suppose that the stimulation of transcellular water exchange in the meristematic region is likely caused by the increase in aquaporin activity in the plasma membrane, whereas the ABA-induced stimulation of water exchange in the elongation zone results from the increase in water permeability of the vacuolar channel in plasmodesmata.

The effect of another chemical stress factor, salicylic acid on the root meristem was manifested in the increase of D_1 coefficient and in the decrease of self-diffusion coefficient determined by PMD method (Fig. 5). In other words, the effect of salicylic acid was analogous to ABA-induced dynamics of water exchange.

The treatment of root elongation zone with salicylic acid produced a considerable decrease in D_2 and D_3 coefficients (Fig. 6). Lyalin [31] showed that even a short-term exposure of roots to salicyclic acid inhibited the xylem transport and that the extent of the inhibition was comparable with the plant response to full drainage of the root zone. Based on our experimental data, we assume that salicylic acid suppresses the symplastic water transport both in the root meristem and the elongation zone. Furthermore, in the elongation zone, both transport pathways of plasmodesmata (cytoplasmic and vacuolar) turn closed.

Therefore, we demonstrated the existence of the vacuolar symplast ensuring the intercellular continuity of plant tissue. While comparing the effects of stress conditions examined, the occurrence of differential mechanisms for controlling the transcellular water exchange in the elongation zone and the root meristem should be noted. In the root meristem the water conductance of the transcellular route increased upon dehydration and the action of chemical stressors. In the elongation zone, the dehydration stress elevated water exchange through the apoplast, while the chemical stressors produced different effects: ABA promoted water conductance of the vacuolar symplast but salicylic acid reduced water conductance of both symplastic systems.

ACKNOWLEDGMENTS

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