Gating of aquaporins by heavy metals in *Allium cepa* L. epidermal cells

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Abstract Changes in the water permeability, aquaporin (AQP) activity, of leaf cells were investigated in response to different heavy metals (Zn^{2+} , Pb^{2+} , Cd^{2+} , Hg^{2+}). The cell pressure probe experiments were performed on onion epidermal cells as a model system. Heavy metal solutions at different concentrations (0.05 μM –2 mM) were used in our experiments. We showed that the investigated metal ions can be arranged in order of decreasing toxicity (expressed as a decrease in water permeability) as follows: $\text{Hg}>\text{Cd}>\text{Pb}>\text{Zn}$. Our results showed that β -mercaptoethanol treatment (10 mM solution) partially reverses the effect of AQP gating. The magnitude of this reverse differed depending on the metal and its concentration. The time course studies of the process showed that the gating of AQPs occurred within the first 10 min after the application of a metal. We also showed that after 20–40 min from the onset of metal treatment, the water flow through AQPs stabilized and remained constant. We observed that irrespective of the metal applied, the effect of AQP gating can be recorded within the first 10 min after the administration of metal ions. More generally, our results indicate that the toxic effects of investigated metal ions on the cellular level may involve AQP gating.

Keywords Epidermal onion cells · Aquaporins · Heavy metals · Cell pressure probe · Half-time of water exchange · β -mercaptoethanol

Abbreviations

AQP	Aquaporin
PPC	Cell pressure probe
$T_{1/2}$	Half-time of water exchange
ME	β -mercaptoethanol

Introduction

Since 1993, when the first aquaporin (AQP) from plants (γ -TIP) was cloned and functionally expressed (Maurel et al. 1993), there has been a growing interest in AQPs and their influence on the biophysics of water flow across plant membranes (Steudle and Henzler 1995; Maurel 1997; Tyerman et al. 2002). Since that time, evidence has been increasingly presented that aquaporins are central components in plant–water relations at all levels of organization (cell, tissue, organ, and whole plant). It is now widely known that most (75–95%) of water transport is mediated by aquaporins (Maurel 1997; Kjellbom et al. 1999; Tyerman et al. 1999; Henzler et al. 2004; Ye et al. 2005). Therefore, the open/close state of AQP and its regulation is essential in maintaining cell water balance and the adjustment of plant–water relations. Because of the integration of AQPs in numerous functions in plant development and adaptations to different living conditions, studies on aquaporins gave us unique insights into various aspects of plant biology (Maurel et al. 2008). Adaptation to heavy metal stress is one of these aspects.

Although there is widespread literature discussing questions related to heavy metals and their effects on plants (van Assche and Clijstres 1990; Ros et al. 1990; Ernst 1990; Manahan 1992; Wierzbicka 1994, 1999; Fodor 2002; Shanker et al. 2004; Wierzbicka et al. 2007), not much attention has been

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paid to studies concerning plant–water relations under heavy metal stress (Poschenrieder and Barcelo 2004). This fact is rather surprising in the context of the primary toxic effects of heavy metals on plasma membrane and the essential role of water relations in plants' growth (e.g., Salisbury and Ross 1992; Kacperska 2004). It is also widely known that plants have to adjust their water balance in response to heavy metals (Shaw 1990; Poschenrieder and Barcelo 2004). Therefore, investigating the influence of heavy metals on water relations in the aspect of cellular plant biophysics and physiological adjustment to stress caused by these metals may result in a new approach to the problem of how heavy metals generally act, how they influence the plant physiology, and how do plants respond to this kind of stressor.

The development of pressure probe techniques (Steudle 1993), which allow the measurement of water relations in single cells, gave the green light to direct studies of the influence of toxic metal ions on water parameters. However, at present, there are only a few studies relevant to the influence of heavy metals on cell water relations that have been employing these techniques. Among them, there are papers which used cell pressure probe (CPP) to study the inhibition of water channels (AQPs) using mercuric as a gating agent (e.g., Daniels et al. 1994; Maurel 1997; Tyerman et al. 1999; Biela et al. 1999; Hukin et al. 2002; Javot and Maurel 2002; Henzler et al. 2004). All the aforementioned studies concerned the mechanisms of water uptake by roots, mechanisms of regulation of water relations in plants, the processes of water diffusion by cell membranes, and functioning of aquaporins. However, research has never been focused on the phenomena of gating of AQPs by mercuric and other heavy metals in connection with the problem of environmental pollution. In other words, in our experiments on AQPs activity in epidermal cells of *Allium cepa*, we used heavy metals not to investigate the physiological details of AQP functioning but to see their role in plant response to heavy metals as an environmental stressor. We should keep in mind that heavy metals belong to the most dangerous pollutants and constitute a serious environmental hazard. To the authors' knowledge, the present work is the first protocol comparing the influence of different heavy metals on AQPs exactly in this context.

The present paper focuses on the sensitivity of aquaporins to toxic metals including lead, zinc, cadmium, and mercury (as a reference point) in *A. cepa* epidermal cells as a model system in a new ecotoxicological approach to the problem. The aim was to examine the effect of selected heavy metals on water relations by investigating water permeability (AQP activity) in *A. cepa* epidermal cells using the cell pressure probe. We focused also on the time course of alterations of the water permeability of the tested cells. This approach gave us a better understanding of the role of AQPs during heavy metal stress in plants.

Materials and methods

Plant material

The experiments were performed on cells from the epidermis of live bulb scales of *A. cepa* L., which were obtained from a local market. The epidermis of *A. cepa* L. makes up one layer of closely arranged, cylindrical cells. Fragments of the epidermis were stripped from the adaxial side of the onion scale with tweezers and placed in control and appropriate heavy metal solutions or fixed directly to the metal sledge of the cell pressure probe. All the basic parameters describing the physical and biophysical properties of the tested cells were summarized in Table 1.

Cell pressure probe experiments

A CPP can be used to measure the half-time of water exchange ($T_{1/2}$), elastic modulus (ϵ), turgor pressure (P), and hydraulic conductivity (L_p) as previously described by Steudle (1993). However, in most cases, the half-time of water exchange can be used as a direct measure of changes of cell L_p . Half-times may be affected by the mechanical properties of the cell wall and water permeability of the plasmalemma. They were the same during swelling or shrinking. The half-time is the time required to shift halfway from the initial to the final volume of the cell. The CPP was filled with silicon oil (type AS4; Wacker, Munich, Germany). An electronic pressure transducer converted the pressure signal into a proportional voltage (more recent type, NJ, USA), which was directly fed into a computer for the calculation of parameters using a specifically designed software, the Pfloek Program, version 1.09 (Pfloek; Department of Plant Ecology, University of

Table 1 Basic parameters describing the physical and biophysical properties of *A. cepa* epidermal cells: dimensions, turgor pressure (P), volumetric elastic modulus (ϵ), half-time ($T_{1/2}$), and hydraulic conductivity (means $\pm$ SD, $n=6$)

Measurements	
Cell dimensions	
Diameter (μm)	55.4 $\pm$ 9.9
Length (μm)	412 $\pm$ 82.1
Volume (10^{-13} m^3)	9.0 $\pm$ 2.4
Water relations parameters	
Turgor pressure (P , MPa)	0.65 $\pm$ 0.1
Volumetric elastic modulus (ϵ , MPa)	5.4 $\pm$ 0.6
(ϵ/V , $10^{13} \text{ MPa m}^{-3}$)	0.6 $\pm$ 2.4
Half time of water exchange ($T_{1/2}$, s)	5.0 $\pm$ 0.5
Hydraulic conductivity (L_p , $10^{-7} \text{ ms}^{-1} \text{ MPa}^{-1}$)	2.5 $\pm$ 1.2

L_p value was calculated for mean value of $T_{1/2}$

Bayreuth, Germany). An oil-filled glass capillary was attached to the probe with a narrow tip of a diameter of around 10 μm , which was introduced into the cell. Magnets were used to fix the epidermis of the onion cells on a metal sledge. During experiments, nutrient or heavy metal solutions flowed along the cells by gravity and were pumped to the top of the sledge. The magnets provided a secure fixation of the piece of tissue, which is a prerequisite for measuring turgor pressure in individual cells without causing leakages around the capillary tip due to vibrations or shaking of the tissue. When a cell was punctured, turgor (P) caused a meniscus to develop between the cell sap and oil within the tip of the capillary. To restore cell sap volume to a value close to the original one, the meniscus was gently pushed back to a position close to the surface of the epidermal cells. When P was stationary, the hydraulic parameters of the cell were determined ($T_{1/2}$). Hydrostatic pressure relaxations were induced by rapidly moving the meniscus and keeping it at the new position until a steady pressure was re-attained. Pressure vs. time curves (relaxations) were recorded by the computer, which evaluated $T_{1/2}$. From the half-time of water exchange ($T_{1/2}$), cell L_p can be calculated according to Azaizeh et al. (1992), namely,

$$L_p = \frac{V}{A} \times \frac{\ln(2)}{T_{1/2}(\varepsilon + \pi^i)}. \quad (1)$$

Here, V is the cell volume and A its surface area. For a cylindrical cell, $V/A = r/2$ (where r is the radius of the cell), provided that the contribution of the ends of cells can be neglected (see above). The osmotic pressure of cell sap is denoted by π^i and the elastic coefficient of the cell by ε (elastic modulus). Knowing the osmotic concentration of the medium, the cell's osmotic pressure was obtained from the stationary turgor pressure of the cells. For a detailed description of the background of Eq. 1, the reader is referred to Steudle (1993) or Ye et al. (2004). Hence, at constant ε (Kim and Steudle 2007), $T_{1/2}$ is a direct measure of hydraulic conductivity (L_p). We used the parameter ($T_{1/2}$) in our results. That allowed us to avoid the effect of error propagation when calculating L_p (e.g., Wan et al. 2004; Ye and Steudle 2006).

Comparison of the toxicity of Zn, Cd, Pb, and Hg cations on aquaporins

To assess the influence of heavy metals on aquaporins, water permeability through the cell membranes was measured by monitoring the change in $T_{1/2}$ (calculated from pressure–relaxation curves, cf. Steudle 1993). To determine the degree of toxicity of mercury, cadmium, lead, and zinc, solutions of HgCl_2 , CdCl_2 , PbCl_2 , and ZnCl_2 were used. Epidermal fragments were incubated for 30 min in

nutrient solutions (control: 1.5 mM KNO_3 , 1 mM CaCl_2 , 1 mM MgSO_4 , 8.1 μM H_3BO_3 , 18 μM FeNaEDTA , 1.5 μM MnCl_2), which contained the following concentrations of heavy metals (in μM): HgCl_2 , 50 or 100; PbCl_2 , 100 or 2,000; CdCl_2 , 50 or 100; and ZnCl_2 , 100 or 2,000. Doses of heavy metals used in our experiments were selected on the basis of our previous research comparing the toxicity effects of cadmium and lead in epidermal cells of *A. cepa* (Wierzbicka et al. 2007). The nutrient solution used was prepared according Henzler and Steudle (1995), but was modified to avoid precipitation of heavy metals. During measurements, the nutrient solution flowed along the cells. Next, to check for the reversibility of changes in $T_{1/2}$ (L_p), the whole procedure was repeated, but directly after incubation in heavy metal solutions and before CPP measurements, the cells were treated for 5 min with 10 mM of the scavenger β -mercaptanol (ME; e.g., Wayne and Tazawa 1990; Henzler and Steudle 1995; Tazawa et al. 1996). A total of 200 cells were tested, i.e., 18 cells for each type of treatment.

Time course of changes in $T_{1/2}$

The time course of changes in water permeability ($T_{1/2}$) was studied. We measured the $T_{1/2}$ during cell treatment by metal ions. The total time for one measurement in a single cell was 60 min for all treatments, except for 100 μM HgCl_2 which rapidly caused irreversible toxic effects, i.e., killed the cells. The trend observed can be approximated by a hyperbola described by Eq. 2. The coefficient of determination (R^2) was computed for each curve as a measure of goodness-of-fit.

$$y = y_0 + \frac{ax}{b + x}. \quad (2)$$

Error considerations

Quantitative measurements of water relation parameters on the cellular level are subject to different sources of error (a) because the measured quantities (volume, surface area, etc.) are rather small and (b) because some of the parameters like ε or L_p are not directly measured but are calculated from other quantities. They could thus accumulate different errors. This is independent of the method used for determining water relation parameters. The basis for the evaluation of accumulation errors is Gauss' law of error propagation (see textbooks of physics and statistic, e.g., Kreyssing 1977), which has been applied in this study.

Statistics

Data were analyzed using Microsoft Office Excel 2003 and Sigma Plot 8 for Windows. The Student's t test was

employed to test for significance ($\alpha=0.05$). The results were presented as means $\pm$ SD. Because of the difficulty of maintaining the cells free of leaks for a sufficiently long period of time, a satisfactory number of measurements were performed on 5–16 cells. Similar number of cells was also used by Tomos et al. (1981), Tyerman and Steudle (1982), and Wei et al. (2001). A total of 251 cells were measured in both types of experiments.

Results

Comparison of the toxicity of Zn, Cd, Pb, and Hg cations

Water permeability was significantly inhibited by all the metal ions tested. The degree of the inhibition depended on

the ion applied, its concentration, as well as on the time of exposure. In order to determine the level of toxicity of the studied ions (Hg^{2+} , Cd^{2+} , Pb^{2+} , and Zn^{2+}), we compared mean values of half-time of the water exchange ($T_{1/2}$), as can be seen in Fig. 1a–d. The graph shows that 30-min treatment with solutions of 100 μM caused different effects measured as an increase in $T_{1/2}$. Treatment with 100 μM solution of Cd^{2+} caused the highest increase in $T_{1/2}$. In the case of Pb^{2+} , the increase in $T_{1/2}$ was smaller when compared with the effect of Cd^{2+} , while the smallest increase in $T_{1/2}$ was observed for the Zn^{2+} solution. The use of 100 μM of Hg^{2+} was not possible as cells treated with this concentration of mercuric ions rapidly lost plasma membrane integrity, which was accompanied by a drop in turgor pressure. This indicated that concentrations of Hg^{2+} higher than 50 μM caused an irreversible leakage of the

Fig. 1 Effects of ZnCl_2 (a), PbCl_2 (b), CdCl_2 (c), and HgCl_2 (d) on cellular water relations (half-time of the water exchange, $T_{1/2}$) in epidermal cells of *A. cepa*. Black bars indicate control, the light gray

bars indicate cell after 30-min treatment with heavy metals, and dark gray bars indicate removal of heavy metals by 10 mM β -mercaptanol. Values were given as mean $\pm$ SD

cells that eventually died (data not shown). It can be seen in Fig. 1a–d that for 100 μM solutions, increases in $T_{1/2}$ were 4.1-fold for Cd, 1.9-fold for Pb, and 1.1-fold for Zn. Application of solutions in the millimolar range was possible only for Zn and Pb, while for Cd and Hg, due to a highly toxic effect, solutions in concentrations lower than 100 μM could be employed. As a general rule, the higher the concentration of heavy metal ions, the higher was the increase in $T_{1/2}$ (Fig. 1a–d). All the observed effects were significant (t test, $p < 0.05$). We conclude that the strongest inhibition of water permeability was recorded for mercury and cadmium, a moderate one for lead, and the weakest for zinc.

To test for reversibility of the observed effect we used ME as a scavenger of heavy metals. Figure 1a–d indicates that 5-min treatment with ME reduced the increases in $T_{1/2}$ (inhibition of water permeability) back to the control level in most treatments (no significant difference with control). For low doses of all tested metals, $T_{1/2}$ after ME treatment always returned to the control level (Fig. 1a–d). Application of ME did not cause $T_{1/2}$ to drop back to the control level in the case of a high dose of lead ions (2 mM solution), whereas in the case of zinc ions (used at the same dose), $T_{1/2}$ decreased completely to the control level (Fig. 1a, b). Thus, ME used as a scavenger reversed the inhibition of water permeability by all investigated metal ions. This means that the observed effect was mainly due to the gating of AQPs by heavy metal ions rather than due to their general toxic effect on plant cell.

Time course of changes in $T_{1/2}$

Figure 2a–h presents the time course of changes of $T_{1/2}$ in response to treatments with different concentrations of heavy metals. Applied metals and doses correspond to treatments used in the first experiment. The process reached saturation after 20–40 min depending on the cation used. Saturation was most rapid in the case of Hg^{2+} and Cd^{2+} . The data suggest that there were lag times following the onset of inhibition which differed for the different metals used. The data were fitted hyperbolically, as shown in Fig. 2a–h. According to the data given in Fig. 2, lag times were short for 50 μM of Cd^{2+} (Fig. 2c), 100 μM of Pb^{2+} (Fig. 2e), and 100 μM of Zn^{2+} (Fig. 2g), but were all around 10 min, suggesting that it takes some time for the heavy metals to get access to the cysteine residues in the AQPs. It should be noted that in all the cases shown in Fig. 2, turgor remained constant at $P = 0.66$ MPa for control and 0.4 MPa for heavy metal treatments.

Treatment with 50 μM of Hg^{2+} (Fig. 2b), 100 μM of Cd^{2+} (Fig. 2d), and 2,000 μM of Zn^{2+} (Fig. 2h) exerted a powerful effect on the volume transport kinetics: After 45 min of

incubation, the $T_{1/2}$ had almost doubled in the case of treatment with cadmium ions (100 μM), and in the case of 2,000 μM of PbCl_2 , the $T_{1/2}$ had even almost triplicated (Fig. 2d, h).

As in our first experiment, the observed $T_{1/2}$ values were higher for higher doses of heavy metals: for Cd (Fig. 2c, d), for Pb (Fig. 2e, f), and for Zn (Fig. 2g, h). We showed in time course experiments that the decrease in water permeability occurred very rapidly after the administration of heavy metal. This means that the reaction of AQPs on heavy metal ions was fast and could be regarded as one of the first responses of plant cells to heavy metal stress.

Discussion

General considerations

Using a standard biophysical technique (CPP), we have measured the water permeability of plant cell membrane. Although there is no direct evidence that observed changes in water permeability can be attributed solely to the activity of AQPs, it is hard to imagine that these rapid changes in $T_{1/2}$ could be due to the alternation of water permeability of the lipid bilayer or those of other transporters (Tyerman et al. 1999; Kim and Steudle 2007). Therefore, in our paper, we interpreted cell water permeability, measured as $T_{1/2}$, in terms of AQP activity. Similar interpretation has been employed by Henzler and Steudle (1995), Ye et al. (2004), and Kim and Steudle (2007).

Our results indicate that heavy metals gate AQPs in the membranes of the epidermal cells of *A. cepa* bulb scale which, in turn, cause a reversible reduction of the overall water permeability of the cell. At the same time, it might seem that since heavy metal ions are known to be disastrous to different kinds of processes in the cell, our observations could be due to a general toxic effect, but not due to AQP gating. To dispel this doubt, we carried out experiments using ME which is well known and widely used as complexation reagent, especially in experiments employing heavy metals (Margetinova et al. 2008). Using ME enabled us to verify whether observed changes in water permeability were due to AQP gating or accounted for the general failure of cell metabolism (including AQP activity) caused by heavy metal ions. The increase in $T_{1/2}$ due to the general toxic effect would not be reversed by ME. Since changes in $T_{1/2}$ were easily reversible by applying ME, the observed increase in $T_{1/2}$ can be attributed to AQP gating by heavy metals rather than to metabolism impairment. It should be noted that ME is routinely used to study the reversibility of AQP gating by mercury and zinc ions (cf. Wayne and Tazawa 1990; Henzler and Steudle 1995;

Fig. 2 Time course of changes in $T_{1/2}$ of epidermal cells of *A. cepa* in response to heavy metals treatment: control (a), 50 μM HgCl_2 (b), 50 μM CdCl_2 (c), 100 μM CdCl_2 (d), 100 μM PbCl_2 (e), 2,000 μM PbCl_2 (f), 100 μM ZnCl_2 (g), and 2,000 μM ZnCl_2 (h). Solid line

indicates control level of $T_{1/2}$ and intercept line indicate level of $T_{1/2}$ after removal of heavy metals by 10 mM ME (according to Fig. 1). For each, hyperbola equation and coefficient of determination (R^2) were given

Tazawa et al. 1996; Philip et al. 2008; Watanabe et al. 2009). We demonstrated, using a cell pressure probe, that heavy metal stress induced a significant increase in the half-time of water exchange of onion epidermal cells. On

the basis of this observation, we put forward the hypothesis that pollution with heavy metals disturbs water transport in plant cell. The present findings seem to agree with this hypothesis.

Heavy metals as an environmental pollutant and plant–water relations

There are only a few studies relevant to the influence of metal toxicity on cell water relations. For example, the impact of aluminum ions on the water permeability of maize roots has been tested by Gunse et al. (1997). There are also several papers which employed CPP to study the inhibition of water channels (AQPs) by mercuric (Maurel 1997; Tyerman et al. 1999; Henzler et al. 2004). In any case, in all these studies, mercury represents one of the very few tools available to evaluate the contribution of AQPs to water transport in plant tissue. As yet, this metal has never been perceived as an environmental problem and a model to study the effects of pollution by heavy metals on plant–water relations.

Therefore, in our study, employing CPP, we decided to test the influence of Hg on AQP gating. In addition, we decided to examine three other highly toxic heavy metals: zinc, cadmium, and lead. All the three metals belong to the most dangerous pollutants (Ernst 1990; Wierzbicka et al. 2007). While relatively much research has addressed the problem of AQPs gating by Hg, other heavy metals have not been systematically studied in this context (Niemietz and Tyerman 2002). Zinc, which has weaker affinity for sulfhydryl groups than Hg, has been reported to inhibit the hydraulic conductivity of *Chara* cells (Rygor et al. 1992; Tazawa et al. 1996). Zelenina et al. (2003, 2004) investigated the impact of Ni, Cu on human AQP3, while Niemietz and Tyerman (2002) proposed to use Au and Ag as blocking agents in studies on plant and human AQPs. All the aforementioned research studies not only proved that other metals can block AQPs but also paved the way for future research in the interactions of heavy metals with AQPs. In our experiments, we followed this in order to show a connection between environmental pollution and the functioning of AQPs in plant cells. It seems that the impact of heavy metals on plant–water relations may depend mostly on their interactions with AQPs.

Toxicity of heavy metals

The investigated metal ions caused a decrease in water flow through the plasma membrane. We showed that the higher the concentration of the tested metal, the lower was the rate of water flow through the plasma membrane. The concentration of 100 μM was the only one common to all the experiments. The selection of other concentrations depended on the power with which the cation gated AQPs. In the case of powerful gating agents (such as Hg^{2+} and Cd^{2+}), we employed concentrations lower than 100 μM . For less powerful ions (Zn^{2+} , Pb^{2+}), concentrations higher than 100 μM were applied. On the basis of this observation, as well as the $T_{1/2}$ values obtained during our experiments, we

showed that the toxicity of heavy metals is related to the amount of reduction in hydraulic conductivity (increase of half-time) by the cations. Thus, the toxicity sequence of metals was: $\text{Hg} > \text{Cd} > \text{Pb} > \text{Zn}$. Similar effects have been shown by Yang et al. (2004) in broad bean guard cells for the following sequence of metals: $\text{Hg} > \text{Pb} > \text{Zn} > \text{La}$. Furthermore, it has to be mentioned that although this order may be different for different organisms, the most widely accepted toxicity sequence of metals is: $\text{Hg} > \text{Cu} > \text{Cd} > \text{Ag} > \text{Zn}$ (Shaw 1990). As the toxicity sequence obtained as a result of our research on AQPs activity is consistent with previous findings (Shaw 1990; Yang et al. 2004), we can therefore conclude that measuring AQP activity provides a suitable and reliable tool in heavy metal toxicity assessments.

The question arises as to why some metals inhibit AQP activity stronger than others and what is the mechanism of this interaction. It seems that this phenomenon can be attributed to the differences in chemical properties and atomic structure of the investigated metals. Pb^{2+} , Zn^{2+} , Hg^{2+} , and Cd^{2+} have similar atomic semi-diameters and valence, so it is possible that they also share the same mechanism to affect water channels. Different researchers (Kozono et al. 2002; Wan et al. 2004, among others) have shown the ability of heavy metals (Hg) to close the aquaporins in consequence of their reaction with group SH of the protein. This process was demonstrated by a reversal of inhibition with ME (Wayne and Tazawa 1990; Steudle and Henzler 1995). According to our results, it seems that the toxicity is related in a simple way to the intensity by which the cations bind to SH groups. It seems that metals with high values of the solubility product constant (K_{sp} 25°C) for sulfides ($K_{\text{spZnS}} = 1.1 \times 10^{-21}$) bond to the AQPs less strongly than metals with lower K_{sp} values ($K_{\text{spHgS}} = 1.6 \times 10^{-52}$).

It is also well described that plant AQPs are gated by cytosolic pH and pCa (Gerbeau et al. 2002; Alleva et al. 2006; Tornroth-Horsefield et al. 2006) as well as by dephosphorylation (Johansson et al. 1998; Guenther et al. 2003; Tornroth-Horsefield et al. 2006; Maurel et al. 2008). These mechanisms cannot be completely excluded as a cause of the observed phenomenon. However, they are less probable than direct gating of AQPs by creating a bond between metal ion and cysteine (189). Since the effect on $T_{1/2}$ was completely reversed by ME, it is highly probable that AQP closure was caused by the interaction of metal ions with the SH cysteine group rather than by the effects on cellular metabolism (like pH and pCa), as described by Zhang and Tyerman (1999).

Time course of changes in $T_{1/2}$

We have also shown that heavy metal effects on plant cells appear very quickly, within the first few minutes. After introducing the tip of the microcapillary of the CPP into the epidermal cells of *A. cepa*, the heavy metals started to be

applied and half-time of water exchange were measured for up to 1.5 h (on average 1 h). The results showed that the time delay of reaction of AQPs (increase in $T_{1/2}$) to heavy metals was very short, up to 10 min. When the half-time was increasing (L_p decrease), the cells required a time of about 25–40 min to reach saturation. This may suggest that the faster the system will become stable, the easier, the better, and the more efficient will the plant–water reaction to heavy metal stress be. Hence, the more effective is the water regulation in plant cell under the heavy metal stress, the better is the adaptation of the whole plant to this kind of stressor.

Summary

Besides the gating of AQPs, it cannot be excluded at present that there are changes in the expression of AQPs in response to heavy metals as well. Additionally, the results suggested that at the cell level, AQP reaction may be one of the first feedbacks to toxic activity of heavy metals. It seems that in plants responding to heavy metal stress, the disturbance of water balance is the primary stress-induced event affecting the opened/closed state of AQPs. Hence, it seems that AQPs may play a significant role in plant cells' response to heavy metals. However, in order to understand the relation between heavy metal stress and water relations in plants, further studies into aquaporins and metal tolerance mechanisms are required. It seems that interesting insights into the phenomenon of metal tolerance would be gained by testing the response of AQPs to treatment with heavy metals in plants adapted to metalliferous soils.

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Conflict of interest The authors declare that they have no conflict of interest.

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