

RESEARCH PAPER

Comparative study of the active cadmium efflux systems operating at the plasma membrane and tonoplast of cucumber root cells

Magdalena Migocka*, Anna Papierniak, Ewelina Kosatka and Grażyna Kłobus

Wrocław University, Institute of Plant Biology, Department of Plant Physiology, Kanonia 6/8, 50-328 Wrocław, Poland

* To whom correspondence should be addressed. E-mail: mmigocka@biol.uni.wroc.pl

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Abstract

The strategies developed by plants to avoid the toxicity of cadmium (Cd) and other heavy metals involve active sequestration of metals into the apoplast and vacuoles. The protein systems excluding heavy metals from the cell cytosol localize to the plasma membrane and tonoplast and are energized either by ATP or by the electrochemical gradient generated by H⁺-ATPase or by V-ATPase and pyrophosphatase (PPase), respectively. In this work, a comparative study on the contribution of both the plasma membrane and tonoplast in the active detoxification of plant cells after treatment with Cd was performed. The studies using plants treated and untreated with Cd reveal that both, H⁺-coupled and MgATP-driven efflux of Cd across plasma membranes and tonoplast is markedly stimulated in the presence of Cd in the environment. Previous studies on plasma-membrane localized H⁺-coupled Cd efflux together with the present data demonstrating tonoplast H⁺/Cd²⁺ antiport activity suggest that H⁺-coupled secondary transport of Cd displays a lower affinity for Cd when compared with Cd primary pumps driven by MgATP. In addition, it is shown that MgATP-energized Cd efflux across both membranes is significantly enhanced by cysteine, dithiothreitol, and glutathione. These results suggest that Cd is excluded from the cytosol through an energy-dependent system as a free ion as well as a complexed form. Although both membranes contribute in the active exclusion of ionized and complexed Cd from the cytosol, the overall calculation of Cd accumulation in the everted plasma membranes and vacuolar vesicles suggests that the tonoplast and vacuole have a major function in Cd efflux from the cytosol in the roots of cucumber subjected to Cd stress.

Key words: Active transport, cadmium, chelators, heavy metals, metal transporters.

Introduction

Heavy metal pollution is of considerable importance due to its obvious impact on human and animal health. Nevertheless, it is particularly dangerous to sessile organisms which cannot avoid metal toxicity by simple migration (Hadjiliadis, 1997; Almeida *et al.*, 2007). Among heavy metals, cadmium (Cd) is one of the most dangerous elements to plants, since elevated level of Cd in the soil solution cause numerous harmful effects. It has been shown that Cd may interfere with mineral nutrition by hampering the uptake and translocation of essential elements (Boussama *et al.*, 1999; Dong *et al.*, 2006). Cd usually affects overall cell metabolism via inhibition of the

key enzymes of essential metabolic pathways, alteration of membrane composition and function, as well as stimulation of free radical production, which ultimately leads to oxidative stress (Gratão *et al.*, 2005). As a consequence, Cd affects the most important physiological processes such as photosynthesis or respiration and thus inhibits plant growth and development (Gallego *et al.*, 1996; Perfus-Barbeoch *et al.*, 2002). Although the toxic effects of Cd on biological systems have been frequently reported, the molecular mechanisms of Cd detoxification are still not completely understood. The broad spectrum of strategies developed by plants to cope with the

Abbreviations: BSA, bovine serum albumin; BTP, BIS–TRIS propane; DAB, 3,3'-diaminobenzidine; DEX, dextran T-500; DTT, dithiothreitol; GSH, glutathione; MTs, metallothioneins; PCs, phytochelatin; PEG, polyethylene glycol; PM, plasma membrane.
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toxicity of Cd and other heavy metals has been well described (Cobbett, 2000; Clemens, 2001; Hall, 2002; Hall and Williams, 2003; Clemens *et al.*, 2002; Sharma and Dietz, 2006). In general, they comprise the precise regulation of the uptake, chelation and distribution of metals among tissues, cells and cell organelles. As a result, the excess of harmful ions is rapidly deposited within the cell wall, vacuoles or in metabolically inactive tissues, to ensure proper function of plant cells upon elevated concentrations of heavy metals (Clemens 2001; Ducić *et al.*, 2005; Yang *et al.*, 2005). Within the cell wall, a number of compounds (e.g. polygalacturonic acids) are present that are capable of binding metal ions, thus partially preventing their influx into the cell. Similarly in cytosol, diverse organic acids, peptides and specific proteins display the ability to chelate metal ions and thus protect other metabolically essential proteins from metal-induced deactivation. The cytosolic metal-chelating compounds include citrate, malate, amino acids such as histidine and cysteine, as well as cysteine-rich amino acid derivatives: glutathione (GSH), metallothioneins (MTs) and phytochelatin (PCs) (Cobbett and Goldsbrough, 2002; Sharma and Dietz, 2006). Previous studies using plant cell membranes showed that Cd complexes, such as GSH–Cd and PC–Cd are deposited within plant vacuoles via an ATP-dependent process (Vögeli-Lange and Wagner, 1990; Salt and Rauser, 1995; Li *et al.*, 1997; Benavides *et al.*, 2005). More recent data from yeast indicate that the accumulation of PC–Cd or GSH–Cd complexes in vacuoles employs proteins of the ABC transporter family (Clemens *et al.*, 2002; Preveral *et al.*, 2009). Plant cells possess a large number of genes encoding ABC proteins but it is still not clear whether any of the plant ABC transporters is specific for Cd ions or Cd complexes. In contrast, growing evidence suggests that the proteins of the P_{1B}-ATPases family (heavy metal ATPases; HMAs) might play a key role in the detoxification of plant cells following Cd excess (Hussain *et al.*, 2004; Verret *et al.*, 2004, 2005; Morel *et al.*, 2009). HMA4, the first cloned and characterized plant divalent transporter of the P_{1B}-ATPase family, has been shown to contribute to zinc (Zn) homeostasis and Cd detoxification in *Arabidopsis thaliana* and *Arabidopsis halleri* (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2004, 2005; Courbot *et al.*, 2007). Plants lacking another P_{1B}-ATPase, HMA2, accumulate more Cd²⁺ when exposed to this metal (Eren and Arguello, 2004). Both proteins are essential for the effective root to shoot translocation of Cd and Zn (Wong and Cobbett, 2009). Furthermore, HMA-catalysed Cd efflux from the cells may be specific for Cd complexes, as the activity of AtHMA2 expressed in yeast was much more stimulated in the presence of both, Cd and cysteine than by Cd alone (Eren and Arguello, 2004). An alternative mechanism of Cd detoxification that relies on Cd²⁺/H⁺ antiport activity has been also reported (Salt and Wagner, 1993; Ortiz *et al.*, 1995; Gonzalez *et al.*, 1999; Burzyński *et al.*, 2005; Migocka and Kłobus, 2007). So far, the proton-coupled Cd efflux has been measured and characterized in membranes isolated from cucumber roots [plasma membranes (PMs)] or tobacco and oat roots (tonoplasts). Protein candidates that may be potentially involved in this process have also already been

proposed. They belong to the cation diffusion facilitator [CDF (MTP)] and CAX families that are probably capable of transporting a broad spectrum of metal ions.

From the above data it may be concluded that transport systems of the PM and tonoplast participating in active Cd efflux from the cytosol exhibit a dual mode of energy coupling. Until now the experimental confirmation of both the ATP-dependent and ΔpH-dependent Cd transport activities in plant cell membranes has been limited to the tonoplast. Cd²⁺/H⁺ antiport activity has recently been shown in PMs isolated from cucumber root cells (Migocka and Kłobus, 2007). To date, the major indications for the presence of ATP-dependent Cd transporters in PMs come from research using *Arabidopsis* transgenic plants and protoplasts or from expression studies of genes encoding plant metal ATPases in bacteria or yeast. Using such systems, it was demonstrated that *hma2* and *hma4* mutants displayed considerably reduced translocation of Cd from roots to shoots and increased Cd sensitivity when compared with wild-type plants (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Wong and Cobbett, 2009). The heterologous expression of PM-localized *AtHMA2*, *AtHMA4* or *AhHMA4* and tonoplast-localized *AtHMA3* in yeast confirmed that the proteins were capable of conferring tolerance to different metals, including Cd (Mills *et al.*, 2003, 2005; Eren and Arguello, 2004; Gravot *et al.*, 2004; Courbot *et al.*, 2007). Nevertheless, the properties of the MgATP-energized (HMA- or ABC-mediated) metal transport across membranes isolated from plant tissues have been studied only for the tonoplast. Moreover, the comparative analysis of the contribution of the PM and tonoplast to the active detoxification of plant cells from Cd excess is still lacking.

A comparative study of the Cd transport activities of the PM and tonoplast is presented in this work. The results confirm previous suggestions that transport systems operating at both membranes can utilize ATP as well as an electrochemical gradient as the source of energy. It is also shown that both systems are markedly stimulated under Cd stress and in the presence of cysteine, dithiothreitol (DTT) and GSH.

Materials and methods

Plant material

Cucumber plants (*Cucumis sativus* L.) were grown in nutrient solution containing 1 mM K₂SO₄, 0.7 mM CaSO₄, 0.33 mM Ca(H₂PO₄)₂, 0.33 mM MgSO₄, and 5 mM KNO₃, and supplemented with the following microelements: 25 μM ferric citrate; 3 μM MnSO₄; 1.7 μM H₃BO₃; 0.3 μM CuSO₄; 0.003 μM ZnSO₄; and 0.017 μM Na₂MoO₄. After 6 d, plants were transferred for 24 h to fresh nutrient solution (pH 5.5) containing or not (control) Cd (CdSO₄) at 50 μM. The plants were grown under a 16 h photoperiod at 25 °C during the day and 22 °C during the night.

Plasma membrane isolation

PMs of good yield and purity were isolated according to Larsson (1985) as modified by Kłobus (1995). The PM vesicles were separated from the total microsomes by two-phase partitioning in a 6.2% dextran–polyethylene glycol (DEX–PEG) system throughout the 5 min centrifugation at 500 g. The membranes were pelleted

and resuspended in 5 mM BIS-TRIS propane-MES (BTP-MES), pH 7.5, 330 mM sorbitol, 5 mM KCl, 0.1 mM EDTA and 0.05% Brij, and incubated for 10 min on ice in order to reverse the membrane orientation. Finally, the inside-out PM vesicles were pelleted and resuspended in 5 mM BTP-MES, pH 7.5, 330 mM sorbitol, 5 mM KCl and 0.1 mM EDTA.

Tonoplast membranes were isolated essentially as described by Kabała and Kłobus (2001). The microsomal fraction was overlaid on a discontinuous sucrose density gradient consisting of 20, 28, 32, and 42% (w/w) sucrose and centrifuged at 80 000 g for 3 h. The tonoplast-enriched fraction as well as other fractions were collected after centrifugation at 80 000 g for 30 min. Following the 30 min centrifugation at 80 000 g, the pellets were resuspended in 200 mM TRIS-MES with 2.5 mM sucrose. The pH of the resuspension buffer was established as 7.5 when ATP-dependent Cd transport was assayed or 6.0 when Δ pH-dependent Cd transport was measured.

Determination of the purity and orientation of membrane fractions

Marker enzyme activities: Marker enzyme activities were determined according to Gallagher and Leonard (1982) and calculated from the differences in the rate of ATP hydrolysis determined in the absence and presence of specific inhibitors: 1 mM NaN_3 (mitochondrial and thylakoid ATPase), 50 mM NaNO_3 (tonoplast ATPase), and 0.1 mM Na_3VO_4 (PM ATPase). The hydrolytic activity of vacuolar H^+ -pyrophosphatase (V-PPase) was measured as described by Maeshima and Yoshida (1989) with $\text{Na}_4\text{P}_2\text{O}_7$ as a substrate. The amount of P_i released during the reactions was determined according to Ames (1966).

The orientation of PM or tonoplast vesicles was estimated from the latencies of H^+ -ATPase and V-ATPase activities, respectively. The assays were conducted according to Murphy and Riley (1962), with or without 0.02% Triton X-100 in the reaction mixtures.

Marker enzyme immunodetection: A 15 μg aliquot of membrane proteins was incubated with 10 mM TRIS, 2% (w/v) SDS, 80 mM DTT, 40% (w/v) glycerol, 5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, and 0.05% (w/v) bromophenol blue for 30 min at room temperature before loading onto 10% (w/v) linear acrylamide mini-gels. The separated microsomal proteins were electroblotted onto 0.45 μm pore nitrocellulose membranes using a Sigma-Aldrich SV10-EB10 blotting apparatus and the transfer buffer containing 25 mM TRIS, 150 mM glycine (pH 8.3), and 10% (v/v) methanol. The membranes were incubated with monoclonal antibodies against PM H^+ -ATPase kindly provided by W Michalke (Universität Freiburg, Germany) or tonoplast V-ATPase (Dr E Fischer-Schliebs, University of Darmstadt, Germany) at 37 °C for 1 h. After repeated washing, nitrocellulose membranes labelled with primary antibodies were further incubated for 1 h with secondary antibodies (conjugated to horseradish peroxidase; Agrisera) and visualized by staining with 3,3'-diaminobenzidine (DAB).

Measurement of Cd transport in vesicles

Δ pH-dependent Cd transport into tonoplast vesicles was measured using two different methods. In the first assay, acridine orange was used as a pH-sensitive probe. The membranes (60 μg of protein) were introduced into the reaction mixture containing 20 mM TRIS-MES, pH 7.5, 2.5 mM sucrose, 50 mM KCl, and 10 μM acridine orange. Following the addition of 3 mM MgATP, the generation of a proton gradient was observed as the dissipation of acridine orange absorbance at 495 nm. After inhibition of proton pumping with 500 nmol bafilomycin, different concentrations of CdSO_4 were added to the membranes and the recovery of acridine orange absorbance was monitored during the next 3 min. In control experiments, equal concentrations of potassium sulphate or glucosamine sulphate were added to the reactions instead of CdSO_4 .

In the second assay, an artificial pH gradient across the membranes was imposed on tonoplasts. The membranes (60 μg of

protein) suspended in 20 mM TRI-MES, pH 6.0, containing 330 mM sorbitol, were introduced into the same buffer, pH 8.0, containing different concentrations of CdSO_4 . After 5 min, the membranes were vacuum filtrated through a 0.45 μm nitrocellulose filter (Millipore) and washed with the same buffer to release metal ions unspecifically bound to the membrane and filter surfaces. Afterwards, the vesicles were disrupted with 2 ml of 3 M HCl and the Cd content in the filtrate was measured using flame atomic absorption spectrometry (AAS). In control experiments, Cd accumulation in tonoplast vesicles without an artificial pH gradient was determined.

ATP-dependent Cd transport was determined in both PMs and tonoplasts using a direct filtration assay and AAS. Membranes (60 μg of protein) resuspended in 5 mM TRIS-MES, pH 7.5, containing 330 mM sorbitol, were introduced into 20 mM TRIS-MES, pH 7.5, containing 330 mM sorbitol and different concentrations of CdSO_4 . The reaction was started by the addition of 3 mM MgATP and, after 5 min, the mixtures were vacuum filtrated. In control experiments, 3 mM MgSO_4 was used instead of 3 mM MgATP. The effect of specific inhibitors (orthovanate, bafilomycin, and nitrates), amino acids (histidine or cysteine), GSH or DTT on the ATP-dependent Cd transport into the PM or the tonoplast was assayed by the addition of these chemicals to the reaction medium along with MgATP or MgSO_4 .

Protein determination

The amount of protein was estimated by the method of Bradford (1976) with bovine serum albumin (BSA) as the protein standard.

Statistical analysis

Data on Cd transport activities were analysed by one-way completely randomized analysis of variance (ANOVA), and comparison of means was performed by Duncan's test at a significance level of 0.05.

Results

Purity and orientation of tonoplast and plasma membrane fractions

The purity of tonoplast and PM vesicles was determined using both, the specific inhibitors of marker membrane enzymes (H^+ -ATPase, V-ATPase, PPase, mitochondrial-ATPase and thylakoid-ATPase) and specific antibodies raised against PM H^+ -ATPase and tonoplast V-ATPase. The results of the assays of ATP and PP_i hydrolysis showed that the NO_3^- -sensitive, VO_4^- -insensitive H^+ -ATPase activity and the PPase activity of the isolated tonoplast vesicles were the highest in the second fraction of the sucrose gradient (Fig. 1A). In contrast, vanadate-sensitive ATP hydrolysis was not considerable in fraction 2 and was significantly increased in the lower fractions of the gradient. Western blots confirmed that tonoplasts and PMs sediment in the upper (1–2) and lower (3–7) phases throughout the sucrose gradient, respectively (Fig. 1C, D). The latency of V-ATPase activity revealed that the tonoplast-enriched fraction 2 contained mostly (75%) the right side-out tonoplast vesicles (Table 1), confirming the usefulness of the obtained membranes for further proton transport measurements. In contrast, considering the latency of H^+ -ATPase (Table 2), the majority of PM membranes contaminating fraction 2 presented the right side-out orientation; therefore, their influence on the results

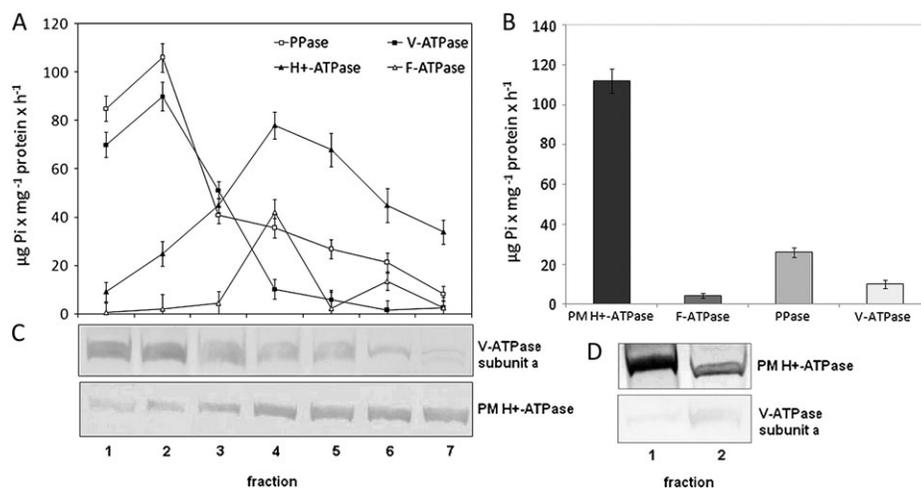


Fig. 1. Evaluation of vacuolar membrane (A, C) and plasma membrane (B, D) purity by enzymatic characterization and western blot analyses. During tonoplast preparation, total membranes were fractionated on a sucrose gradient and the resulting fractions were subjected to the analyses of marker enzyme activity (A) and blotted with antibodies specific for V-ATPase and PM H⁺-ATPase (C). Fraction 2 was used in the further assay since it was enriched in tonoplast vesicles of good yield and purity. In contrast, the fractionation of the total membranes on the two-phase system allowed two phases to be obtained, with the upper phase (fraction 1) enriched in pure plasma membranes (D). Marker enzyme activities confirmed the purity of the plasma membranes in fraction 1 (B).

Table 1. Effect of 0.02% Triton X-100 on the hydrolytic ATPase activity of V-ATPase and PM H⁺-ATPase in the fraction 2 enriched in tonoplast membranes. Enzyme activity was calculated from a difference between the amount of Pi produced in and out of presence of specific inhibitors (nitrate for V-ATPase or orthovanadate for PM H⁺-ATPase). The values presented are average with S.E. of three replications.

Detergent	V-ATPase hydrolytic activity ^a	PM H ⁺ ATPase hydrolytic activity ^a
+ Triton X-100	90 ± 4.2	25 ± 3.9
- Triton X-100	68 ± 5.3	6.3 ± 1.9

^a µg Pi mg⁻¹ protein h⁻¹

Table 2. Effect of 0.02% Triton X-100 and 0.05% Brij 58 on the hydrolytic ATPase activity of PM H⁺-ATPase in the upper phase (fraction 1) of the PEG/DEX two-phase system enriched in plasma membranes. Enzyme activity was calculated from a difference between the amount of Pi produced in and out of presence of orthovanadate. The values presented are average with S.E. of three replications.

Detergent	PM H ⁺ ATPase hydrolytic activity ^a
+ Triton X-100	110.6 ± 3.9
+ Brij 58	115.36 ± 4.6
- Triton X-100/- Brij 58	11.5 ± 1.3

^a µg Pi mg⁻¹ protein h⁻¹

of metal and proton transport assays in tonoplast vesicles was additionally limited.

The separation of the PMs on the DEX-PEG two-phase system also yielded pure, mainly right side-out (90%) oriented PM vesicles (Table 2, Fig. 1B, D). The membranes were immediately used for the metal transport measurements following incubation with 0.05% Brij 58 that allowed entirely inside-out orientated PM vesicles to be obtained (Johansson *et al.*, 1995).

Effect of inhibitors, ionophores, histidine and sulphhydryl group-containing agents on MgATP-energized Cd efflux

The MgATP-dependent uptake of Cd by the everted PMs and right side-out tonoplast vesicles was significantly influenced by protonophores and inhibitors (orthovanadate, an inhibitor of PM H⁺-ATPase, ABC transporters and HMAs; and bafilomycin or nitrate, inhibitors of tonoplast V-ATPase). However, despite the presence of agents dissipating the transmembrane

proton gradient, the substantial accumulation of metal still occurred and was almost completely diminished only when MgATP was excluded from the assay (Fig. 3). The effect of orthovanadate on Cd transport into membranes was dependent on the concentration of the inhibitor. Cd uptake by PM membranes was only slightly reduced (up to 75%) or even stimulated (up to 120%) by 0.1 mM Na₂VO₄ in the control and Cd-treated plants, respectively, whereas it was almost completely reduced in the presence of 1 mM Na₂VO₄. Interestingly, the relevant process observed in the tonoplasts was strongly reduced by both, 0.1 mM (up to 40% in control and Cd-stressed plants) and 1 mM Na₂VO₄ (up to 25% and 20% in control plants and stressed plants, respectively). The rate of MgATP-energized Cd accumulation within vesicles was significantly higher in membranes isolated from Cd-treated plants when compared with membranes isolated from control plants (Fig. 2A-F) and dependent on the concentration of the metal introduced into the incubation medium. As shown in Fig. 2, the phenomenon exhibited Michaelis-Menten saturation

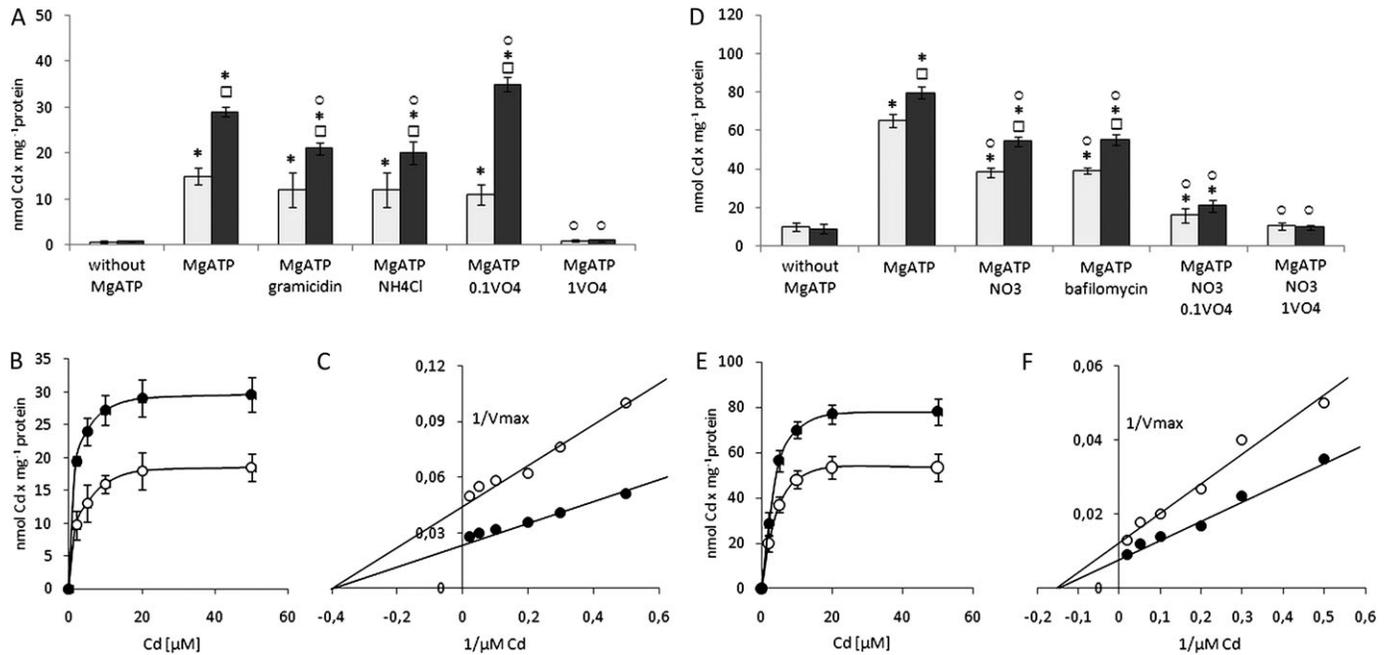


Fig. 2. The properties of MgATP-energized Cd efflux across the plasma membrane (A–C) and tonoplast (D–F). Effect of MgATP, proton pump inhibitors and protonophores on Cd transport into everted plasma membranes (A) and right side-out tonoplasts (D) isolated from control (open squares) and Cd-treated (filled squares) plants. Cd was introduced into the incubation medium at a final concentration of 5 μM . The measurement was performed with use of AAS followed by a direct filtration assay. Presented values are means \pm SD of three independent experiments, with each experiment done in triplicate. Squares indicate a significant difference ($P < 0.05$) between control and Cd-treated plants, and asterisks indicate a significant difference ($P < 0.05$) between Cd transport in the presence of MgATP and corresponding Cd transport measured without MgATP in the incubation medium. Circles indicate a significant difference ($P < 0.05$) between Cd transport in the presence of MgATP and corresponding Cd transport measured with MgATP and protonophores, bafilomycin, nitrate or vanadate in the incubation medium. The Cd concentration dependence of MgATP-energized Cd transport into the plasma membranes (B) and tonoplast (E) isolated from control (open circles) and Cd-treated (filled circles) plants with apparent K_m values of 2.5 μM and 6.6 μM estimated for plasma membrane-catalysed (C) and tonoplast-catalysed (F) Cd transport, respectively. The presented values are representative of the results obtained in 3–4 independent experiments with each experiment done in triplicate.

kinetics with an apparent K_m of 2.5 μM and 6.6 μM for PMs (Fig. 2B, C) and tonoplasts (Fig. 2E, F), respectively. The pre-treatment of plants with Cd did not affect the estimated K_m values for MgATP-energized Cd efflux. In contrast to K_m values, V_{max} values of Cd efflux across both membranes changed significantly due to Cd pre-treatment of plants and the MgATP-energized Cd efflux at tonoplasts displayed a markedly higher V_{max} (90 nmol and 125 nmol of Cd for membranes from control and Cd-treated plants respectively) than the MgATP-energized Cd efflux across PMs (22.2 nmol and 40 nmol of Cd for membranes from control and Cd-treated plants, respectively).

It has been reported that the cytoplasmic free heavy metal concentrations appear to be in the picomolar range under physiological conditions (Rae *et al.*, 1999; Outten and O'Halloran, 2001; Eren and Arguello, 2004), suggesting that the vast majority of heavy metals present within the cells exist in complexed forms. Hence, the effect of potent heavy metal-chelating agents (cysteine, histidine, GSH and DTT) on the ATP-dependent Cd efflux across tonoplasts and PMs was tested to give a better insight into the mechanism and specificity of the process.

The effect of metal-chelating agents was different in the PMs obtained from the control and Cd-treated plants.

Namely, the uptake of Cd by everted PMs isolated from control plants was markedly stimulated only by 5 mM DTT (up to 160%) whereas 50 mM cysteine and 5 mM GSH did not affect the transport activity significantly (Fig. 3A). In contrast, the stimulatory effect of GSH and cysteine on the Cd transport across PMs derived from Cd-treated plants was similar to the effect of DTT, reaching a 200% and 170% increase in the presence of GSH/cysteine or DTT, respectively, in comparison with the measurements not including chelating agents (Fig. 3A). The effect of DTT on MgATP-energized Cd transport was not dependent on the DTT concentration (Fig. 4B), whereas cysteine- and GSH-mediated stimulation of the metal efflux increased slightly (1.5-fold) with the increase of cysteine or GSH in the reaction medium from 10 mM up to 50 mM or from 1 mM up to 10 mM, respectively (Fig. 4A, C). Moreover, the stimulatory effect of cysteine and DTT was maintained in the presence of 0.1 mM vanadate, whereas GSH-induced stimulation of MgATP-dependent Cd efflux across the PM was eliminated by the inhibitor (Fig. 3A). Orthovanadate at 1 mM almost completely inhibited the MgATP-energized Cd efflux in the presence and absence of chelators (Fig. 3A).

In the case of tonoplasts, the differences among control and Cd-treated plants in MgATP-dependent Cd translocation into

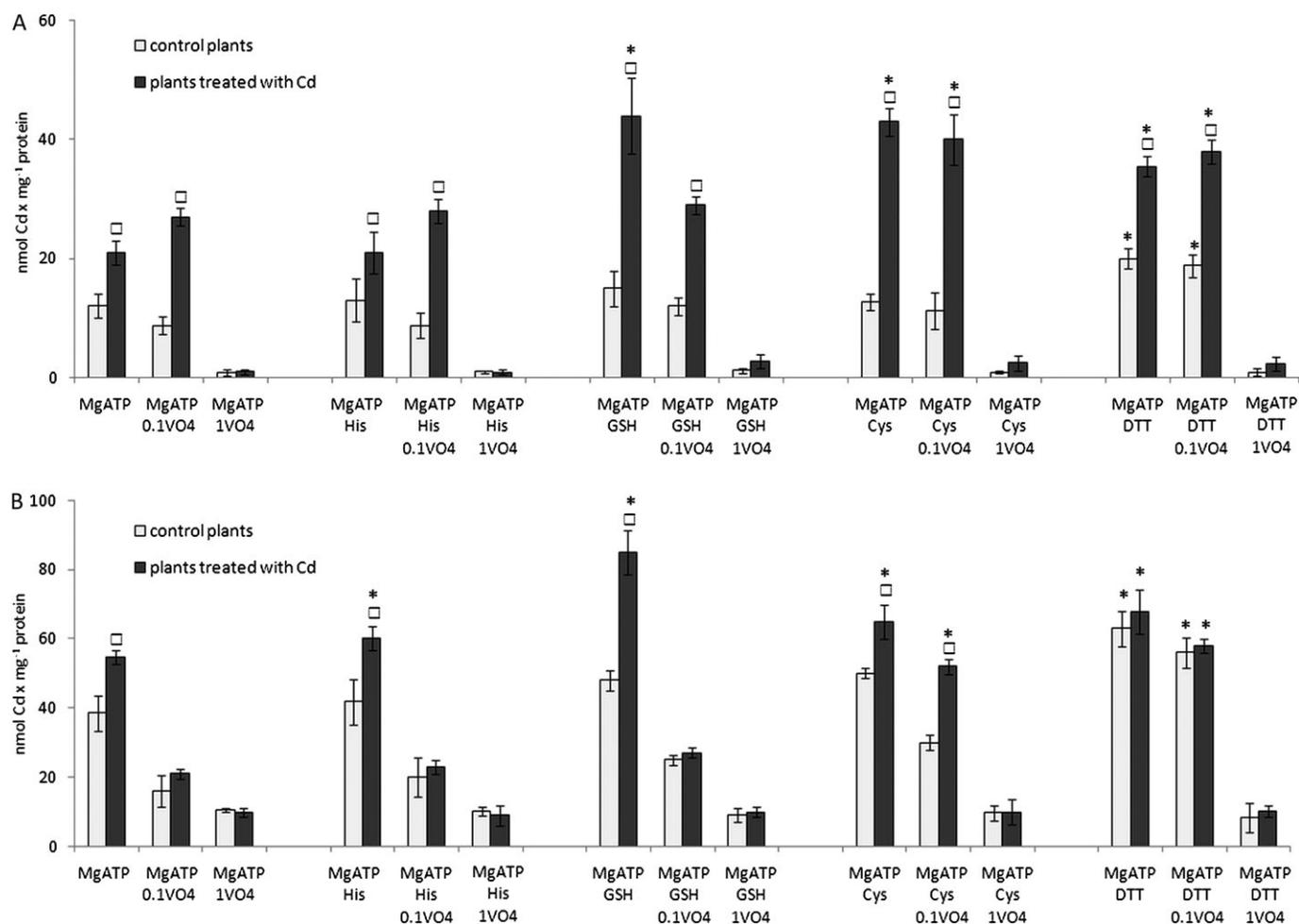


Fig. 3. The effect of 50 mM cysteine, 50 mM histidine, 5 mM GSH and 5 mM DTT on MgATP-dependent Cd transport into everted plasma membranes (A) and tonoplasts (B) isolated from control (open squares) and Cd-stressed (filled squares) plants. Orthovanadate was introduced into the reactions at a final concentration of 0.1 mM (0.1VO4) or 1 mM (1VO4). All incubation media with tonoplast and plasma membranes contained 50 mM nitrate or 5 μ M gramicidin, respectively. Cd was introduced into the incubation media at a final concentration of 5 μ M. The measurement was performed with the use of AAS followed by a direct filtration assay. The presented values are means \pm SD of three independent experiments with each experiment done in triplicate. Squares indicate a significant difference ($P < 0.05$) between control and Cd-treated plants and asterisks indicate a significant difference ($P < 0.05$) between Cd transport in the presence of cysteine, histidine, GSH or DTT and corresponding Cd transport measured without these chemicals in the incubation media.

the vacuole in the presence of metal-chelating agents were not as striking as for PMs. Still GSH, cysteine and DTT displayed the greatest stimulatory effect on the active metal accumulation within vesicles (Fig. 3B). Explicitly, metal transport activity increased up to 125, 130 and 165% under 5 mM GSH, 50 mM cysteine and 5 mM DTT, respectively, in the membranes derived from the control plants. In comparison, Cd transport across tonoplasts obtained from Cd-treated plants was stimulated up to 160, 120 and 125% by 5 mM GSH, 50 mM cysteine and 5 mM DTT, respectively. The increase in DTT or cysteine concentrations in the reaction medium did not affect the overall stimulation rate of MgATP-energized Cd efflux across the tonoplast (Fig. 4E, F). In contrast, GSH-mediated stimulation of metal transport into the vesicles increased up to 70% with the increase of GSH concentration from 1 mM up to 5–10 mM (Fig. 4D). The stimulatory effect

of GSH and cysteine on active Cd accumulation within vesicles was markedly reduced and almost completely eliminated in the presence of 0.1 mM or 1 mM orthovanadate, respectively. Interestingly, 0.1 mM vanadate did not affect DTT-induced Cd accumulation within tonoplasts obtained from control and Cd-treated plants (Fig. 3B), whereas 1 mM vanadate markedly inhibited this process.

Accumulation of Cd in the tonoplast vesicles imposing a pH gradient

As shown in the Fig. 2D, Cd transport into tonoplast vesicles was higher in the presence of MgATP than in the presence of MgATP combined with the inhibitors preventing proton gradient formation. Since the formation of a Δ pH stimulates Cd accumulation in tonoplast vesicles it may be assumed

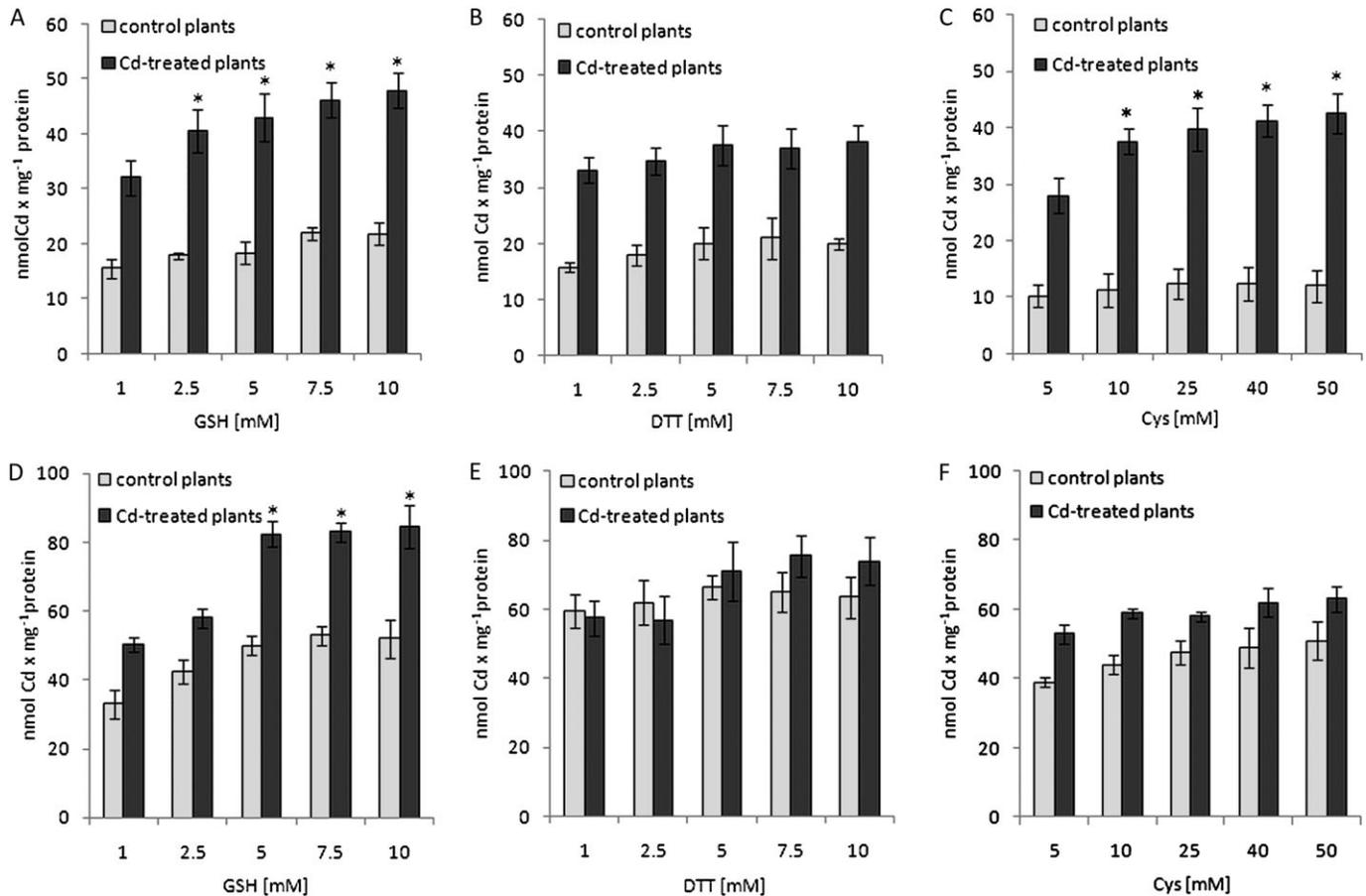


Fig. 4. The effect of different concentrations of cysteine, GSH, and DTT on MgATP-dependent Cd transport into everted plasma membranes (A–C) and tonoplasts (D–F) isolated from control (open squares) and Cd-stressed (filled squares) plants. All incubation media with tonoplast and plasma membranes contained 50 mM nitrate or 5 μ M gramicidin, respectively. Cd was introduced into the incubation media at a final concentration of 5 μ M. The measurement was performed with the use of AAS followed by a direct filtration assay. The presented values are means \pm SD of 4–6 independent experiments with each experiment done in triplicate. Asterisks indicate a significant difference ($P < 0.05$) between Cd transport in the presence of the lowest and the higher concentrations of cysteine, GSH or DTT in the incubation media.

that beside MgATP, the proton motive force generated by V-ATPase could also be a source of energy for Cd deposition within plant cell vacuoles. Consistent with previous findings (Salt and Wagner, 1993; Ortiz *et al.*, 1995; Gonzalez *et al.*, 1999), the results confirm that a Cd²⁺/H⁺ antiporter activity operates at the tonoplasts. A similar system of Cd efflux has also been described recently for PMs (Burzyński *et al.*, 2005; Migocka and Kłobus, 2007). To investigate the effect of Cd on ATP-driven intravesicular acidification of isolated tonoplasts, the previously described method using acridine orange as a pH-sensitive probe was used (Burzyński *et al.*, 2005; Migocka and Kłobus, 2007). In preliminary experiments, Cd concentrations up to 500 μ M did not affect the absorbance of acridine orange in the presence or absence of vesicles or ATP (data not shown). As demonstrated in Fig. 5A, in the presence of ATP a drop in acridine orange absorbance was recorded that indicated intravesicular acidification mediated by the V-ATPases present in the vesicle membrane. Bafilomycin stopped the further development of intravesicular acidification whereas the addition of Cd to the reaction mixture caused an

instantaneous, concentration-dependent dissipation of the Δ pH generated by the ATP-dependent proton pump (Fig. 5A, B). The rate of Cd-induced proton gradient dissipation was significantly higher in membranes isolated from Cd-treated plants when compared with membranes isolated from control plants (Fig. 5A–D). As shown in Fig. 5C, the phenomenon exhibited saturation kinetics with an apparent K_m of 30 μ M and 25 μ M in tonoplasts isolated from control and Cd-treated plants, respectively. Nonetheless, the kinetic curve for Δ pH-dependent Cd efflux was different from the saturation kinetics of MgATP-dependent Cd accumulation within vesicles, suggesting positive cooperativity in Cd binding by tonoplast-localized Cd²⁺/H⁺ antiporter. The dissipation of the proton gradient in vesicles was caused only by Cd ions since neither glucosamine sulphate nor potassium sulphate at concentrations up to 10 mM affected the Δ pH in tonoplast vesicles in a way similar to CdSO₄ (data not shown). Nonetheless, to confirm that the Cd-induced Δ pH dissipation in the membranes was strictly related to Cd transport into tonoplast vesicles, the direct accumulation of Cd inside vesicles

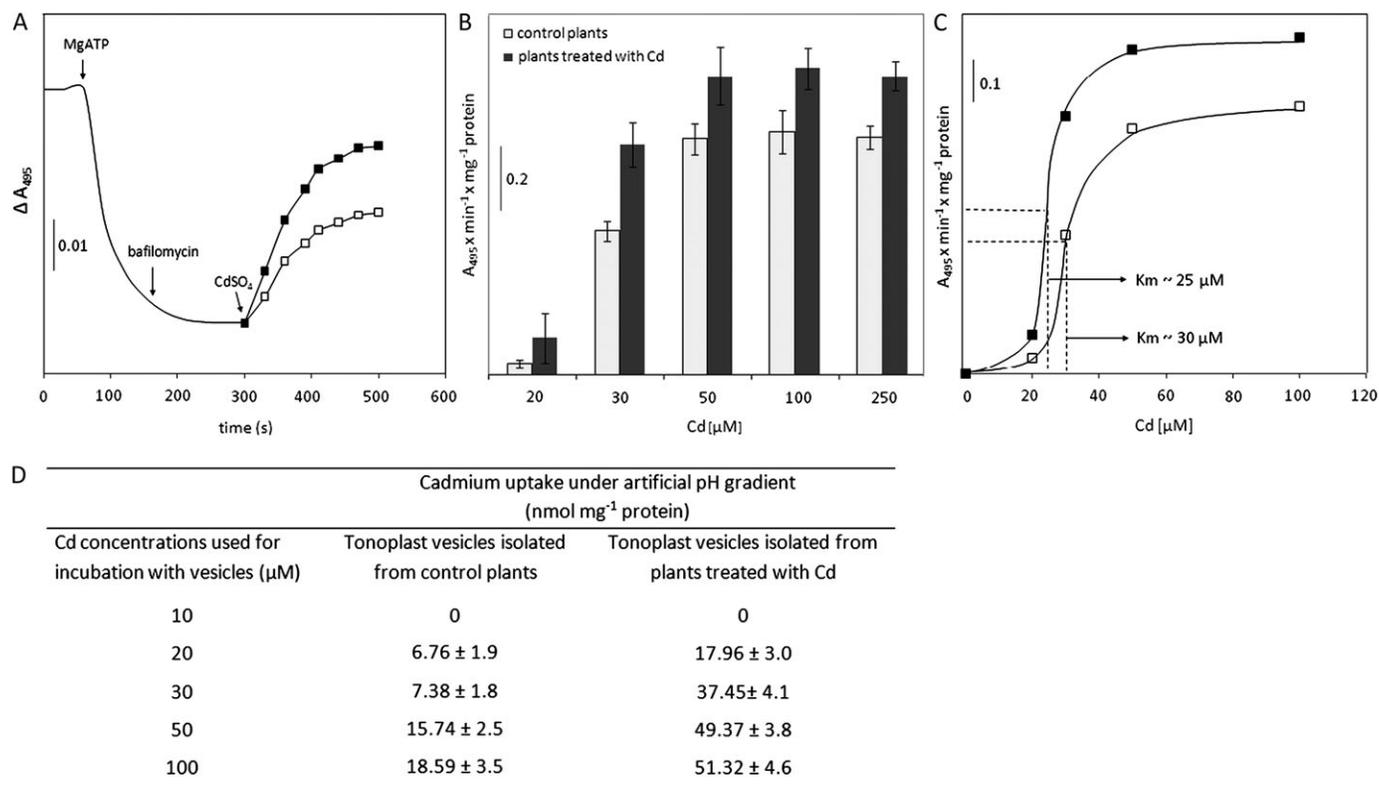


Fig. 5. The properties of Cd transport into tonoplast vesicles imposing an electrochemical proton gradient. (A) Effect of 30 μM CdSO₄ on the proton gradient generated by V-ATPase in tonoplasts isolated from control plants (open squares) and plants treated with Cd (filled squares). (B) Effect of different Cd concentrations on acridine orange absorbance change measured in vesicles imposing a ΔpH generated by V-ATPase. (C) Cd concentration dependence of Cd transport into tonoplast vesicles isolated from control (open squares) and Cd-treated (filled squares) plants with K_m values given. (D) The average net uptake of Cd for vesicles imposing an artificial pH gradient and subjected to direct vacuum filtration and AAS analyses. The values of Cd uptake were corrected for the amount of metals unspecifically bound to tonoplasts measured in control conditions without a pH gradient (data not shown). The presented values are representative of the results obtained in 3–4 independent experiments with each experiment done in triplicate (B–D) except for A which presents values corresponding to a representative experiment of at least four independent assays.

with an artificially created transmembrane pH gradient was measured using AAS (Fig. 5D). A direct filtration assay confirmed the results obtained with acridine orange as the pH-sensitive dye. Indeed, the Cd content within the vesicles was dependent on the presence of a ΔpH across the membranes and the transport of metal increased with the external concentration of the metal exhibiting saturation kinetics (Fig. 5D).

Discussion

The results provide direct evidence for an important role for both PM and tonoplast transport in detoxification of Cd of cucumber root cells. It has been previously reported that the transport of heavy metals into tonoplast vesicles isolated from oat roots occurs in an MgATP- and proton-dependent manner (Salt and Wagner, 1993; Salt and Rauser, 1995; Gries and Wagner, 1998). Also tonoplast vesicles isolated from the tolerant ecotype of *Silene vulgaris* accumulated Zn using two sources of energy: the electrochemical gradient and MgATP (Verkleij *et al.*, 1998). This finding strongly supports the hypothesis that the tonoplast could play an

essential role in plant heavy metal tolerance. Indeed, the results presented here confirm that Cd accumulation within plant vacuoles is mediated by H⁺-coupled Cd antiport as well as by the ATP-energized Cd pumps. Since the K_m value of H⁺-coupled Cd transport into tonoplast vesicles was slightly changed after plant exposure to Cd, it may be assumed that more than one protein antiporter is engaged in an active sequestration of Cd within vacuoles. Moreover, the kinetic curve of H⁺/Cd²⁺ antiport suggests allosteric properties of Cd antiporters, indicating that either the proteins with multiple Cd binding sites participate in H⁺-coupled Cd efflux across tonoplasts or Cd antiporters form protein polymers consisting of subunits, each containing a separate Cd binding site. It has been previously demonstrated that metal antiporters of different families are localized at tonoplasts. They comprise members of the CAX and CDF (MTP) families that contribute to the active, H⁺-coupled influx of heavy metal ions [Cd and manganese (Mn)] into vacuoles. It has been shown that Cd²⁺ can be directly transported into the vacuoles by the cation exchangers CAX2 and CAX4 (Hirschi *et al.*, 2000; Korenkov *et al.*, 2007). In addition, the overexpression of

AtCAX2 or *AtCAX4* in tobacco enhanced both Cd and Zn transport into root tonoplast vesicles as well as Cd accumulation in roots of plants exposed to Cd (Korenkov *et al.*, 2007). Growing evidence indicates that members of CAXs display broad specificity for a wide range of metals including Cd, Mn, calcium (Ca) and nickel (Ni) (Hirschi *et al.*, 2000; Cheng *et al.*, 2002; Korenkov *et al.*, 2007), hence the family may be regarded as the major contributor to vacuolar accumulation/sequestration of metal ions. There is also growing data confirming the involvement of CDF proteins in detoxification of Cd in plant cells. TcMTP1, the CDF transporter in heavy metal hyperaccumulator *Thlaspi goesingense*, is accumulated to high levels at the tonoplast of shoot cells, participating in the enhanced tolerance of the plant to Zn and Cd (Persans *et al.*, 2001; Gustin *et al.*, 2009). Also in another metallophyte, *Brassica juncea*, CDF transporters seem to be essential for Cd tolerance. It was recently shown that *BjCET2* belonging to the CDF family is remarkably up-regulated by Cd and Zn ions and confers increased tolerance to these metals in *Brassica* plants (Xu *et al.*, 2009). Therefore, the CDF transporters family appear to display a quiet broad specificity for heavy metals, including Cd. In addition, both types of proteins, CAX and CDF transporters, have been shown to form homo-oligomers as well as hetero-oligomers (Bloss *et al.*, 2002; Blaudez *et al.*, 2003; Cheng *et al.*, 2005; Zhao *et al.*, 2008; Fukunaka *et al.*, 2009), which could explain the saturation kinetics observed for H⁺-coupled Cd efflux across tonoplasts of cucumber root cells (Fig. 5C).

The H⁺/Cd²⁺ antiport into tonoplasts isolated from cucumber roots was considerably stimulated when Cd was present in the external solution (Fig. 5). This may indicate that the protein catalysing Cd transport across tonoplasts is regulated by Cd at the transcriptional and/or post-translational level, leading to enhanced Cd sequestration within the vacuole under heavy metal excess. It has been evidenced for the CAX proteins that they may be up-regulated by metals via enhanced expression of *CAX* mRNA (Cheng *et al.*, 2002) or post-translationally, through modification of the N-terminal part of the protein (Pittman and Hirschi, 2001; Pittman *et al.*, 2002). A similar mode of transcription regulation may also be attributed to plant MTPs. The steady-state mRNA levels of MTP1 were found to be enhanced significantly in *B. juncea* seedlings after exposure to Ni²⁺, Cd²⁺ or Zn²⁺ (Muthukumar *et al.*, 2007). The activity of plant CDF proteins may also be modulated post-transcriptionally, through re-localization of transporters between the PM and vacuolar membrane (Kim *et al.*, 2004).

In this study, an additional ATP-dependent vanadate-sensitive system excluding Cd from the vacuole has also been measured in cucumber root cells (Fig. 2D–F). The MgATP-energized Cd efflux was dependent on the Cd concentration and followed Michaelis–Menten saturation kinetics (Fig. 2E, F). In comparison with the H⁺-coupled Cd efflux at the tonoplast, MgATP-driven Cd pumping exhibited a markedly higher affinity for Cd ($K_m \sim 25\text{--}30 \mu\text{M}$ versus $6.6 \mu\text{M}$, respectively) (Fig. 2D–F, 5), since both

systems were induced at quite different Cd concentrations in the incubation medium: $20 \mu\text{M}$ or $1 \mu\text{M}$, respectively (Fig. 2E, F, 5B, D). A similar MgATP-driven Cd transport system has been described previously in yeast where Ycf1, a tonoplast ABC-pump belonging to the MRP subfamily, sequesters GSH-conjugated Cd and other metals in the vacuole (Li *et al.*, 1997). Until now only one plant ABC transporter (AtMRP3) has been shown to be potentially involved in vacuolar Cd accumulation, as its expression in the *ycf1* strain partially complemented the mutant phenotype (Rae *et al.*, 1998). Besides MRP3, a member of the P_{1B}-ATPases (HMA3) has been shown to be involved in Cd/lead (Pb) transport across the tonoplast of plant cells (Gravot *et al.*, 2004; Morel *et al.*, 2009). An *Arabidopsis* *HMA3* knock-out mutant was found to be more sensitive to Zn and Cd whereas ectopic overexpression of the gene improved plant tolerance to Cd, cobalt (Co), Pb and Zn (Morel *et al.*, 2009).

The results presented in this work clearly demonstrate that ATP-dependent Cd translocation into tonoplast vesicles is sensitive to 0.1 mM vanadate and stimulated by external Cd (Fig. 3B). Furthermore, GSH and cysteine significantly enhanced ATP-energized vacuolar Cd accumulation only in plants exposed to Cd excess whereas DTT stimulated this activity in both, Cd-treated and untreated plants (Fig. 3B). Interestingly, only GSH stimulation increased with increasing concentration of this agent in the incubation medium whereas DTT and cysteine affected MgATP-driven Cd transport in a concentration-independent manner (Fig. 4D–F). In contrast to the Cd-translocating bacterial ZntA, primary Cd transporters of cucumber root cell tonoplasts required a much higher cysteine:Cd and GSH:Cd concentration ratio to enhance Cd transport activity significantly. In the studies of Sharma *et al.* (2000), metal-induced ZntA hydrolytic activity increased with increasing concentrations of cysteine or GSH, reaching a maximum for a metal ion:thiolate ratio of 1:2. Also ZntA transport activity increased 5-fold in the presence of cysteine at 2-fold the concentration of Cd²⁺ (Sharma *et al.*, 2000). In this study, millimolar concentrations of cysteine and GSH were required to induce maximal transport activity of Cd primary transporters at the tonoplast in the presence of $5 \mu\text{M}$ Cd²⁺ in the incubation medium (Fig. 4D, F). Similar, high (millimolar) concentrations of cysteine in the incubation medium were required to induce the maximal activity of plant HMA2 in the presence of $1 \mu\text{M}$ Cd²⁺ or Zn²⁺ (Eren and Argüello, 2004) and Ag⁺/Cu⁺-ATPase from *Archaeoglobus fulgidus* (Mandal *et al.*, 2002). Hence, the mode of metal delivery and binding to various metal pumps might be different in different organisms. GSH-dependent stimulation of ATP-energized Cd transport at the tonoplast was abolished by vanadate whereas the stimulatory effect of cysteine was only slightly affected by this inhibitor. These results suggest that at least two differentially regulated protein systems might catalyse the direct ATP-dependent Cd transport into vacuoles: ABC transporters of GSH–Cd conjugates sensitive to low concentrations of vanadate or a P_{1B}-ATPase similar to HMA3. It has been previously demonstrated that metal

ATPases are less sensitive to vanadate than other P-ATPases and they can even be stimulated in the presence of a low concentration of the inhibitor. Hence, at least 0.2 mM vanadate was required to inhibit the formation of acylphosphate by human Menkes ATPase (copper ATPase) (Voskoboinik *et al.*, 2001). Similarly in plants, Na₂VO₄ inhibited AtHMA2 activity with a slightly high IC₅₀ (0.15±0.05 mM; Eren and Argüello, 2004). Moreover, the activity of HMA2 was significantly stimulated by heavy metals (Zn and Cd) and cysteine or other amino acids containing sulphhydryl sites that are likely to bind metal ions (Eren and Argüello, 2004). Since cysteine-induced and 0.1 mM vanadate-insensitive MgATP-dependent Cd accumulation in tonoplasts was observed only in Cd-treated plants, it might be assumed that the protein involved in this process is synthesized/activated only under Cd stress. On the other hand, GSH stimulation of MgATP-dependent Cd sequestration within the vacuole was more remarkable when compared with the cysteine effect: >80 nmol Cd min⁻¹ was accumulated in vesicles in the presence of MgATP-GSH whereas >60 nmol and 50 nmol of Cd min⁻¹ was transported into the same vesicles under ATP-cysteine or under an artificial electrochemical gradient, respectively. These data suggest that the main contributor to Cd detoxification in root cells belongs to the ABC subfamily of GSH-Cd transporters and explain the highest impact of GSH on the MgATP-energized Cd accumulation within vacuoles. As for the cysteine and DTT effect, a few studies have clearly demonstrated the dependence of HMA activity on the presence of thiolates (Sharma *et al.*, 2000; Mitra and Sharma, 2001; Mandal *et al.*, 2002; Eren and Argüello, 2004) and/or reducing agents (Voskoboinik *et al.*, 1998; Rensing *et al.*, 2000). It is well known that cysteine residues are capable of reacting with heavy metal ions (Zn²⁺, Cd²⁺, Pb²⁺, Hg²⁺ and Ag⁺) because of the high affinity between the soft sulphide and the soft metal. Since within cells heavy metals have been shown to be delivered to target proteins by specific metal-binding chaperones containing sulphhydryl groups (Tehseen *et al.*, 2010), the metal substrates for metal transporters *in vivo* are probably metal-thiolate complexes rather than the free metals (Sharma *et al.*, 2000). It could explain the positive effect of cysteine and GSH on MgATP-driven Cd accumulation within vacuoles. To date, DTT as a reducing agent was only shown to be required for human and bacterial Cu-ATPase activity to maintain the metal in its Cu¹⁺ redox state (Voskoboinik *et al.*, 1998; Rensing *et al.*, 2000; Mandal *et al.*, 2002). In this study, the stimulatory effect of DTT on MgATP-driven Cd transport across the tonoplast was the same for control plants as for Cd-treated plants (Fig. 3B). It may be assumed then that DTT affects metal transport through its impact on a wide range of proteins that could be involved in Cd transport by the protection of their sulphhydryl groups. Thus DTT might have influenced the primary as well as the secondary transport of Cd through the transporters present at the tonoplast, by affecting the redox state of their thiol groups and/or the processes of their homo- or hetero-oligomerization that could be significant to Cd transport activity (Blaudez *et al.*, 2003).

In contrast to the tonoplast, the role of the PM in heavy metal detoxification currently remains unclear. It has been previously suggested that plants developed at least two strategies for avoiding heavy metal build-up within the cell that employ the PM: the reduction of the uptake of heavy metals into the cell or the stimulation of the efflux of metals that have already entered the cytosol (Hall, 2002). The efflux of Cd, Pb, Cu, Mn and Ni across the PMs of cucumber roots that was energized by the proton motive force generated by PM ATP-ases was previously revealed (Burzyński *et al.*, 2005; Migocka and Kłobus, 2007). This was the first indication proving that the metal/H⁺ antiporters similar to tonoplast exchangers reside at the PM and are significantly stimulated in the presence of metal ions in the environment (Migocka and Kłobus, 2007). The results presented here may be the first kinetic confirmation of previous genetic studies indicating that Cd may be excluded from plant cells via both an ATP-dependent and ΔpH-dependent ways. Since CDF and CAX proteins have been shown also to be localized to the PM (Luo *et al.*, 2005; Qi *et al.*, 2005; Kim *et al.*, 2007) it might be assumed that Cd²⁺/H⁺ antiport at the PM is directed by members of these families. However, the evidence demonstrating PM location of CAX or CDF proteins is relatively scarce and further research is needed to confirm this idea.

On the other hand, the ATP-dependent metal transporters have been localized in plant PMs but direct ATP-energized Cd exclusion across the PM of plant cells has not been demonstrated yet. Proteins involved in such a process have been identified in bacteria and yeast. It has been shown in yeast that PM P_{1B}-ATPase Pca1 actively excretes Cd out of the cells (Adle *et al.*, 2007). A similar process in bacteria is catalysed by the heavy metal P-ATPase, CaD (Tsai *et al.*, 1992). Homologous proteins that could detoxify plant cells after exposure to Cd have been also identified. They include PM-localized P_{1B}-ATPases (HMA2, HMA4 and HMA5) and ABC transporters (PDRs). Thus far, only one PM ABC transporter (AtPDR8) has been shown to be important for Cd detoxification (Kim *et al.*, 2007). Nevertheless, it seems to be primarily involved in pathogen defence. More evidence implicates HMA proteins in active Cd exclusion across the PM. In *A. thaliana* HMA2 and HMA4 participate in both, the essential Zn homeostasis and Cd detoxification (Hussain *et al.*, 2004). As has already been mentioned, more detailed analysis of HMA2 revealed that the pump activity is stimulated in the presence of cysteine (Eren and Argüello, 2004). Furthermore, cysteine has been shown to increase dramatically the activities of heavy metal-transporting P-ATPases of *A. fulgidus* (CopA-Cu), *Escherichia coli* (ZnTA-Cu) and *Saccharomyces cerevisiae* (Pca1-Cd) (Yang *et al.*, 2007).

ATP-dependent Cd transport across PMs of cucumber root cells that is demonstrated in this study, displayed significantly lower capacity to remove Cd from the cytosol than the relevant activity measured in tonoplasts. Figures 2 and 3 clearly show that everted PMs accumulated ~2-fold less Cd through energy-dependent transport systems. The MgATP-driven Cd efflux across PMs displayed

a significantly lower V_{\max} of Cd transport with a higher K_m for Cd when compared with the corresponding process observed at the tonoplast (Fig. 2). It may be suggested that the primary Cd transport system at PMs had a higher affinity for Cd but a much lower capacity for Cd efflux in comparison with the MgATP-driven uptake of Cd into vacuoles. However, both membrane systems seem to be regulated in a similar manner. Cysteine, GSH and DTT significantly stimulated ATP-energized Cd transport across PMs isolated from Cd-treated plants (Fig. 3A). However, in the experiments including PMs, both GSH and cysteine induced primary Cd efflux in a GSH or cysteine concentration-dependent manner (Fig. 4A, C). Still, relatively high concentrations of GSH (5–10 mM) or cysteine (10–50 mM) were required for the MgATP-driven Cd efflux to reach its maximal activity (Fig. 4A, C). Similarly to the tonoplast, DTT stimulation of primary Cd efflux was not affected by the alterations in the DTT concentration (Fig. 4B). Moreover, the GSH-mediated induction of Cd efflux was abolished in the presence of 0.1 mM vanadate which may suggest that a protein of the ABC family could be involved in the transport of GSH–Cd complexes across the PM. Conversely, the lack of sensitivity of ATP-dependent Cd transport to 0.1 mM vanadate in the presence of cysteine indicates the contribution of P_{1B} -ATPases (HMAs) in the cysteine–Cd efflux from root cells. Since both GSH and cysteine stimulation of Cd transport was observed only in the Cd-treated plants, it may be assumed that the proteins responsible for these activities (possibly members of PDRs and HMAs) are highly expressed during heavy metal stress. The slightly or considerably enhanced stimulation of Cd transport across the PM was observed in membranes isolated from control and Cd-treated plants, respectively, when DTT was included in the reaction medium (Fig. 3A). Similarly to the tonoplast, DTT could stimulate the activity of proteins involved in Cd transport through reduction of their sulphhydryl groups. Nevertheless, it could also serve as a chelator for Cd and thus improve its 0.1 mM VO_4^{3-} -insensitive transport by HMA-like protein.

In comparison with cysteine, GSH and DTT, the effect of histidine on the Cd transport in the tonoplast and PM was minute, which may indicate that this amino acid exhibiting high affinity for Ni (Ingle *et al.*, 2005) does not participate in Cd chelation.

Conclusions

In this work, PMs and tonoplasts isolated from cucumber roots were used to determine the energy-dependent transport of both, ionized Cd and complexed Cd out of cells. Consistent with previous findings from studies on cucumber, other plants, animals, bacteria and yeast, it is shown that active Cd transport exhibits two modes of energy coupling: MgATPase (transport of Cd^{2+} or Cd–cysteine/GSH) and antiporter (H^+ /Cd $^{2+}$ antiport) using the electrochemical gradient formed by the membrane proton pumps. Both MgATP-driven Cd efflux systems are markedly stimulated in the presence of Cd in the external solution as well as by cysteine, DTT and GSH. Thus

it may be suggested that Cd induces the expression and/or post-translational modification of the proteins responsible for Cd efflux, resulting in the increased detoxification of cells after Cd excess. It may also be assumed that Cd thiolates and not free Cd ions are the main substrates for primary Cd transporters localized at the PM and tonoplast. Despite the contribution of both the PM and tonoplast to active Cd efflux from the cytosol of root cells, it seems that in the non-hyperaccumulator cucumber, the tonoplast and vacuole have the major function of detoxification of cytosol during Cd stress. The tonoplast primary Cd efflux system exhibits lower affinity but much greater capacity for Cd uptake into the vacuole than the relevant PM-localized primary Cd efflux. It may be suggested then that tonoplast-localized Cd active transporters operate at higher velocity and capacity than the corresponding systems at the PMs. To our knowledge, this report represents the first direct demonstration of MgATP-dependent Cd transport activity in PMs of plant cells and the first comparison of PM and tonoplast primary Cd efflux activities.

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