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RESEARCH PAPER

Different effect of cadmium and copper on H^+-ATPase activity in plasma membrane vesicles from *Cucumis sativus* roots

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Abstract

The effect of heavy metals on plasma membrane (PM) H^+-ATPase (EC 3.6.3.14) activity in cucumber (*Cucumis sativus*) roots was studied. The aim of this work was to explain the mechanism of modification of the PM H^+-ATPase activity in plants subjected to heavy metals. Plants were treated with 10 μM Cd or Cu for 6 d. After 3 d exposure to the heavy metals, some of the plants were transferred to control conditions for a further 3 d (3/3 plants). The activity of PM H^+-ATPase was found to be increased in plants treated with heavy metals. The highest activity measured as proton transport was observed in 3/3 plants. Estimation of transcript levels of *C. sativus* PM H^+-ATPase in roots indicated that the action of Cd, but not Cu, affected the gene expression level. Transcript levels of *C. sativus* PM H^+-ATPase (*CsHA2*, *CsHA3*, *CsHA4*, *CsHA8*, and *CsHA9*) genes increased in roots treated with Cd. Moreover, Western blot analysis with antibody against phosphothreonine and 14-3-3 protein indicated that increased activity of PM H^+-ATPase under heavy-metal stress resulted from phosphorylation of the enzyme. It was found that Cu markedly increased the activity of catalase and ascorbate peroxidase and reduced the level of H_2O_2 in cucumber roots. In contrast, Cd did not affect these parameters. These results indicate that Cd and Cu can, in different ways, lead to modification of PM H^+-ATPase activity. Additionally, it was observed that treatment of plants with heavy metals led to an increased level of heat-shock proteins in the tissues. This suggests that the plants had started adaptive processes to survive adverse conditions, and increased PM H^+-ATPase activity could further enhance the repair processes in heavy-metal-stressed plants.

Key words: Cd, Cu, H^+-ATPase activity, heavy metals, plasma membrane.

Introduction

Among the heavy metals, cadmium (Cd) is widely acknowledged as one of the most phytotoxic agents (Sanita di Toppi and Gabbrielli, 1999). Cd has no known function as a nutrient and seems to be highly toxic to plants. The heavy metal copper (Cu) is essential for plant growth and development but can be toxic at high concentrations (van Hoof *et al.*, 2001; Hall, 2002). The plant plasma membrane (PM) can be regarded as the first structure that is a target for heavy-metal toxicity. An increase in permeability related to membrane damage is observed in plants that have been subjected to heavy-metal stress (De Vos *et al.*, 1991, 1992; Demidchik *et al.*, 1997, 2001; Murphy and Taiz, 1997; Murphy *et al.*, 1999). One of the physiological responses to excess Cu is K^+ efflux from the roots. Rapid K^+ efflux has been interpreted as a symptom of toxicity resulting from Cu-induced oxidative damage to the PM. Intracellular free Cu ions can react with water to produce free-radical hydroxyls, which in turn react to cause membrane lipid peroxidation (De Vos *et al.*, 1991, 1992). It is well known that metal ions are easily bound to both the sulfhydryl groups of proteins and hydroxyl part of phospholipids (Devi and Prasad, 1999). They can also replace the Ca^{2+}

Abbreviations: APX, ascorbate peroxidase; BTP, Bis-Tris propane; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; HSP, heat-shock protein; PM, plasma membrane.

ions at essential sites of cell membranes (Breckle and Kahle, 1991). All these events result in disruption of membrane integrity and ionic homeostasis of cells. Thus, tolerance may involve the protection of plasma membrane integrity against heavy-metal damage and maintaining ionic balance. A factor that may be important in the maintenance of PM integrity in the presence of heavy metals could be enhanced membrane repair after damage (Salt *et al.*, 1998). This could involve heat-shock proteins (HSPs). HSPs can be expressed in response to a variety of stress conditions, including heavy metals, because they may function in the protection and repair of proteins under stress conditions. Moreover, maintaining ionic balance and replenishing the loss of essential substances in repair processes is an important issue under such conditions. Support of active transport of ions and organic compounds through the PM requires increased generation of a proton gradient by the PM proton pump. Generation of an electrochemical gradient across the membrane results in a proton-motive force that is used by passive transport for assimilation of various nutrients, as well as for releasing ions and toxic substances from cells. Besides regulation of growth and development processes, PM H^+-ATPase also plays a role in adaptation of plants to stress conditions (Janicka-Russak, 2011). A few observations have indicated that H^+-ATPase activity was changed under heavy-metal stresses (Lindberg and Wingstrand, 1985; Kennedy and Gonsalves, 1989; Ros *et al.*, 1992*a*,*b*; Fodor *et al.*, 1995; Demidchik *et al.*, 1997; Astolfi *et al.*, 2003; Burzyński and Kolano, 2003; Astolfi *et al.*, 2005; Shen *et al.*, 2005; Janicka-Russak *et al.*, 2008; Kabała *et al.*, 2008). To date, data concerning heavy-metal action on the PM H^+-ATPase are contradictory. Mechanisms of Cu^{2+}-induced H^+-ATPase suppression have been investigated *in vitro* (Vara and Serrano, 1982; Serrano *et al.*, 1985; Serrano, 1990). According to these authors, Cu^{2+} ions are one of the most powerful inhibitors of PM H^+-ATPase, producing half-maximal inhibition at 2–5 μM and complete inhibition at 10–20 μM. The inhibitory effect of Cu^{2+} seems to be related to its interaction with sulfhydryl groups at the active site of the ATPase. The effect of metals on PM H^+-ATPase activity depends on time of exposure of plants to heavy metals, the type and concentration of heavy metal, and the plant species. In an earlier investigation, we found that in cucumber seedling roots, a 2 h treatment of plants with Cd and Cu led to inhibition of PM H^+-ATPase activity (Janicka-Russak *et al.*, 2008). However, a longer time of treatment of plants with these heavy metals led to increased activity of the enzyme in rice (Ros *et al.*, 1992*a*,*b*). In contrast, Astolfi *et al.* (2003, 2005) noted inhibition of activity of PM H^+-ATPase in oat and maize roots treated for a few days with Cd.

The PM H^+-ATPase enzyme is encoded by a multigene family (Portillo, 2000; Arango *et al.*, 2003). Many studies have shown changes of in gene expression of the PM H^+-ATPase in response to a variety of environmental factors, including salt stress (Binzel, 1995; Janicka-Russak and Kłobus, 2007), dehydration (Surowy and Boyer, 1991), light conditions (Harms *et al.*, 1994), mechanical stress (Oufattole *et al.*, 2000), and low temperature (Janicka-Russak *et al.*, 2011). It is known that, besides the genetic regulation of the proton pump, its activity might be fast modulated post-translationally at the protein level, mainly through reversible phosphorylation (Schaller and Sussman, 1988; Portillo, 2000). Data suggest that activation of the plant PM H^+-ATPase is mediated through phosphorylation of Thr-948 in the enzyme, which allows the binding of 14-3-3 protein (Svennelid *et al.*, 1999; Palmgren, 2001; Arango *et al.*, 2003). Additionally, activity of PM H^+-ATPase may depend on the membrane redox state. An oxidoreductase, whose activity depends on NAD(P)H, is located in the PM of plant cells. This protein does not participate directly in proton transport across the PM but has an impact on the process by modifying activity of the PM H^+-ATPase proton pump (Kłobus and Buczek, 1995). PM oxidoreductase, by transporting electrons across the PM, simultaneously acidifies the cytoplasm (Lüthje *et al.*, 1997). A low pH in the cytoplasm and membrane depolarization stimulate the activity of PM H^+-ATPase (Hager and Moser, 1985; Rubinstein and Stern, 1986; Kłobus, 1995). Moreover, heavy metals were found to produce oxidative stress (Howlett and Avery, 1997; Schützendübel and Polle, 2002; Pál *et al.*, 2005).

Therefore, this study aimed to investigate the mechanism of Cd and Cu action on PM H^+-ATPase activity in cucumber roots under long-term heavy-metal stress (6 d). The hydrolytic and transporting activities of the proton pump were measured in parallel with the expression of genes encoding this enzyme. Using specific antibodies against phosphothreonine and 14-3-3 protein, immunoblot analyses were performed to verify whether modulation of enzyme activity, observed under heavy-metal stress, resulted from changes in phosphorylation of its protein. In addition, the effect of Cd and Cu on the antioxidant enzyme activities, PM oxidoreductase and the level of hydrogen peroxide in the roots were investigated. Moreover, because HSPs play a crucial role in protecting plants against stress by re-establishing normal protein conformation, stabilization of protein and membranes, the level of HSPs in cucumber seedlings subjected to 6 d of Cd or Cu stress were studied.

Material and methods

Cucumber seeds (*Cucumis sativus* L. var. Krak), germinated in darkness at 25 °C for 48 h, were transferred to a nutrient medium containing 10 μM Cd or Cu for 6 d. After 3 d, some of the plants exposure to heavy metals were transferred to control conditions (nutrient solution without 10 μM Cd or Cu) for another 3 d (3/3 plants). The nutrient solution (pH 5,5) contained: 1.7 mM KNO_3, 1.7 mM $Ca(NO_3)_2$, 0.33 mM KH_2PO_4, 0.33 mM $MgSO_4$, and the microelements 75 μM ferric citrate, 10 μM $MnSO_4$, 5 μM H_3BO_4, 1 μM $CuSO_4$, 0.01 μM $ZnSO_4$, and 0.05 μM Na_2MoO_4. The plants were grown hydroponically with a 16 h photoperiod (180 μmol m^{-2} s^{-1}) at 25 °C during the day and 22 °C at night. The relative humidity in the light and dark was 70%.

PM vesicles were isolated from cucumber root microsomes by phase partitioning according to the procedure of Larsson (1985), as modified by Kłobus (1995). An 8 g phase system containing

6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 330 mM sorbitol, 5 mM KCl, and 5 mM Bis-Tris propane (BTP)/MES (pH 7.5) was used. The PMs obtained by this procedure were composed mainly of right-side-out vesicles and were used to determine the hydrolytic ATPase activity. Some of the vesicles were turned to the inside-out-oriented form by the method of Johansson *et al.* (1995) and used for measurements of ATP-dependent H^+ transport in the PM.

The hydrolytic activity of the vanadate-sensitive ATPase (PM H^+-ATPase) was determined according to the procedure of Gallagher and Leonard (1982), as modified by Sze (1985). The reaction mixture contained 50 μg of protein (PM), 33 mM TRIS-MES (pH 7.5), 3 mM ATP, 2.5 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 0.1 mM Na_2MoO_4, and 50 mM $NaNO_3$, with or without 200 μM Na_3VO_4 and 0.02% Triton X-100. PM H^+-ATPase activity was expressed as the difference between the activity measured in the absence and presence of Na_3VO_4. The amount of P_i released during the reaction was determined according to the method of Ames (1966) with 0.2% (w/v) SDS included to prevent precipitation (Dulley, 1975).

H^+ transport activity was measured spectrophotometrically as the change in acridine orange absorbance at 495 nm (A_{495}) according to the method of Kłobus and Buczek (1995). The assay medium contained PM vesicles (about 50 μg of protein), 25 mM BTP-MES (pH 7.5), 330 mM sorbitol, 50 mM KCl, 0.1% BSA, 10 μM acridine orange, and 0.05% Brij 58. Proton transport was initiated by the addition of 3 mM Mg-ATP. For every combination, passive proton movement through the membrane was determined without ATP in the reaction medium.

To evaluate expression of the genes encoding the PM H^+-ATPase, *CsHA2* (GenBank accession no. EU735752), *CsHA3* (EF375892), *CsHA4* (HO054960), *CsHA8* (HO054964), *CsHA9* (HO054965), and *CsHA10* (HO054966), real-time PCR was performed using the LightCycler® 2.0 system from Roche Diagnostics. For the normalization of expression of each *CsHA* gene, a gene encoding TIP41-like protein (GW881871) was used as the internal standard. Total RNA was isolated from 50 mg of frozen root tissue using Tri Reagent (Sigma) according to the manufacturer's instructions. Total RNA yield was determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific) and the $A_{260/280}$ ratio showed the expected values between 1.9 and 2.0. To avoid any DNA contamination, the RNA samples were treated with RNase-free DNase I (Fermentas) and then reverse transcribed into first-strand cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. The cDNA was then used as the template for PCR amplification with a RealTime 2× PCRMaster Mix SYBR® (A&A Biotechnology) kit. The gene-specific primers used for PCR were carefully designed using the LightCycler Probe Design program. Expression of *CsHAs* was analysed with the following primer pairs: 5′-ACCCGAGTCGACAAACATCT-3′ (forward) and 5′-CTTGGCACAGCAAAGTGAAA-3′ (reverse) for *CsHA2*; 5′-AAGTTTCTGGGGTTCATGTGGAAT-3′ (forward) and 5′-GTAACAGGAAGTGACTCTCCAGTC-3′ (reverse) for *CsHA3*; 5′-CTACAGCTTGGTAACATACATTC-3′ (forward) and 5′-GTTGTAGTCCATGTAATGTCCTC-3′ (reverse) for *CsHA4*; 5′-CTCATGCGCAAAGAACATTAC-3′ (forward) and 5′-CTGAATTGTGTCAATGTCAAGTC-3′ (reverse) for *CsHA8*; 5′-AAACCAGAAGTGCTGGAG-3′ (forward) and 5′-CTCAGCACCCTCACTAGTAA-3′ (reverse) for *CsHA9*; 5′-GACATAATCAAGTTTGCAATCAGATA-3′ (forward) and 5′-TTCTGTATAAGTTGTGCGGT-3′ (reverse) for *CsHA10*; and 5′-CAACAGGTGATATTGGATTATGATTATAC-3′ (forward) and 5′-GCCAGCTCATCCTCATATAAG-3′ (reverse) for TIP41-like protein. The following amplifications conditions were applied: 30 s at 95 °C; 45 cycles of 10 s at 95 °C, 10 s at 58 °C, and 12 s at 72°C, and a final melting for 15 s at 65°C.

For Western blot analysis, 10 μg of PM protein was incubated in SDS buffer containing 2% (w/v) SDS, 80 mM dithiothreitol, 40% (w/v) glycerol, 5 mM PMSF, 10 mM Tris/HCl (pH 6.8), 1 mM EDTA, and 0.05% (w/v) bromophenol blue for 30 min at room temperature and separated by 7.5% SDS-PAGE (Laemmli, 1970). After 1 h of electrophoresis at 25 mA, the proteins were electrotransferred (60 V, 200 mA) for 1.5 h to nitrocellulose using an SV10-EB10 blotting apparatus (Sigma-Aldrich). The transfer buffer contained 25 mM Tris/HCl (pH 8.3), 150 mM glycine, and 10% (v/v) methanol.

To identify the PM H^+-ATPase, the blots were incubated overnight (8 °C) with monoclonal antibody against PM H^+-ATPase (46E5B11D, kindly provided by W. Michalke, Universität Freiburg, Germany). The antiserum was diluted 2000-fold. After repeated washing, the nitrocellulose membrane was incubated at room temperature for 1 h with 1:4000-diluted secondary antibody (anti-mouse, conjugated to horseradish peroxidase (HRP); Sigma-Aldrich) and visualized by staining with 3,3′-diaminobenzidine (DAB).

Phosphorylation of the PM H^+-ATPase was detected with a rabbit polyclonal anti-phosphothreonine antibody (Abcam) used at a concentration of 2 μg/ml after overnight incubation (8 °C). The membranes were rinsed and incubated for 1 h at room temperature with 10 000-fold secondary antibody conjugated to HRP (polyclonal goat anti-rabbit IgG; Abcam). The results were visualized by staining with DAB.

Detection of 14-3-3 protein was performed with a rabbit polyclonal anti-14-3-3 antibody (Abcam). The antiserum was diluted 1000-fold. After overnight incubation (8 °C), the membranes were rinsed and incubated for 1 h at room temperature with 10 000-fold secondary antibody conjugated to HRP (polyclonal goat anti-rabbit IgG; Abcam). The results were visualized by staining with DAB.

Detection of HSPs was performed with rabbit polyclonal anti-HSP17.6, anti-HSP17.7, anti-HSP70, and anti-HSP101 (Agrisera). The antisera were diluted 500-, 1000-, 6000-, and 500-fold, respectively. After overnight incubation (8 °C), the membranes were rinsed and incubated for 1 h at room temperature with 6000-fold secondary antibody conjugated to HRP (polyclonal goat anti-rabbit IgG; Abcam). The results were visualized by staining with DAB.

The activity of PM oxidoreductase was assayed according to the method of Kłobus (1995). Reduction of ferricyanide by NADH in PM vesicles was measured spectrophotometrically as the change in A_{420}. The assay medium contained PM vesicles (about 50 μg of protein), 25 mM BTP-Mes (pH 7.5), 250 mM sorbitol, 50 mM KCl, 3.75 mM $MgSO_4$, 0.5 mM NADH, 0.02% Triton X-100, and 0.5 mM $K_3Fe(CN)_6$.

To measure H_2O_2 levels, 1 g of cucumber root was ground with a mortar and pestle in liquid nitrogen. Next, 3 ml of 50 mM Mops (pH 7.2) was added. Samples were centrifuged at 10 000 *g* for 10 min. The supernatant was used for measurement of H_2O_2. The reaction mixture contained 50 mM Mops, 0.2 μg/l of pyranine, 30 U/ml of peroxidase (VI-A; Sigma), and supernatant. The H_2O_2 level was determined fluorometrically (excitation at 405 nm and emission at 510 nm) using a TD-20/20 Fluorometer (Turner Designs).

Catalase (EC 1.11.1.6) activity was determined as described by Aebi (1984). The decomposition of H_2O_2 was followed by measuring the decrease in A_{240} for 150 s and was calculated per 60 s. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), plant extract, and 10 mM H_2O_2. One unit of catalase is defined as the amount of enzyme that breaks down 1 μmol of H_2O_2/min.

Ascorbate peroxidase (APX) activity was determined in a mixture containing 100 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2, and enzyme extract (Chen and Asada, 1989). Oxidation of ascorbate was followed by measuring the decrease in A_{290}. The conversion was assumed as the molar absorption coefficient value of 2.8 mM^{-1} cm^{-1}.

Protein was measured according to the method of Bradford (1976) in the presence of 0.02% Triton X-100 with BSA as the standard.

For each of at least three independent protein and RNA extractions, measurements of enzyme activity and gene expression were obtained in triplicate and the means ±SD of these values are presented in the figures. The quantitative PCR data were analysed by the $\Delta\Delta C_T$ method using LightCycler Software 4.1 (Roche).

Results

In the present study, alteration in PM H^+-ATPase activity in cucumber roots treated for 6 d with 10 μM Cd or Cu (Cd 6 and Cu 6 plants) was examined. Some of the plants, following 3 d exposure to heavy metals, were transferred to control conditions (without heavy metals in the nutrient medium) for the next 3 d (Cd 3/3 and Cu 3/3 plants). Treatment of cucumber seedlings with Cd or Cu stimulated the hydrolytic activities of the PM H^+-ATPase by about 40% (Fig. 1). Both metals affected the transporting activity of the PM proton pump in a similar way (as hydrolytic activity) in plants treated with them constantly for 6 d (Fig. 2). However, the highest stimulation (about 90%) of the proton transport across the PM was observed in 3/3 plants (Fig. 2). This result clearly indicated an enhanced H^+/ATP coupling ratio of H^+-ATPase in PM obtained from plants in which the heavy metal was removed from the nutrient solution.

The effect of Cd or Cu on the PM proton pump activity could involve the expression levels of genes. Six of the isoforms of PM H^+-ATPase are expressed in cucumber roots: *CsHA2*, *CsHA3*, *CsHA4*, *CsHA8*, *CsHA9*, and *CsHA10* (A. Wdowikowska, unpublished data). To assess the expression level of these PM H^+-ATPase genes in cucumber roots treated with heavy metals, a real-time PCR assay was performed. The relative expression of PM H^+-ATPase genes in cucumber roots was differentially affected as a result of Cd and Cu treatment. The transcript levels of *CsHA2*, *CsHA3*, *CsHA4*, *CsHA8*, and *CsHA9* in roots treated with Cd was higher than those in the control plants (Fig. 3). The transcript level of the proton pump genes was affected in a similar manner by both plant treatments: treatment with the heavy metal for 6 d and when the heavy metal was withdrawn after 3 d treatment from the nutrient solution. In contrast, Cu had no effect on the transcript level of any of the investigated isoforms of the PM H^+-ATPase genes.

The effect of Cd and Cu on the PM oxidoreductase activity in cucumber seedlings was also examined. The results of the reduction of ferricyanide by NADH in PM vesicles isolated from control and heavy-metal-treated cucumber roots are presented in Fig. 4. Oxidoreductase activity determined in PM isolated from Cd 6 or Cd 3/3 cucumber roots was the same as in the control plants, whereas activity of the enzyme measured in membranes obtained from Cu-treated plants was higher (by about 40%) than in the control plants.

Cd and Cu had different effects on H_2O_2 accumulation and activity of the antioxidant enzymes catalase and APX.

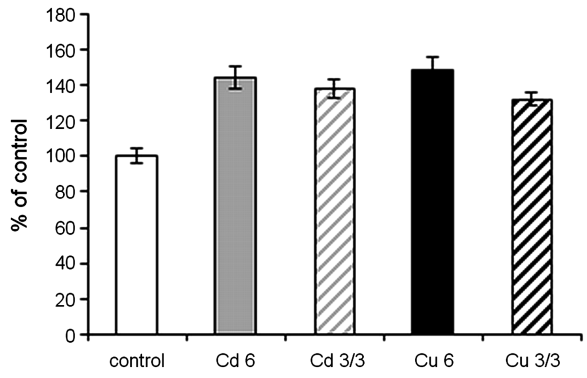

Fig. 1. Effect of Cd and Cu on the hydrolytic activity of H^+-ATPase in PM vesicles. PM was isolated from control roots (control) and from roots treated for 6 d with 10 μM Cd (Cd 6) or Cu (Cu 6). In some plants, after 3 d treatment with Cd or Cu, the metal was withdrawn from nutrient (Cd 3/3 and Cu 3/3). The hydrolytic activity of PM H^+-ATPase was measured as described in Materials and methods. The results are shown as means ±SD of three independent experiments, with each experiment performed in triplicate.

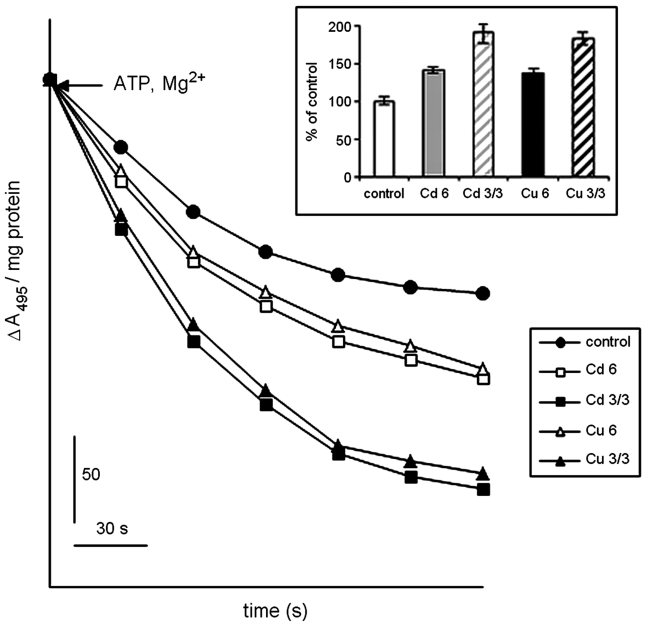

Fig. 2. Effect of Cd and Cu on the proton transport activities measured in the PM of vesicles. PMs (50 μg of protein) were isolated from control seedling roots, from roots treated for 6 d with 10 μM Cd (Cd 6) or Cu (Cd 6), or from plants in which the heavy metal was withdrawn after 3 d (Cd 3/3 and Cu 3/3). After equilibration of the PM with the reaction medium (for at least for 5 min), vesicle acidification was initiated by the addition of Mg-ATP to a final concentration of 3 mM. The formation of a ΔpH gradient in the vesicles was monitored as the change in acridine orange absorbance (A_{495}). The values presented are representative of results obtained in three independent experiments each carried out in triplicate. The inset graph (the steady state of H^+ transport) shows the percentage of the control value for each of the treated seedling roots as means ±SD from the three independent experiments.

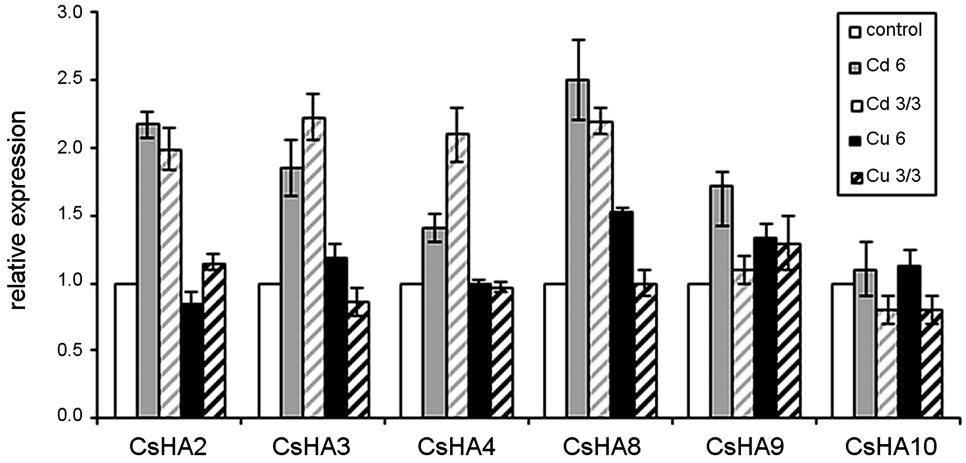

Fig. 3. Relative expression of PM H^+-ATPase genes in cucumber roots exposed to heavy metals. To determine the expression of PM H^+-ATPase genes, real-time PCR analysis was performed as described in Materials and methods. RNA was isolated from the control roots, from roots treated for 6 d with 10 μM Cd (Cd 6) or 10 μM Cu (Cu 6), or from plants in which the heavy metal was withdrawn after 3 d (Cd 3/3 and Cu 3/3). Results are shown as means ±SD of three replications.

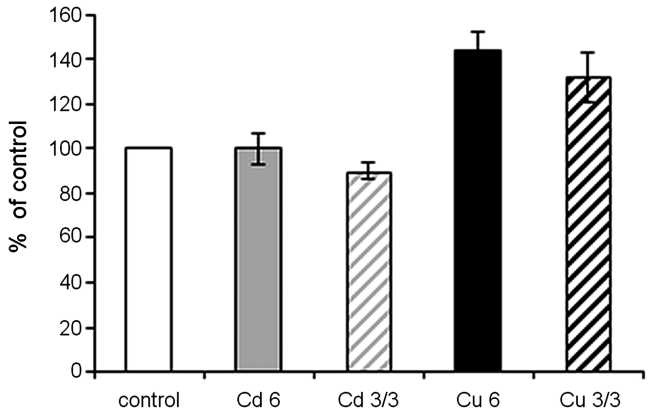

Fig. 4. Ferricyanide reduction by NADH in PM vesicles. Reduction of ferricyanide by NADH in PM vesicles was measured spectrophotometrically as the change in A_{420} as described in Materials and methods. PM was isolated from control roots and from roots treated for 6 d with 10 μM Cd (Cd 6d) or 10 μM Cu (Cu 6d) or from plants in which the heavy metal was withdrawn after 3 d (Cd 3/3 and Cu 3/3). Results are expressed as the percentage of enzyme activity (PM oxidoreductase) determined in the PM of control roots; 100% PM oxidoreductase activity was 975 μmol $K_3Fe(CN)_6$ mg^{-1} protein min^{-1}. The values presented are means ±SD of four independent experiments each performed in triplicate.

Table 1. Effect of heavy metals on H_2O_2 level, and catalase and APX activity

H_2O_2 level and catalase and APX activities were determined as described in Materials and methods. Results are means ±SD of three independent experiments each carried out in triplicate, and are given as the percentage of the control (±SD).

Heavy metal	H_2O level	Catalase	APX
Control	100	100	100
Cd 6d	105 (±4)	81 (±12)	98 (±5)
Cd 3/3	98 (±6)	93 (±8)	94 (±3)
Cu 6d	35 (±11)	147 (±6)	156 (±11)
Cu 3/3	30 (±7)	138 (±7)	138 (±7)

Cd did not significantly change these parameters, but treatment of cucumber seedlings with Cu changed both the accumulation of H_2O_2 and the activity of catalase and APX (Table 1). The level of H_2O_2 was lower than in the roots of control plants, while the activity of the investigated antioxidant enzymes, which are efficient scavengers of H_2O_2, was stimulated by Cu.

As the activity of H^+-ATPase can also be altered rapidly through reversible protein phosphorylation, Western blotting with an anti-phosphothreonine antibody was performed (Fig. 5a). To verify the specificity of the antibody against phosphoamino acids, a control with antibody 46E5B11D against PM H^+-ATPase was carried out (Fig. 5b). In plants treated with Cd or Cu, the level of phosphorylation was higher than in the controls and was similar to that in fusicoccin-treated plants. Fusicoccin is a fungal toxin that induces phosphorylation of the penultimate threonine of PM H^+-ATPase in plants.

Additionally, to confirm the phosphorylation of PM H^+-ATPase in heavy-metal-treated plants, Western blotting with anti-14-3-3 antibodies was performed (Fig. 6). It was found that the abundance of these proteins was higher in PM from stressed plants than in PM from control plants. The increase in abundance of 14-3-3 proteins was more visible in 3/3 plants.

HSPs can be considered as markers of stress in plants. The level of four HSPs was investigated in the roots of cucumber seedlings treated with heavy metals. This research was performed by Western blotting using antibodies against HSPs 17.6, 17.7, 70, and 101. Cd and Cu significantly elevated the level of HSPs 17.6, 17.7, and 101 compared with the control plants, where the level of these proteins was almost undetectable (Fig. 7). The level of HSP 70 was high in both the control and the heavy-metal-treated roots, but under stress conditions the abundance of this protein was higher (Fig. 7).

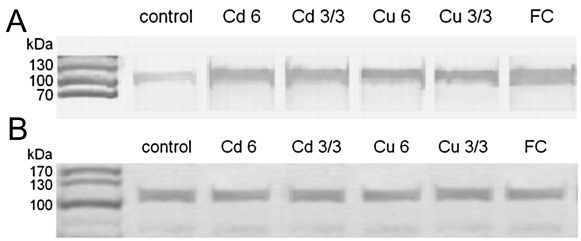

Fig. 5. Western blot of PM protein obtained from control plants, from plants treated for 6 d with 10 μM Cd (Cd 6) or Cu (Cd 6), or from plants in which the heavy metal was withdrawn after 3 d (Cd 3/3 and Cu 3/3), with antibodies raised against phosphothreonine (A) and PM H^+-ATPase (B). FC indicates plants treated with fusicoccin, which induces phosphorylation of the penultimate threonine of PM H^+-ATPase. The positions of molecular mass standards are indicated on the left. The blots are representative of results obtained in three independent experiments.

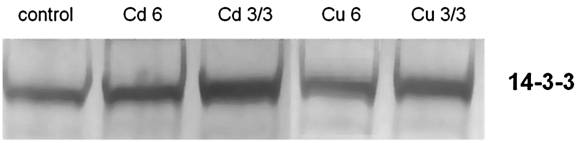

Fig. 6. Western blot of PM protein obtained from control plants, from plants treated for 6 d with 10 μM Cd (Cd 6) or Cu (Cd 6), or from plants in which the heavy metal was withdrawn after 3 d (Cd 3/3 and Cu 3/3), with antibodies raised against 14-3-3 proteins. The blot is representative of results obtained in four independent experiments.

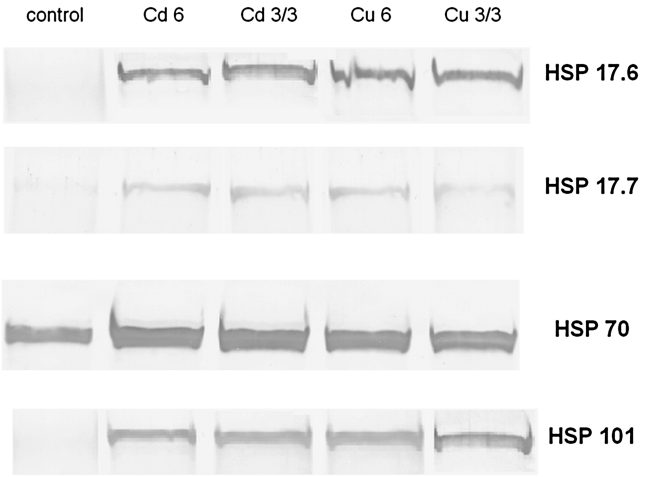

Fig. 7. Western blot of PM protein obtained from control plants, from plants treated for 6 d with 10 μM Cd (Cd 6) or Cu (Cd 6), or from plants in which the heavy metal was withdrawn after 3 d (Cd 3/3 and Cu 3/3), with antibodies raised against HSPs 17.6, 17.7, 70, and 101. The blot is representative of results obtained in three independent experiments.

Discussion

The plant PM may be regarded as the first structure that is a target for heavy-metal toxicity. An increase in permeability related to membrane damage is observed in plants that have been subjected to heavy-metal stress (Fodor *et al.*, 1995). Membrane reconstruction, which consists of stabilization of proteins and membranes, is very important in adaptation of plants to heavy metals. Maintaining proteins in their functional conformation and preventing the aggregation of non-native proteins is important for survival under stress conditions, and HSPs play a major role in this stage. HSPs are key components contributing to cellular homeostasis in cells under both optimal and adverse growth conditions. Here, it was demonstrated that, in plants treated for 6 d with 10 μM Cd or Cu and in plants transferred after 3 d treatment with heavy metals to nutrient solutions without Cd or Cu for another 3 d, the levels of HSPs 17.6, 17.7, 70, and 101 increased. HSPs are known to be expressed in plants when they are exposed to high temperatures as well as in response to other abiotic stresses, such as salinity, water stress, cold, oxidative stress, and heavy-metal stress (reviewed by Sun *et al.*, 2002; Wang *et al.*, 2004). Moreover, it was shown that HSP 101 is produced during the post-stress phase, implying a role in the recovery of normal conditions after stress (Agarwal *et al.*, 2003). The results presented in this work suggest that in Cd- or Cu-stressed cucumber plants, there appeared to be repair processes that enabled the plants to survive these adverse conditions. The plants initiated an adaptive state, and increased PM H^+-ATPase activity could further enhance the repair processes in heavy-metal-stressed plants. Membrane damage, caused by heavy metals, leads to cell ionic disturbance. Maintaining ionic equilibrium and replenishing lost substances is also important under such conditions. H^+-ATPase is the only proton pump operating in PMs, playing a central function in the regulation of ion homeostasis. Maintaining active transport of ions and organic compounds across the PM requires increased generation of a proton gradient by PM H^+-ATPase. Therefore, the role of PM H^+-ATPase is important in plants treated with heavy metals. There are a few reports that indicate changes in PM H^+-ATPase activity as a result of Cd or Cu. In our previous investigation, we found that, in cucumber seedling roots, brief treatment of the plants with Cd and Cu led to inhibition of PM H^+-ATPase activity (Janicka-Russak *et al.*, 2008). A similar inhibitory effect of short-term treatment of plants with Cd and/or Cu on the activity of H^+-ATPase in roots of different plants was observed by Kennedy and Gonsalves (1989), Fodor *et al.* (1995), and Burzyński and Kolano (2003). Moreover, Astolfi *et al.* (2003, 2005) reported inhibition of activity of PM H^+-ATPase in oat and maize roots after long-term treatment (7 and 21 d) with Cd. However, Burzyński and Kolano (2003) and Ros *et al.* (1992*ab*) showed that longer treatment time of plants with Cd or Cu led to increased activity of the enzyme in cucumber, maize, and rice. Our results confirm this observation. In the present work, it was found that, in plants treated for 6 d with heavy metals, or in plants where Cd or Cu treatment lasted only 3 d and plants were then transferred to nutrient solution without heavy metals for the next 3 d, the activity of PM H^+-ATPase increased significantly. Notably pronounced was the increase in

proton transport across the PM observed for 3/3 plants. Withdrawal of the adverse factor intensified the PM proton pump activity. This result clearly indicated that, in post-stressed (3/3) plants, heavy metals could enhance coupling between ATP hydrolysis and proton transport at the PM. Thus, regulation of the activity of plant PM H^+-ATPase could involve modulation of the H^+/ATP coupling ratio. Kerkeb *et al.* (2002) also observed an enhanced H^+/ATP coupling ratio of PM H^+-ATPase in tomato cells under osmotic stress. The results presented in this paper show that, under heavy-metal stress, cucumber cells increase PM H^+-ATPase activity, but in post-stressed plants, withdrawal of the heavy metals from the environment contributes to higher H^+ pumping activity through the PM H^+-ATPase with only light stimulation in ATP hydrolysis. Moreover, the increase in H^+-ATPase activity in post-stressed plants might be related to removal of the inhibitory action of the heavy metal. According to Serrano (1990), Cu^{2+} ions are one of the most powerful inhibitors of PM H^+-ATPase.

As it is known that the activity of PM H^+-ATPase could be regulated at the expression level of genes as well as the enzyme protein level, it seemed interesting to determine the mechanism of stimulation of these enzyme genes in plants treated with heavy metals. The cucumber genome has been sequenced and submitted to GenBank by Huang *et al.* (2009). Based on the genome sequence, the cucumber genome potentially encodes ten isoforms of PM H^+-ATPase. Six of the isoforms are expressed in cucumber roots: *CsHA2*, *CsHA3*, *CsHA4*, *CsHA8*, *CsHA9*, and *CsHA10* (A. Wdowikowska, unpublished data). The relative expression of PM H^+-ATPase genes in cucumber roots was affected by Cd treatment. The transcript levels of *CsHA2*, *CsHA3*, *CsHA4*, *CsHA8*, and *CsHA9* in roots treated with Cd was higher than in the control plants. In contrast, alteration of PM proton pump activity in cucumber roots stressed with Cu appeared not to involve gene expression levels, because the transcript levels were unchanged in all of the enzyme isoforms expressed in cucumber roots. These results are in agreement with findings showing that Cd is an effective modulator of plant gene expression (Fusco *et al.*, 2005; Kovalchuk *et al.*, 2005). It was shown that, in mustard greens (*Brassica juncea*), 73 transcript-derived fragments were identified as Cd responsive (Fusco *et al.*, 2005).

It was shown here that the activity of PM H^+-ATPase directly depends on the activity of NAD(P)H-dependent oxidoreductase. This enzyme does not participate in proton transport directly but contributes to the activation of PM H^+-ATPase (Kłobus, 1995). PM oxidoreductase, by transporting electrons across the PM, simultaneously acidifies the cytoplasm (Lüthje *et al.*, 1997). It is known that a low pH in the cytoplasm and membrane depolarization stimulate the function of PM H^+-ATPase. Thus, stimulation of PM H^+-ATPase activity under Cu stress may result from an increase in the activity of PM oxidoreductase under such conditions. However, it is important to remember that there is no clear opinion about the impact of oxidoreductases on the important changes in membrane potential. Nevertheless, a significantly higher reduction in ferricyanide by NADH in PM vesicles isolated from Cu-treated plants was observed. The impact of Cd and Cu on PM H^+-ATPase activity was similar. However, there is an important difference between these two metals: Cu can change the redox properties of the cell, while Cd is classified as a non-redox-reactive heavy metal (Schützendübel and Polle, 2002). Under the conditions of Cd stress presented in this work, Cd did not affect the H_2O_2 content or the antioxidant enzyme activities in the cucumber roots, while Cu significantly changed these parameters. It was found that 6 d treatment of seedlings with Cu led to a visibly decreased accumulation of H_2O_2. The level of H_2O_2 correlated with catalase and APX activity, and both of these enzymes are efficient scavengers of H_2O_2. In plants treated with Cu, the activity of these antioxidant enzymes increased and the level of H_2O_2 decreased. In contrast to the results obtained in this study, Cd has been found to produce oxidative stress (Shaw, 1995; Cho and Seo, 2004), but, in contrast to other heavy metals such as Cu, it does not seem to act directly on the production of reactive oxygen species via the Fenton and/or Haber Weiss reaction (Sanita di Toppi and Gabbrielli, 1999; Benavides *et al.*, 2005). Varying responses to Cd-induced oxidative stress are probably related to the levels of Cd supplied.

The increase in PM H^+-ATPase activity in plants treated with Cd and Cu was correlated with higher levels of phosphorylation of this enzyme obtained from heavy-metal-stressed plants. Based on these results, it can be assumed that the changes in activity of H^+-ATPase under conditions of heavy-metal stress are due to reversible protein phosphorylation, which allows the binding of 14-3-3 protein. Additionally, it was shown that both Cd and Cu contributed to the increase in levels of 14-3-3 proteins in the PM. This result further confirms the role of fast post-translational modification of ATPases under heavy-metal stress. The highest level of 14-3-3 proteins was detected in root tissues of post-stressed 3/3 plants. In the PM from these plants, the highest level of ATP-dependent proton transport and enhanced H^+/ATP coupling ratio were also recorded. Kerkeb *et al.* (2002) showed that the formation of a 14-3-3/H^+-ATPase complex leads to an enhanced H^+/ATP coupling ratio of the PM proton pump in tomato cells under osmotic shock. It could be speculated that, in plants where heavy metals have been withdrawn from the environment, the modification of PM H^+-ATPase by phosphorylation and formation of a 14-3-3/H^+-ATPase complex was highest, leading to the creation of a large gradient of protons across the membrane. In turn, this gradient could be used by the plant to replenish the loss of essential substances in repair processes, or, as seems to be more important, to remove excess toxic ions from the cytoplasm to the outside of cells on the basis of proton/heavy-metal antiport. In plants, transporters of the cation diffusion facilitator (CDF) family seem to mediate such cytoplasmic efflux of heavy-metal cations. These proteins have been named metal tolerance proteins (MTPs). Among this family, at least one, MTP8, could participate in the efflux of Cd and Cu from the cytosol, as it has been shown that

MTP8 is upregulated in response to Cd and Cu in roots of *Arabidopsis halleri* (Talke *et al.*, 2006).

In conclusion, our results may indicate participation of the PM proton pump in repair processes soon after the abiotic stress is withdrawn or when the stress factor acts on the plant for a long time. Heavy-metal stress leads to disturbances in the structure and functioning of the PM and accumulation of toxic metals in the cytoplasm. Support of active transport of ions and organic compounds through the PM requires increased generation of a proton gradient by H^+-ATPase. The results presented in this paper show increased PM H^+-ATPase activity by Cd and Cu in plants stressed for 6 d. Both metals led to post-translational modification by reversible enzyme phosphorylation. Additionally, the data demonstrated that, in Cd-stressed plants, stimulation of the PM proton pump could be explained by high expression of *CsHA* genes.

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