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ORIGINAL ARTICLE

Cadmium and zinc activate adaptive mechanisms in *Nicotiana tabacum* similar to those observed in metal tolerant plants

Rosario Vera-Estrella[1] · María F. Gómez-Méndez[1] · Julio C. Amezcua-Romero[1,2] · Bronwyn J. Barkla[3] · Paul Rosas-Santiago[1] · Omar Pantoja[1]

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Abstract

Main conclusion **Tobacco germinated and grew in the presence of high concentrations of cadmium and zinc without toxic symptoms. Evidence suggests that these ions are sequestered into the vacuole by heavy metal/H^+ exchanger mechanisms.**

Heavy metal hyperaccumulation and hypertolerance are traits shared by a small set of plants which show specialized physiological and molecular adaptations allowing them to accumulate and sequester toxic metal ions. *Nicotiana tabacum* was used to test its potential as a metal-accumulator in a glass house experiment. Seed germination was not affected in the presence of increasing concentrations of zinc and cadmium. Juvenile and adult plants could concentrate $CdCl_2$ and $ZnSO_4$ to levels exceeding those in the hydroponic growth medium and maintained or increased their leaf dry weight when treated with 0.5- or 1-mM $CdCl_2$ or 1-mM $ZnSO_4$ for 5 days. Accumulation of heavy metals did not affect the chlorophyll and carotenoid levels, while variable effects were observed in cell sap osmolarity. Heavy metal-dependent H^+ transport across the vacuole membrane was monitored using quinacrine fluorescence quenching. Cadmium- or zinc-dependent fluorescence recovery revealed that increasing concentrations of heavy metals stimulated the activities of the tonoplast Cd^{2+} or Zn^{2+}/H^+ exchangers. Immunodetection of the V-ATPase subunits showed that the increased proton transport by zinc was not due to changes in protein amount. MTP1 and MTP4 immunodetection and semiquantitative RT-PCR of *NtMTP1*, *NtNRAMP1,* and *NtZIP1* helped to identify the genes that are likely involved in sequestration of cadmium and zinc in the leaf and root tissue. Finally, we demonstrated that cadmium and zinc treatments induced an accumulation of zinc in leaf tissues. This study shows that *N. tabacum* possesses a hyperaccumulation response, and thus could be used for phytoremediation purposes.

Keywords Cadmium · Hyperaccumulation · Metal tolerance · Metal transporters · Phytoremediation · Zinc

Abbreviations

MTP	Metal tolerance protein
NRAMP	Natural resistance-associated macrophage protein
ZIP	ZRT, IRT-like protein family

Introduction

High and rapid increases in urbanization and industrialization activities have augmented the quantity of pollutants in the environment. Cadmium and zinc are two of the most common toxic heavy metals released into the environment (Kabir et al. 2012; Wiseman et al. 2013). Zinc is a micronutrient essential for many physiological and

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✉ Rosario Vera-Estrella
rosario@ibt.unam.mx

[1] Instituto de Biotecnología, UNAM, A.P. 510-3, Colonia Miraval, 62250 Cuernavaca, Morelos, Mexico

[2] Escuela Nacional de Estudios Superiores, Unidad León, UNAM, Blvd. UNAM 2011, Comunidad de los Tepetates, 37684 León, Guanajuato, Mexico

[3] Southern Cross Plant Science, Southern Cross University, Lismore, NSW 2480, Australia

metabolic processes in plants and animals, but becomes toxic when it accumulates to high levels (reviewed in Guerinot and Eide 1999; Anjum et al. 2015). In plants, high concentrations of zinc compete with other biologically important ions like Fe^{2+}, inducing iron deficiency leading to reduced chlorophyll synthesis, and a general reduction in plant growth (Broadley et al. 2007; Yrela 2013). Likewise, high levels of cadmium are toxic and limit yield of most plants. Accumulation of cadmium in plant cells induces lipid peroxidation, inactivates enzymes involved in CO_2 fixation, and disturbs nitrogen and sulfur metabolism causing general tissue damage, stunted growth, chlorosis, leaf epinasty, alteration of chloroplast structure, and inhibition of both pollen germination and tube growth (reviewed in Gallego et al. 2012).

In nature, some plants have developed special mechanisms for metal ion homeostasis and detoxification, which allow them to tolerate toxic levels of specific metals in their aboveground tissue. Among these plants are *Noccaea caerulescens* (cadmium and zinc), *N. goesingense* (cadmium, zinc and nickel), *N. brachypetalum* (zinc, cadmium and lead), *Arabidopsis halleri* (zinc, cadmium and lead), *Pandiaka metallorum* (cobalt), *Eugenia clusioides* (manganese), *Pearsonia matallifera* (niquel), *Perus vittata* (arsenic), and *Astragalus bisulcatus* (selenium and sulfur) (Krämer 2010; Maestri et al. 2010). These metal hyperaccumulator plants are not only able to grow and develop in heavy metal contaminated soils without symptoms of toxicity, but also to accumulate more than 100 to 1000 times the concentration of such metals in their shoots when compared to non-tolerant plant species (Assunção et al. 2003; Krämer 2010; Maestri et al. 2010; Rascio and Navari-Izzo 2011).

Metal transporters in plant cell membranes play a major role in heavy metal hyperaccumulation and tolerance. Metal transporters are encoded by multiple genes including the natural resistance-associated macrophage protein (NRAMP) family contributing to iron and manganese homeostasis (Curie et al. 2000; Thomine et al. 2000), the cation exchanger (CAX) family involved in Ca^{2+}/H^+, Cd^+/H^+, or Mn^{2+}/H^+ exchange (Hirschi et al. 2000), the Irt-like protein (ZIP) family involved in transporting a variety of divalent cations including zinc, iron, manganese and cadmium (Guerinot 2000), and the cation efflux (CDF) family including members from *Arabidopsis thaliana* involved in zinc homeostasis (van der Zaal et al. 1999). These proteins have been grouped into Zn-CDF, Fe/Zn-CDF, and Mn-CDF according to their major metal substrate (Montanini et al. 2007; Gustin et al. 2011). In plants, all CDF members are named metal tolerance proteins—MTPs (Mäser et al. 2001; Gustin et al. 2011). Plant MTPs have been divided into seven groups, based on MTP sequences from *A. thaliana* (Gustin et al. 2011). Physiological and biochemical characterization of members from Group 1 has demonstrated that this family is involved in Zn and Co transport (Kobae et al. 2004; Arrivault et al. 2006; Desbrosses-Fonrouge et al. 2005); however, using yeast complementation experiments, *Os*MTP1 was characterized to transport not only Zn and Co but also Fe and Cd with lower affinity (Menguer et al. 2013). Reconstitution of *At*MTP1 into proteoliposomes facilitated zinc transport (Bloß et al. 2002), and a mutant (*mtp1*) displayed enhanced sensitivity to high zinc concentrations (Kobae et al. 2004). Genes encoding MTPs involved in zinc tolerance and homeostasis have been also reported in diverse plant species like *Medicago truncatula* (*MtMTP1*; Chen et al. 2009), *Oryza sativa* (*OsMTP1*; Yuan et al. 2012), *A. halleri* (*AhMTP1*; Dräger et al. 2004), *N. goesingense* and *N. montanum* (*NgMTP1* and *NmMTP1*; Kim et al. 2004), *Populus trichocarpa* (*PtMTP1*; Montanini et al. 2007), *Sedum aldredii* (*SaMTP1*; Zhang et al. 2011), *Cucumis sativus* (*CsMTP1* and *CsMTP4*; Migocka et al. 2015), and *Brassica juncea* (*BjCET3* and *BjCET4*; Lang et al. 2011).

It has long been known that tobacco plants can absorb trace elements from the soil and to accumulate them in the leaves with different cultivars showing diverse uptake efficiencies (Clarke and Brennan 1989; Vasiliadou and Dordas 2009). This ability has characterized the tobacco plant as an efficient cadmium accumulator and, alarmingly, studies of cigarettes produced from tobacco leaves cultivated around the world have measured large quantities of this heavy metal (Musharraf et al. 2012). Despite the inherent ability of tobacco plants to take up and accumulate heavy metals, little is known about the biochemical and molecular mechanisms that allow it to grow and develop on heavy metal contaminated soils. The present study investigated the ability of tobacco plants to tolerate Cd and Zn and highlighted the transport mechanisms that may be involved in the cellular accumulation of these two heavy metals.

Materials and methods

Plant materials and growth conditions

Nicotiana tabacum L. plants were grown from seeds (cultivar KY14 were a gift from Kendall Hirschi, Baylor College of Medicine, Houston, TX, USA) in soil (Metro mix 510, Scotts, Marylsville, OH, USA, supplemented with perlite 1:3) in a propagation tray with daily watering using tap water. Three weeks following germination, individual seedlings were transferred to 15-L black plastic containers (40 cm × 25 cm × 15 cm) filled with 14 L of ½ strength Hoagland's medium prepared in tap water (Hoagland and Arnon 1938). The solution was changed weekly, and on the

third week, solution was changed to full strength Hoagland's. Metal treatment was initiated on juvenile plants (6-week-old; 0.5-mM $CdCl_2$ or 1-mM $ZnSO_4$) or adult plants (10-week-old; 1-mM $CdCl_2$ or $ZnSO_4$) by inclusion of the indicated metal in the Hoagland's medium. Plants were grown in a glasshouse under natural irradiation and photoperiod. Minimum temperatures ranged from 20 to 24 °C and maximum temperature was maintained at 25 °C.

To measure the ability of the *N. tabacum* to germinate in the presence of either $CdCl_2$ or $ZnSO_4$, the seeds were surface-sterilized by immersion in 100% (v/v) ethanol for 1 min followed by gentle shaking in a 5% sodium hypochlorite solution for 10 min and then by five rinses with sterile MilliQ water. The sterile seeds were placed onto sterile culture plates containing ½ strength Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with different concentrations of $CdCl_2$ or $ZnSO_4$ at 25 °C and 1.5% bacteriological agar and placed in a growth room at 25 °C with a photoperiod of 16-h light and 8-h dark.

Dry weight (DW) measurements were obtained following drying the plant material at 80 °C for 14 h.

Membrane isolation and enrichment

Tonoplast used for hydrolytic, H^+-transport assays, and Western blot analysis was isolated by sucrose (Suc) density gradient centrifugation at 4 °C. Leaves from juvenile or adult *N. tabacum* plants were harvested and sliced into small pieces. Leaf material (30-g fresh weight from a mixed central leaf blade) was placed into 300 mL of ice cold homogenization medium (400-mM mannitol, 10% (w/v) glycerol, 5% (w/v) PVP-10, 0.5% (w/v) BSA, 1-mM PMSF, 30-mM Tris, 2-mM DTT, 5-mM EGTA, 5 mM $MgSO_4$, 0.5-mM butylated hydroxytoluene, 0.25-mM dibucaine, 1-mM benzamidine, 26-mM K^+-metabisulfite, and 5% (w/v) insoluble-PVP adjusted to pH 8.0 with H_2SO_4). Leaf tissue was homogenized in a commercial blender, filtered through four layers of cheesecloth, and centrifuged at 10,000*g* (20 min at 4 °C) using a JA25.5 rotor (Beckman) in a super-speed centrifuge (Avanti J301, Beckman). Pellets were discarded and the supernatants were centrifuged at 80,000*g* (50 min at 4 °C) using a fixed angle rotor (45 Ti, Beckman) in an ultracentrifuge (L8-M, Beckman). The supernatant was aspirated, and the microsomal pellet was resuspended and homogenized in suspension medium (400-mM mannitol, 10% (w/v) glycerol, 6-mM Tris/Mes pH 8.0, and 2-mM DTT). The microsomal suspension was then layered onto a discontinuous Suc gradient consisting of a top layer of 9 mL of 16% (w/v) Suc, over 9 mL of 34% (w/v) Suc, on a cushion of 9 mL of 38% (w/v) Suc. Gradients were centrifuged at 100,000*g* (2 h at 4 °C) using an SW 28 swinging bucket rotor in a Beckman L8-M ultracentrifuge. On a discontinuous Suc gradient, tonoplast from *N. tabacum* separates at the 0/16% Suc interface. Bands from the gradient were collected, diluted in suspension medium and re-centrifuged at 80,000*g* using a 55.2 Ti rotor (Beckman) in an L8-M ultracentrifuge (Beckman) to concentrate. The resuspended pellets were collected, frozen in liquid N_2, and stored at −80 °C.

Protein determination

The protein content of isolated and enriched tonoplast was measured by a modification of the dye-binding assay (Bradford 1976) in which membrane protein was partially solubilized with 0.5% (v/v) Triton X-100 for 5 min before the addition of the dye reagent concentrate (Bio-Rad). BSA was employed as the protein standard.

H^+-transport assays and hydrolytic activity

Tonoplast V-ATPase and V-PPase hydrolytic activities were measured by the release of Pi according to the method of Ames (1966), as previously described (Vera-Estrella et al. 1994; Belogurov and Lahti 2002), using isolated membrane fractions. The values are presented as μmol Pi released mg^{-1} membrane protein h^{-1}. The enrichment of tonoplast vesicles was estimated by measuring the degree of inhibition of ATPase hydrolytic activity by 50-mM KNO_3 or 1-mM sodium vanadate (Na_3VO_4), to estimate the contribution of the vacuolar type V-ATPase or the plasma membrane type P-ATPase, respectively.

Proton transport activities were measured by following the fluorescence quenching of quinacrine (6-chloro-9-{[4-(diethylamino) -1-methylbutyl] amino}-2-methoxyacridine dihydrochloride) to monitor the formation of inside-acid pH gradient across tonoplast vesicles as previously described (Barkla et al. 1995), with some modifications. Enriched tonoplast vesicles (30 μg of protein) were added to 500 μL of buffer consisting of 250-mM mannitol, 10-mM Tris/Mes (pH 8.0), 50-mM TMA-Cl, 6-mM $MgSO_4$, and 3-mM ATP. Fluorescence quenching was monitored in a thermostated cell at 25 °C using a fluorescence spectrometer (model LS-50, Perkin-Elmer) at excitation and emission wavelengths of 427 and 495 nm, respectively, both with a slit width of 5 nm. For measurements of cadmium- or zinc-dependent dissipation of a preformed, inside-acid pH gradient, the ATP-dependent H^+ transport activity was inhibited by the addition of 200-nM bafilomycin A_1 (Bowman et al. 1988) in 0.001% (v/v) DMSO, 250-mM mannitol, and 10-mM Tris/Mes (pH 8.0). After a constant level of fluorescence was obtained, aliquots of $CdCl_2$ or $ZnSO_4$ (as indicated) were added to the vesicles and the initial rate of cadmium- or zinc-

dependent fluorescence recovery was determined. As shown by Bennett and Spanswick (1983), the rate of fluorescence quench recovery is directly proportional to proton efflux. Thus, the initial rate of fluorescence quenching or recovery represents the initial rates of proton transport.

Total RNA isolation and semiquantitative RT-PCR

Plant tissue (a mixed sample of the central leaf blade or roots) was ground to a powder in liquid nitrogen followed by RNA extraction using Trizol according to the manufacture's procedure (Invitrogen, GE Healthcare). Total RNA was treated with RNase-free DNase (Fermentas, México) for 30 min at 37 °C, and then, 5 µg was used to synthesize the first strand cDNA with the addition of 0.5-µg oligo(dT) and 200 units RevertAid H Minus M-MuLC reverse transcriptase (Fermentas) and the reaction was incubated at 42 °C for 1 h. PCR was performed in a volume of 50 µL containing 1.25 units *Taq* DNA polymerase (Fermentas), 0.2-mM dNTP mix, 2-mM $MgCl_2$, and 0.4-µM gene-specific primers. Primer sequences used for the different transporters are shown in Supplemental Table S1. PCR mixtures were initially denatured at 95 °C for 2 min. Cycling parameters were as follows: denaturing at 94 °C for 1 min, primer annealing at 60 °C (*NtUBQ, NtMTP1*, *NtNRAMP1*, and *NtZIP1*) for 1 min, and extension at 72 °C for 1.5 min. The number of cycles was 31 for *NtUBQ*, *NtNRAMP1*, and *NtMTP1*, and 35 for *NtZIP1*. Finally, all PCR mixtures were extended at 72 °C for 10 min. Primer specificity was determined by sequencing of the PCR amplification products for each gene. PCR products were size separated by electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide. Gene expression was normalized to the expression of *NtUBQ* for each time and treatment.

Primary and secondary antibodies

Antibodies against the *A. thaliana* V-ATPase subunits (VHA-A, 60 kDa, cat. #AS09467; VHA-B, 55 kDa, cat. #AS09503; VHA-c, 16 kDa, cat. #AS09468; VHA-E, 29 kDa, cat. #AS08294), MTP1 (43 kDa, cat. #AS09485), and MTP4 (46 kDa, cat. #AS09474) were purchased from Agrisera (Vännäs, Sweden).

SDS-PAGE and protein immunoblotting

Protein was precipitated by dilution of the samples 50-fold in 1:1 (v/v) ethanol: acetone and incubated overnight at −30 °C following the method of Parry et al. (1989). Samples were then centrifuged at 13,000*g* for 20 min at 4 °C using an F2402 rotor in a GS-15R table-top centrifuge (Beckman). Pellets were air dried, resuspended with Laemmli (1970) sample buffer (2.5% (w/v) SDS final concentration), and heated at 60 °C for 2 min before loading onto 10% (w/v) linear acrylamide mini-gels. After electrophoresis, the gels were prepared for immunoblotting. SDS-PAGE separated proteins were electrophoretically transferred onto nitrocellulose membranes (ECL, GE Healthcare) as previously described (Vera-Estrella et al. 1999). Following transfer, membranes were blocked with TBS (100-mM Tris, 150-mM NaCl) containing 0.02% (w/v) Na-azide, and 5% (w/v) fat-free milk powder (Svelty, Nestlé, México) for 2 h at room temperature. Blocked membranes were incubated for a minimum of 3 h at room temperature with the appropriate primary antibodies, followed by the addition of a 1:5000 dilution of secondary antibodies (goat anti-rabbit) conjugated to horse radish peroxidase (Agrisera). Immunodetection was carried out using the chemiluminescent LuminataTM Crescendo procedure (Millipore). Mean intensity of the immunodetected protein bands was calculated using molecular weight markers as loading control standards (Fermentas). Images were captured using a C-Digit Blot Scanner and the Image Studio Lite Western blot analysis software (Li-Cor, Lincoln, NE, USA).

Osmolarity, zinc and cadmium measurements

Leaves were collected and washed twice with deionized water. Tissue (2 g) cut in small pieces was loaded into a 5-mL syringe containing a 1-cm Whatman No. 1 filter disk. The material was frozen at −30 °C and the cell sap was obtained in thawed samples by centrifugation at 1200*g* for 15 min using an S4180 rotor in a GS-15R table-top centrifuge (Beckman). The osmolarity of the cell sap was measured in 50-µL samples with a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany). Heavy metals were extracted from leaves by microwave digestion (MARSX-CEM; CEM, Matthews, NC, USA) according to the EPA 3051 and measured by flame atomic absorption spectrometry at the Instituto Mexicano de Tecnología del Agua (IMTA, Jiutepec, México).

Chlorophyll and carotenoid measurement

Chlorophyll was measured according to Porra et al. (1989). Leaves from untreated and metal-treated *N. tabacum* plants were collected, washed, cut in small pieces, ground in a mortar with 1 mL of 80% (v/v) acetone, and incubated overnight. Samples were centrifuged at 12,000*g* for 10 min and the supernatant collected. Chlorophyll was measured using a Diode Array spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). Absorbance of the chlorophyll *a* extract was detected at 648 nm, chlorophyll *b* at 664 nm, and carotenoids at 470 nm. The values were calculated

using the molar extinction coefficients 20.2, 8.02, and 9.48 for chlorophyll *a, b,* and carotenoids, respectively.

Confocal microscopy

Fluorescence microscopy was performed using an upright multiphotonic confocal microscopy (Olympus BX61WI) equipped with a 25× W/1.05 objective. Sinpyr-1 fluorescence was visualized by excitation with a multi-line Argon laser at 492 nm and spectral detector set 527 nm for the emission.

Statistical analysis

Origin software, version 8.5.1 for Windows (SPSS Inc.), was used to evaluate statistical significance using Student's *t* test for pairwise comparison and two-way analysis of variance (ANOVA) for determining how a response was affected by two factors. All results are presented as mean ± SE. A probability level of 0.01 (indicated by asterisks, ***) was considered highly significant.

Results

Characterization of *N. tabacum* seed germination and plant growth under the presence of either $CdCl_2$ or $ZnSO_4$

Nicotiana tabacum seeds were germinated on petri plates with different concentrations of $CdCl_2$ or $ZnSO_4$, and seedlings growth was monitored (Fig. 1a–d). A high percentage of seeds germinated in the presence of either $CdCl_2$ (90–96%; Fig. 1b) or $ZnSO_4$ (90–97%; Fig. 1b). However, changes in seedling size (2-week-old seedlings) were observed above 0.25-mM $CdCl_2$ and 0.5-mM $ZnSO_4$ (Fig. 1c, d). In the presence of 0.5-mM $CdCl_2$, a reduced growth of the whole seedlings was observed (Fig. 1a). Tobacco seeds did not germinate in $CdCl_2$ concentrations higher than 0.5-mM. Tobacco seedlings showed a higher tolerance to $ZnSO_4$, as they could grow at concentrations up to 1-mM $ZnSO_4$ without symptoms of toxicity (Fig. 1a); a small but significant reduction in seedling size was observed at 0.5- and 1-mM $ZnSO_4$ (Fig. 1d).

As the response to cadmium and zinc can differ between juvenile and adult plants, the responses of tobacco plants at different growth phases were determined. Juvenile plants (6-week-old) grown in hydroponics and treated with either 0.5-mM $CdCl_2$ or 1-mM $ZnSO_4$ for 5 days (Fig. 2a) did not show any visual damage, but possessed a significantly higher dry weight (1.42- and 1.43-fold; respectively; Fig. 2b). To determine possible subtle changes caused by zinc or cadmium, leaf pigment content was monitored (total chlorophyll and carotenoids). After 5 days of $ZnSO_4$ or $CdCl_2$ treatment, the pigment content was maintained at levels measured in the untreated plants (Fig. 2c). It has been demonstrated that exposure to high concentrations of cadmium and zinc can induce a decrease in leaf water potential (Barceló et al. 1986). To determine the effects of cadmium and zinc treatment on leaf water potential, the osmolality of the cell sap was measured in leaves from plants treated with 0.5-mM $CdCl_2$ or 1-mM $ZnSO_4$. Osmolality increased in leaves of plants treated with $ZnSO_4$, while no difference was detected in leaves from plants treated with $CdCl_2$ (Fig. 2d), in comparison to untreated plants.

When adult tobacco plants (10-week-old) were treated with either 1-mM $CdCl_2$ or 1-mM $ZnSO_4$ for 5 days, they also grew without any visible symptoms of toxicity (Fig. 3a). Like juvenile plants, plant biomass (dry weight; Fig. 3b) or total pigment content (chlorophyll *a* and *b* and carotenoids) did not change upon heavy metal treatment (Fig. 3c). Similarly, the leaf osmolarity from heavy metal-treated adult plants was not significantly different to those values found in leaves from untreated plants (Fig. 3d).

Cadmium and zinc content in leaf tissue of tobacco plants

As shown in Table 1, the content of cadmium and zinc in tobacco leaves changed in response to the presence of the heavy metals in the hydroponic solution. Untreated juvenile and adult plants grown in a hydroponic solution in the absence of added cadmium accumulated 7 and 4.5 mg kg^{-1} cadmium in the leaf tissue, respectively, values that were surprisingly high compared to the physiological non-toxic levels reported for most plants (0.5–2.0 mg kg^{-1}; Baker et al. 2000; Assunção et al. 2003). These data may be explained by the fact that the tap water supply at our location contains between 0.05 and 0.75 mg/L of cadmium (Sánchez Pineda 2012). The concentration of zinc in untreated juvenile and adult plants was 31.5 and 21.4 mg kg^{-1}, respectively, amounts that were within the physiological non-toxic levels reported for most plants (20–150 mg kg^{-1}) and reflects, most likely, the presence of the essential micronutrient zinc in the Hoagland's media. The concentration of cadmium in the leaves of juvenile and adult plants growing in the presence of $CdCl_2$ (0.5 or 1.0 mM, respectively) was 355 and 328 mg kg^{-1}, while the concentration of zinc in the leaves of these cadmium-treated plants was 193 and 73 mg kg^{-1}, respectively. Juvenile and adult plants grown in the presence of 1-mM $ZnSO_4$ accumulated high levels of zinc in their leaves (1525 and 849 mg kg^{-1}, respectively; Table 1), values that are considered toxic for most plants (Broadley et al. 2007). In these zinc-treated juvenile and adult plants, 70 and 26 mg kg^{-1} of cadmium was also measured in leaves,

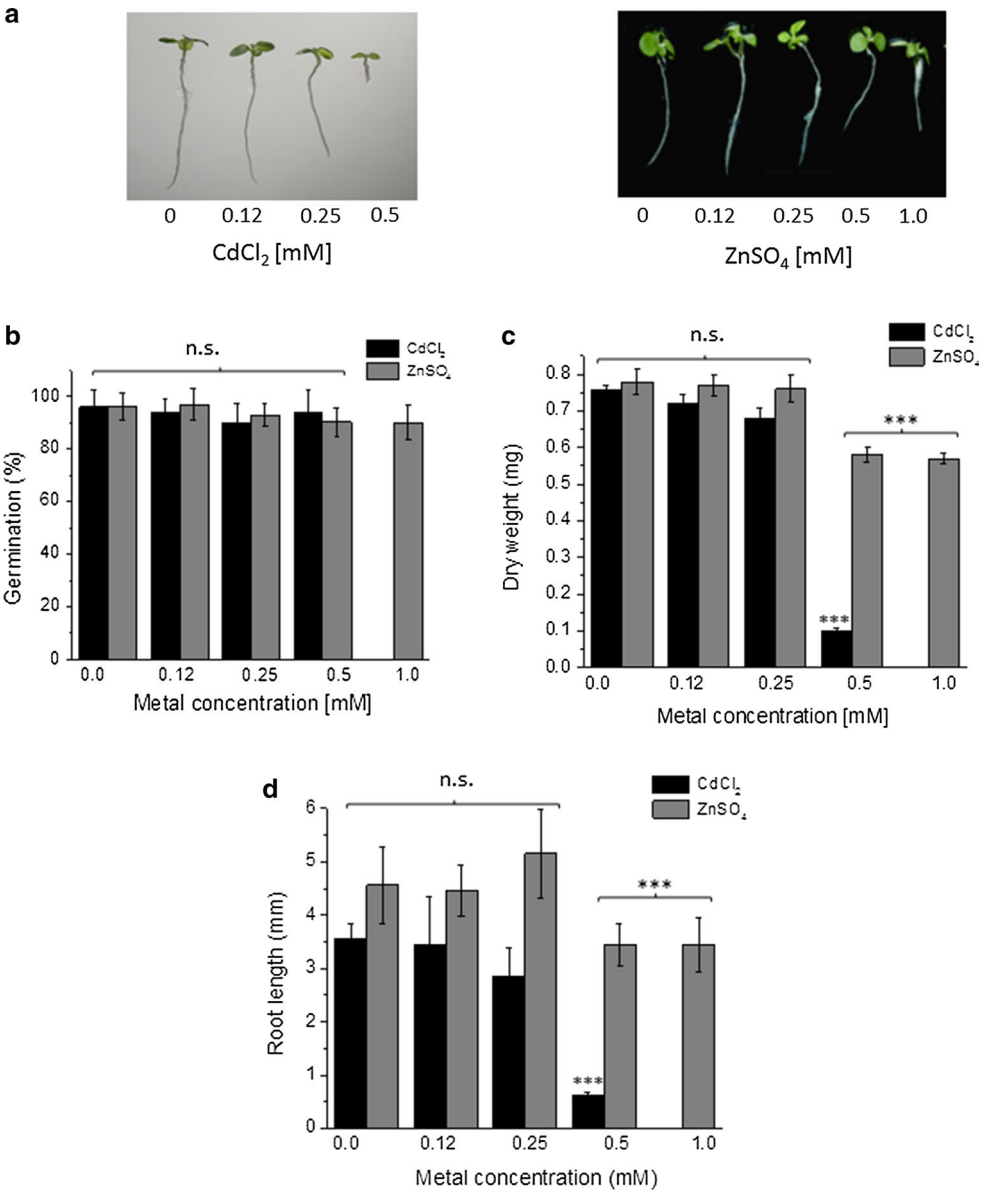

Fig. 1 Effect of different concentrations of $CdCl_2$ or $ZnSO_4$ on tobacco seed germination and seedling growth. Tolerance of young seedlings (2-week-old) derived from seeds sown directly on ½ MS supplemented with different $CdCl_2$ and $ZnSO_4$ concentrations grown in Petri dishes for 14 days. **a** Representative seedlings germinated and grown in different concentrations of $CdCl_2$ or $ZnSO_4$. **b** Seed germination percentages. **c** Dry weight of seedlings. **d** Root length on various concentrations of $CdCl_2$ and $ZnSO_4$. Values are mean ± SE of four independent experiments with three replicates: each replicate represents a Petri dish containing 100 seeds; ($n = 5$; ***significantly different from untreated ($P < 0.01$). *n.s.* not statistically significant

respectively; these data suggest that tobacco leaves can accumulate cadmium and zinc and both metals may stimulate the accumulation of the other ion, a characteristic that has been reported for *T. caerulescens* (Assunção et al. 2003) and *B. juncea* (Salt et al. 1995).

To visualize the accumulation of zinc, leaves of juvenile tobacco plants were immersed in 20-µM Zinpyr-1 (a membrane permeable fluorescent sensor for zinc; Sinclair et al. 2007) and examined under confocal laser microscopy (CLSM). The fluorescence signal of cadmium- and zinc-treated plants was followed in the different leaf tissues. In the untreated plants, a faint autofluorescence signal was detected in the midrib and epidermis (Fig. 4a). In the cadmium- and zinc-treated plants, the signal was highest in the midrib (Fig. 4b, c). In leaves from zinc-treated plants, zinc was accumulated in the midrib, parenchyma, epidermis, and trichomes (Fig. 4c), while in cadmium-treated plants, zinc was also accumulated in the midrib, parenchyma and epidermis but appeared absent from the trichomes (Fig. 4b). These results corroborate findings that cadmium treatment can induce zinc accumulation from the hydroponic solution in leaf tissue of tobacco plants (Fig. 4b).

Effect of cadmium and zinc on tonoplast H^+-V-ATPase, H^+-V-PPase, and metal transport activities

The enrichment of the tonoplast vesicles in the 0/16 fractions was estimated by measuring the H^+-ATPase hydrolytic activity in the presence of inhibitors that differentiate specific types of H^+-ATPases. The V-ATPase is sensitive

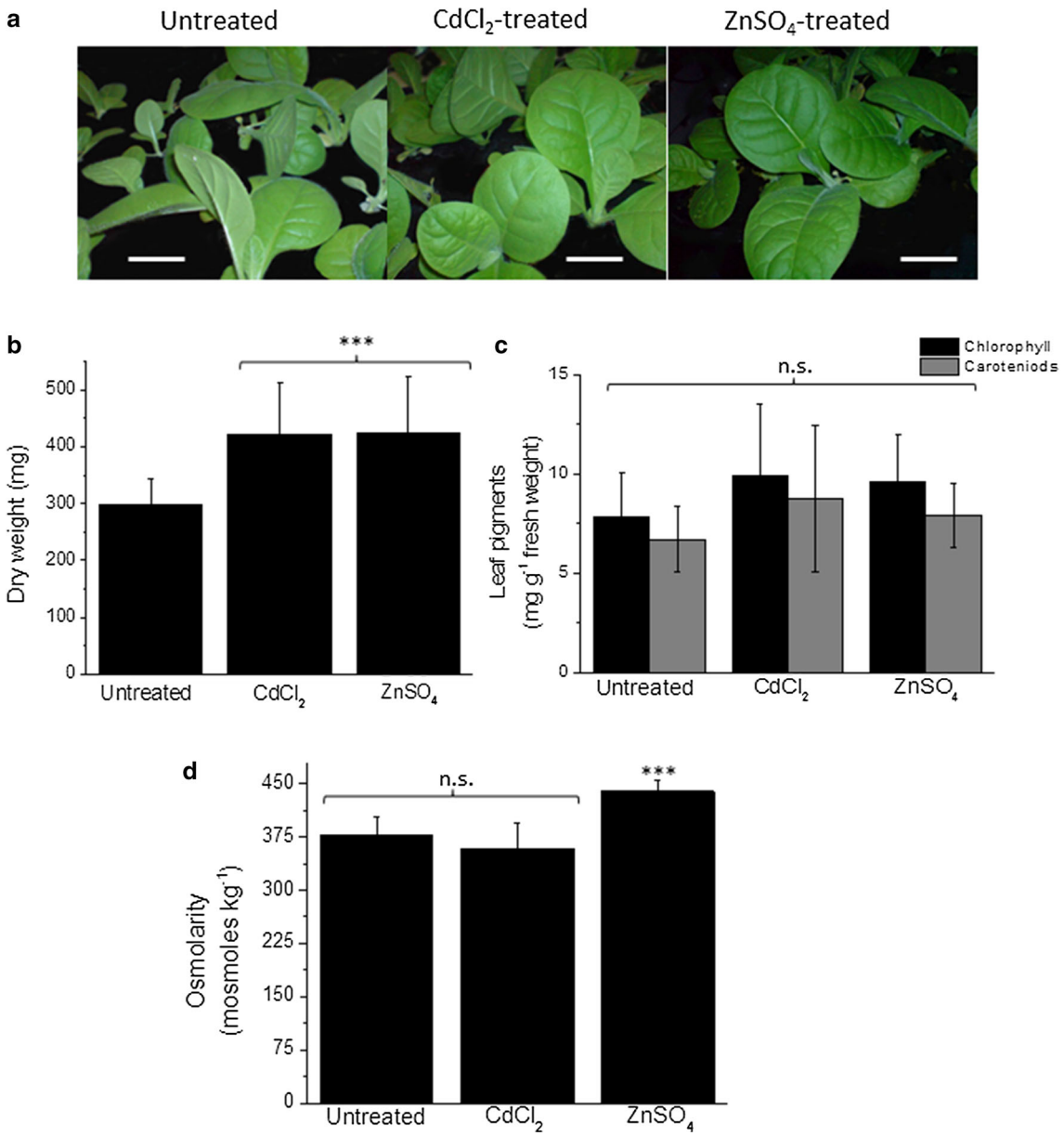

Fig. 2 Cadmium and zinc tolerance of juvenile tobacco plants (6-week-old). Seeds were sown in soil for 14 days, transferred to hydroponic cultures and after two weeks of transfer started treatment with either 0.5-mM $CdCl_2$ or 1-mM $ZnSO_4$ for 5 days. **a** Representative tobacco plants after 5 days of treatment. Pictures are representative of four independent experiments with 30 replicates. **b** Leaf dry weight. **c** Leaf pigment content. **d** Osmolarity from leaves of untreated-, cadmium-, or zinc-treated plants. Data represent the mean of four independent experiments. Each replicate experiment was performed using independent plants. Value significant different (Student's *t* test) from untreated (control) plants is highlighted by *two asterisks* (**) $P < 0.001$. *n.s.* not statistically significant. *Bar* 1 cm

to nitrate and insensitive to vanadate (Na_3VO_4). Vanadate is an inhibitor of the plasma membrane located P-type H^+-ATPase. V-ATPase activity, measured as nitrate-sensitive and vanadate-insensitive H^+-translocation activity, was observed in membrane fractions collected at the 0/16% interface of the discontinuous sucrose gradient. In these fractions, the H^+-ATPase activity after addition of vanadate was like that measured in its absence, while addition of nitrate reduced the H^+-ATPase activity to 19 and 22% in juvenile and adult plants, respectively (Suppl. Table S2). Moreover, the degree of inhibition was the same in $CdCl_2$- or $ZnSO_4$-treated and untreated plants, indicating that the metal treatment did not affect the composition of the isolated membrane fractions (Suppl. Table S2).

Quinacrine fluorescence quenching and recovery was used to measure the proton transport rate of the V-ATPase and the rate of metal-dependent proton transport after the establishment of an inside-acidic pH gradient in tonoplast

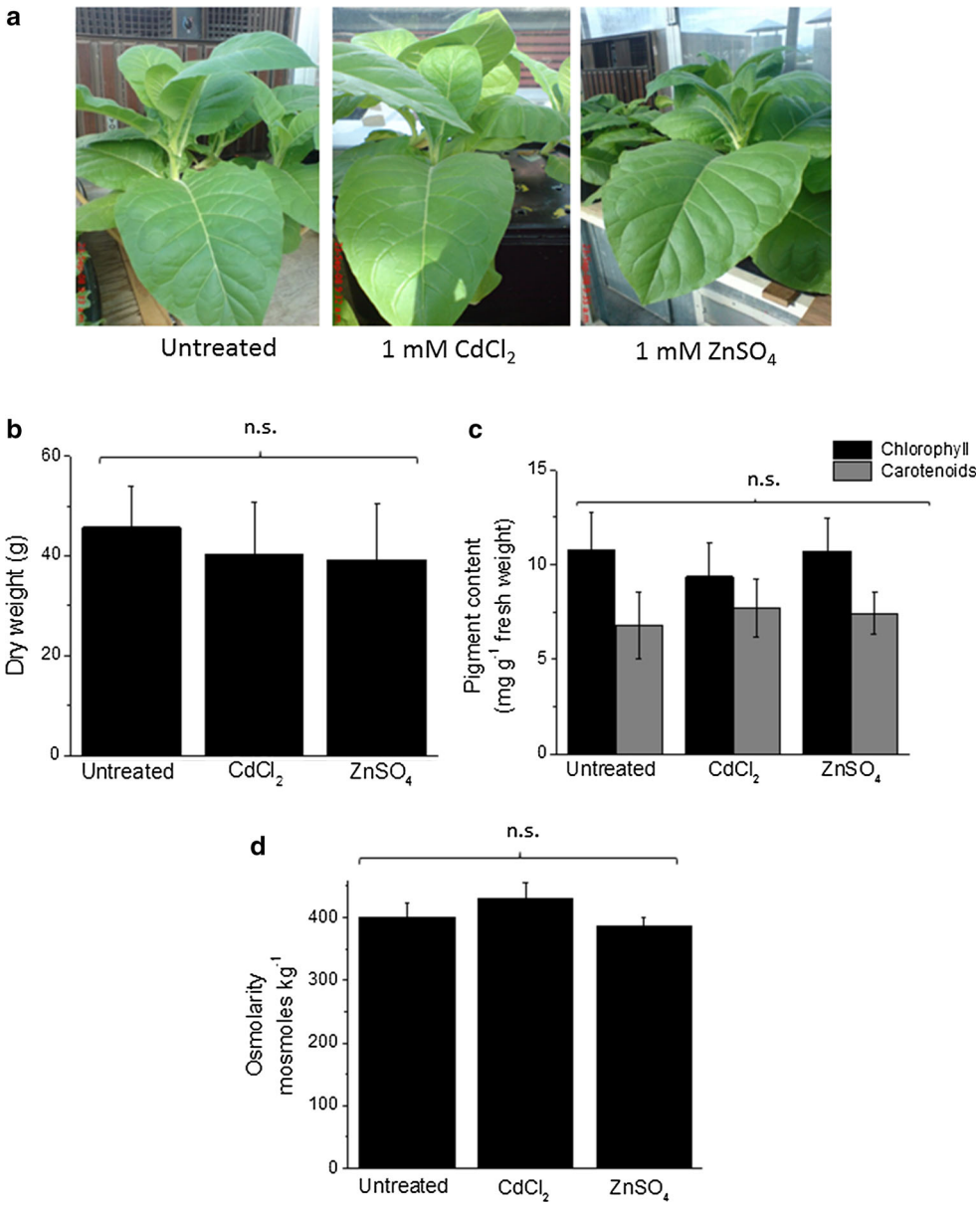

Fig. 3 Cadmium and zinc tolerance of adult tobacco plants. Tolerance of adult plants (10-week-old) grown in hydroponics for four weeks and treated for 5 days with 1.0-mM $ZnSO_4$ or 1.0-mM $CdCl_2$. **a** Representative picture of adult plants after 5 days of treatment. Dry weight (**b**), and total chlorophyll and carotenoid (**c**) content. **d** Osmolarity of leaf tissue from untreated-, and $CdCl_2$- or $ZnSO_4$-treated adult plants. Values are mean ± SE ($n = 30$). Pictures are representative of four independent experiments with six replicates

vesicles following activation of the tonoplast H^+-V-ATPase in sealed and enriched tonoplast vesicles isolated from leaves of juvenile and adult tobacco plants. The total fluorescence quenching in untreated and $CdCl_2$-treated plants was approximately 70% of the initial fluorescence level (Fig. 5a, c), indicating that the final, steady-state pH gradients generated by the pump were not affected by cadmium treatment; however, upon $ZnSO_4$ exposure, a much greater quenching was obtained, indicating that the initial rate of proton transport was higher and the final fluorescence steady-state level was substantially lower. The initial rates of proton transport were calculated from the quenching rates of quinacrine fluorescence taken during the first 40 s following the addition of ATP. Proton transport activity of the V-ATPase of tonoplast vesicles isolated from leaves of juvenile tobacco plants treated with 0.5-mM $CdCl_2$ was not significantly different to that of the untreated plants (Fig. 5a, b); similar results were observed for the hydrolytic activity (Suppl. Table S3). A significant increase in proton transport activity was observed in leaves of $ZnSO_4$-treated juvenile plants (Fig. 5a); while no increase was detected in the hydrolytic activity of the same plants (Suppl. Table S3). In adult plants, $CdCl_2$ treatment did not induce any effect on either the V-ATPase hydrolytic activity (Suppl. Table S3) or the proton transport activity in tobacco leaves (Fig. 5d). In contrast, a reduced hydrolytic activity (1.2-fold) was observed upon $ZnSO_4$ treatment of adult plants, while a 264-fold increase in proton transport activity was detected (Fig. 5c, d; Suppl. Table S3). In plants, two types of V-PPases have been

Table 1 Cadmium and zinc content in leaves from untreated-, $CdCl_2$-, or $ZnSO_4$-treated tobacco plants

Growth phase	Treatment	Cd^{2+} (mg kg^{-1})	Zn^{2+} (mg kg^{-1})
Juvenile plants (6-week-old)	Untreated	7 ± 5.5	31.5 ± 4.1
	0.5-mM $CdCl_2$	355 ± 34.0	193.6 ± 2.5
	1.0-mM $ZnSO_4$	70.4 ± 1.9	1525.6 ± 41.0
Adult plants (10-week-old)	Untreated	4.5 ± 2.8	21.4 ± 5.0
	1.0-mM $CdCl_2$	328 ± 12.1	73 ± 2.2
	1.0-mM $ZnSO_4$	26 ± 3.8	849 ± 103
Physiological (non-toxic)		0.5–2.01[a,c]	20–150[b,c]
Toxic		5–700[c]	>100[c]

The quantity of both metals in plant tissue after 5 days of treatment was determined by atomic absorption spectrometry

[a] Baker et al. 2000; Assunção et al. 2003

[b] Broadley et al. 2007

[c] Pugh et al. 2002

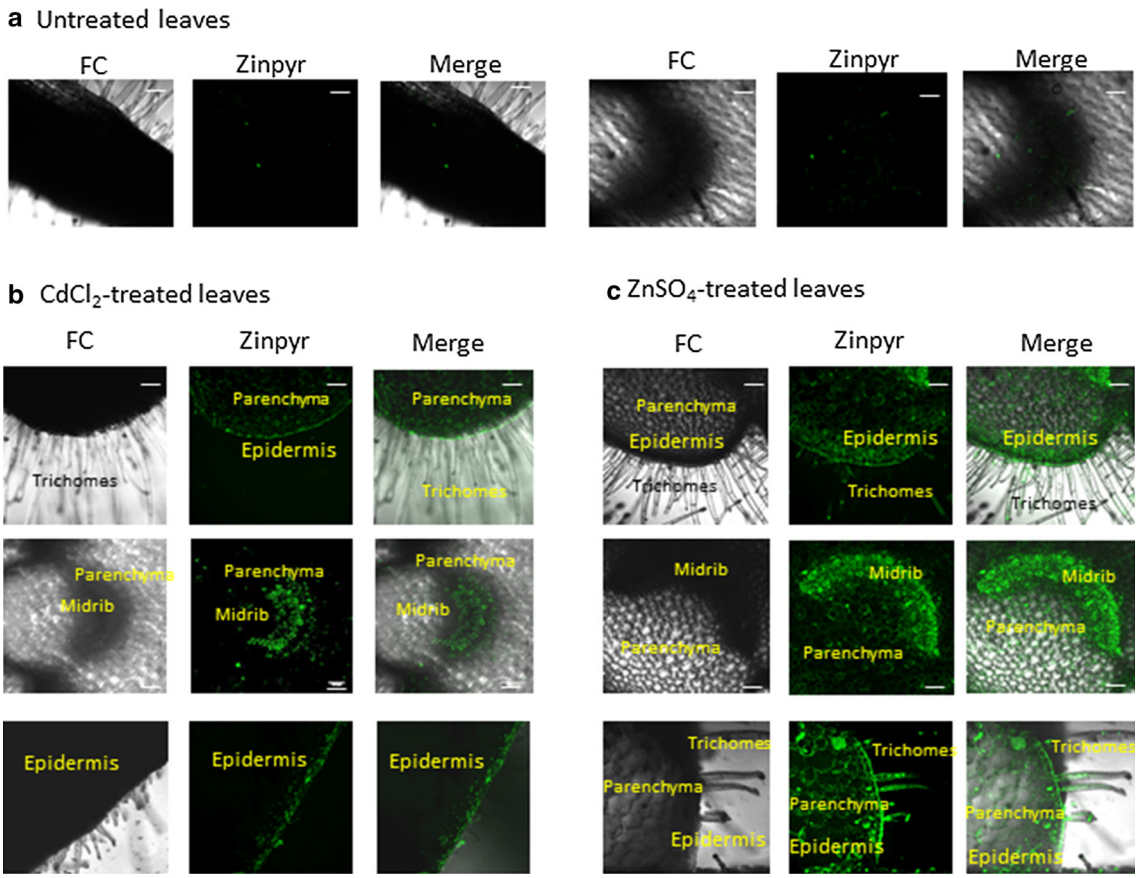

Fig. 4 Detection of zinc levels in different sections of tobacco leaves from juvenile plants. Fluorescence associated with zinc from intact leaves of untreated- (**a**), $CdCl_2$- (**b**), and $ZnSO_4$- (**c**) treated juvenile tobacco plants. Plants were stressed with either 0.5-mM $CdCl_2$ or 1-mM $ZnSO_4$ for 5 days and stained with Zinpyr-1 for 1 h, washed with Ca/Mg PBS solution, and observed by confocal microscopy, employing λ_{ex} 492 nm; λ_{em} 527 nm. *Bar* 127 µm

identified, which differ in their sensitivity to potassium: K^+-stimulated (Type I) and K^+-insensitive (Type II) V-PPases (Belogurov and Lahti 2002). Type I is nearly insensitive to inhibition by Ca^{2+} and depends on K^+ for the activation. Type II is highly influenced by Ca^{2+} and is K^+-insensitive (Belogurov and Lahti 2002). We tested the hypothesis that one of these may be responsible for vacuolar acidification and generation of the proton motif force

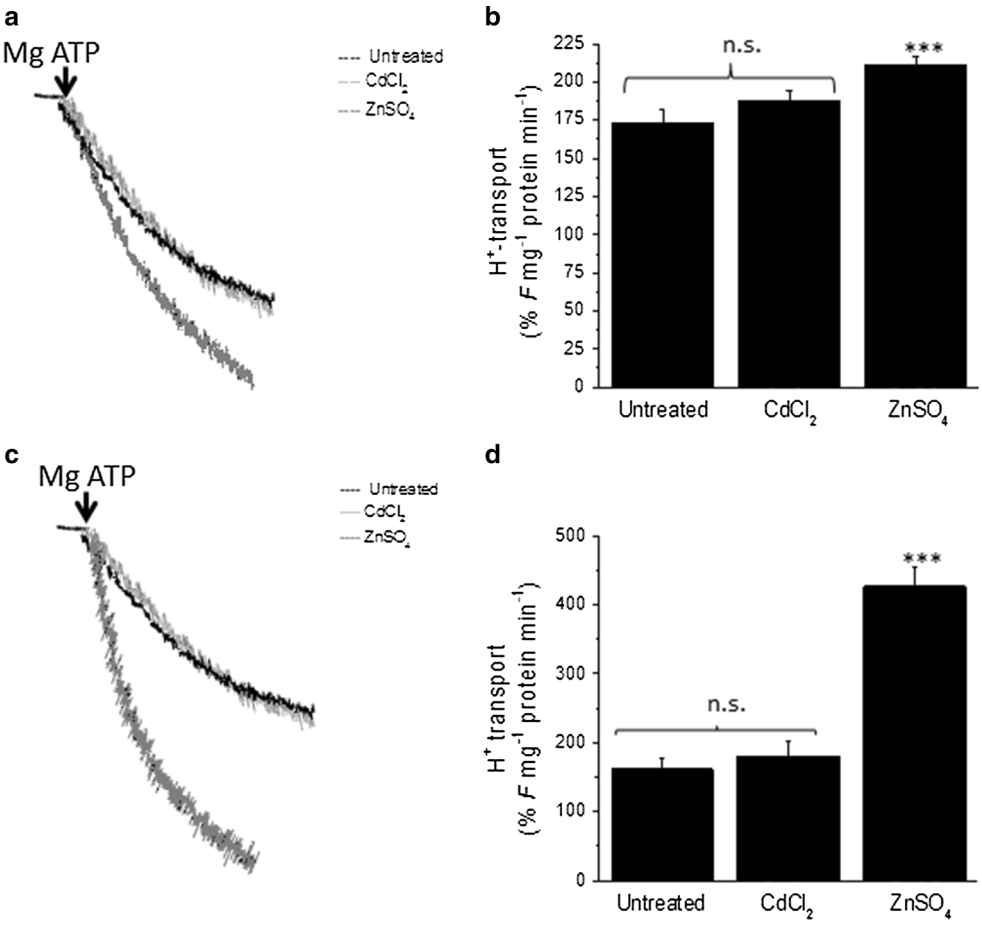

Fig. 5 ATP-dependent H^+-transport into tonoplast vesicles isolate from leaves of 6-week-old (**a**, **b**) or 10-week-old (**c**, **d**) untreated, $CdCl_2$- or $ZnSO_4$-treated tobacco plants. Vesicle acidification was monitored by the quenching of quinacrine fluorescence (see "Materials and methods"); **a** and **c** in the presence of tonoplast vesicles (30 µg of protein) isolated from untreated, and $CdCl_2$- or $ZnSO_4$- treated tobacco plants. The traces in **a** are representative of the results obtained in four independent experiments with each experiment done in tree independent assays and summarized in **b**. Value significant different (Student's *t* test) from untreated (control) plants is highlighted by *three asterisks* (***) $P < 0.001$; *n.s.* not statistically significant. *F* fluorescence intensity

needed for cadmium uptake in tonoplast vesicles isolate from cadmium- or zinc-treated plants. $CdCl_2$ or $ZnSO_4$ treatment resulted in no changes in the hydrolytic activity of the type I V-PPase (Suppl. Table S3); however, measurement of the type II V-PPase showed an increased hydrolytic activity in tonoplast vesicles isolated from both, juvenile and adult plants, under cadmium or zinc treatments (Suppl. Table S3).

Following the generation of a preset, inside-acid pH gradient, by activation and subsequent inhibition of the V-ATPase (Barkla et al. 1995), increasing concentrations of $CdCl_2$ or $ZnSO_4$ were added to the reaction media and the initial rate of quinacrine fluorescence recovery was measured over the first 100 s. The concentration-dependent kinetics of leaf proton-dependent cadmium and zinc uptake into the tonoplast vesicles isolated from untreated, $CdCl_2$- or $ZnSO_4$-treated juvenile, and adult plants was then analyzed by fitting the data to the Michaelis–Menten equation (Van Winkle 1999; Suppl. Table S4).

The initial rate of cadmium exchange in tonoplast vesicles from leaves of untreated juvenile plants (Fig. 6a) increased with increasing concentrations of cadmium reaching saturation above 1-mM $CdCl_2$. In plants grown in the presence of $CdCl_2$, the exchange rate was significantly higher compared to that of untreated plants (Fig. 6a). Cd^{2+}/H^+ exchange was also significantly higher in vesicles isolated from $ZnSO_4$-treated juvenile plants. The Michaelis–Menten kinetics for $CdCl_2$ uptake yielded saturation kinetics for Cd^{2+} influx. The Michaelis constant (K_m) for Cd^{2+}-dependent transport was 0.25, 0.27, and 0.21 mM from untreated, $CdCl_2$-treated and $ZnSO_4$-treated juvenile plants, respectively (Suppl. Table S4), indicating a little change in the affinity for the metal. The estimated V_{max} for Cd^{2+} increased 1.6-fold in tonoplast vesicles isolated from plants exposed to $CdCl_2$ (Suppl. Table S4) and 1.3-fold for plants grown in the presence of $ZnSO_4$ (Suppl. Table S4), suggesting an increase in the transport turnover.

The Zn^{2+}/H^+ exchange activity measured in tonoplast vesicles isolated from 0.5-mM $CdCl_2$-treated juvenile plants was not significantly different to that measured in tonoplast of untreated plants. However, a significant increase was obtained using vesicles isolated from $ZnSO_4$-treated plants (Fig. 6b). The calculated kinetic values for the K_m and V_{max} for Zn^{2+}-dependent H^+ transport indicated that affinity for the metal (K_m) did not significantly change (0.29, 0.25, and 0.24 mM; Suppl. Table S4), while

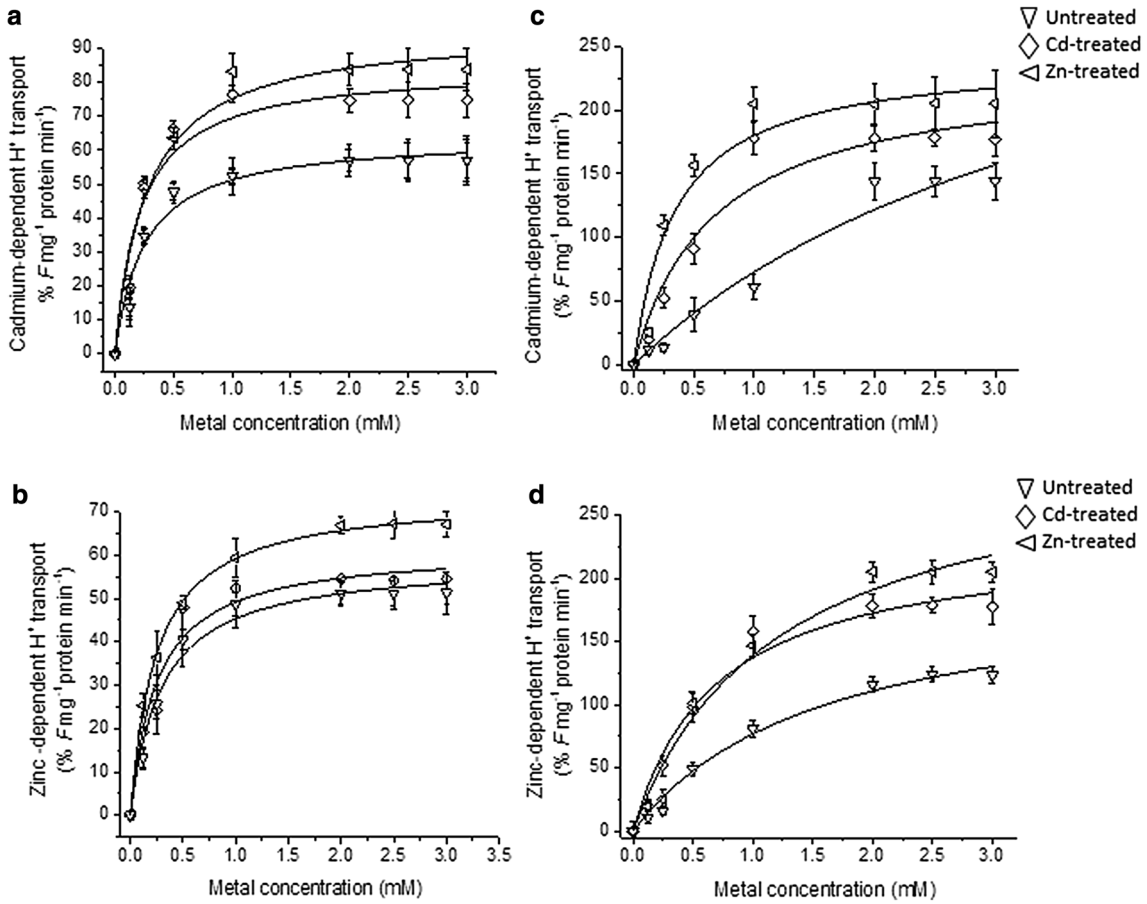

Fig. 6 Cadmium and zinc transport kinetics. **a** $CdCl_2$- and $ZnSO_4$-dependent H^+-efflux from tonoplast vesicles isolated from leaves of untreated (Δ), or 5-days $CdCl_2$ (*diamonds*)- or $ZnSO_4$- (*inverted triangles*) treated juvenile (**a**, **b**) or adult (**c**, **d**) tobacco plants. The pH gradient (inside-acid) was monitored by the quenching of quinacrine fluorescence (see "Materials and methods") by activating the tonoplast H^+-ATPase. The recovery of quinacrine fluorescence was measured upon addition of increasing concentration of $CdCl_2$ (**a**, **c**) or $ZnSO_4$ (**b**, **d**) (0.125–3-mM). Data are the mean ± SD from four independent experiments with each experiment done in tree independent assays. Curves were fitted by non-linear least-square analysis. Value significant different (Student's *t* test) from untreated plants is highlighted by *three asterisks* (***) $P < 0.001$. *n.s.* not statistically significant, *F* fluorescence intensity

a 1.25-fold increase in V_{max} was measured, suggesting that only in the presence of $ZnSO_4$, there was an increase in transport turnover.

The concentration-dependent kinetics of Cd^{2+}- or Zn^{2+}-dependent H^+ transport from tonoplast vesicles isolated from adult plants was complex. Data from tonoplast vesicles isolated from untreated tobacco plants did not fit the Michaelis–Menten equation but did show saturation at high concentrations (Fig. 6c, d). In contrast, data from Cd- or Zn-treated adult plants followed classic Michaelis–Menten kinetics for both Cd- and Zn-dependent H^+ transport (Fig. 6c, d). The estimated V_{max} for Cd transport was 192.5 (% F mg^{-1} min^{-1}) for untreated, 186.8% F mg^{-1} min^{-1} for Cd^{2+}-treated, and 207.1% F mg^{-1} min^{-1} for Zn-treated adult plants, with a K_m of 1.31, 0.43, and 0.26 mM, for untreated, and Cd- and Zn-treated plants, respectively (Suppl. Table S4). While the V_{max} for Zn-dependent H^+ transport was 142.5, 186.8, and 221.4% F mg^{-1} min^{-1} for untreated, and Cd- and Zn-treated adult plants, with a K_m of 1.31, 0.44, and 0.62 mM, respectively (Suppl. Table S4). The high micromolar K_m values may indicate that Cd and Zn are being transport through low affinity transporters. The higher values of V_{max} may be an indication that an increased turnover of the transporter in adult plants occurs when they are treated with either $CdCl_2$ or $ZnSO_4$. The activity in the untreated plants may be the result of metal influx through diverse divalent cation transporters, while in the $CdCl_2$ or $ZnSO_4$ treated plants, the increased abundance of the specific metal/proton transporters allowed measurements of the saturation kinetics of the transporters involved. The dissipation of the proton gradient in vesicles was caused only by cadmium and zinc ions, since neither potassium chloride or sulfate at concentrations up to 1 mM affected the ΔpH in tonoplast vesicles in a way similar to $CdCl_2$ or $ZnSO_4$ (data not shown).

Western-blot analysis of tonoplast H^+-ATPase, and the metal exchangers MTP1 and MTP4 from juvenile and adult tobacco plants in the absence or presence of cadmium or zinc

The observed increase in proton transport activity of the V-ATPase at the tonoplast in both juvenile and adult plants by $ZnSO_4$ treatment suggested that there may be changes in abundance of this multisubunit enzyme at the protein level. To confirm this, changes in the levels of abundance of V-ATPase subunits were studied by Western-blot analysis using antibodies raised specifically against the VHA-A, VHA-B, VHA-c, and VHA-E subunits of the tonoplast H^+-ATPase. Figure 7 shows representative images of Western blots and the histogram plot of the relative band intensities. In tonoplast isolated from leaves of juvenile plants, while the abundance of subunits VHA-B and VHA-E did not change, a significant increase in the amount of VHA-A and VHA-c subunits (12.5- and 6.96-fold, respectively) with 1-mM $ZnSO_4$ treatment was observed (Fig. 7a). In adult plants, the VHA-A, VHA-B, and E subunits did not change in abundance, while an increase in the VHA-c (8.3-fold) subunit was detected when the plants were treated with 1-mM $ZnSO_4$ (Fig. 7b). All data were subjected to unpaired two-tailed Student's *t* test and the changes were found to be significant ($P \leq 0.01$) as indicated.

To investigate the possible involvement of the vacuolar metal/H^+ exchangers MTP1 and MTP4 in the metal transport activity measured in isolated tonoplast vesicles of juvenile and adult tobacco leaves, we used antibodies raised against these transporters to detect the proteins in isolated tonoplast fractions. In juvenile plants treated with either $CdCl_2$ or $ZnSO_4$, a significant increase in the abundance of MTP1 (5- and 7.2-fold, respectively) and MTP4 (10.8- and 12.5-fold, respectively) was detected (Fig. 7c). In adult plants, a 35-fold increased abundance of MTP1 was detected when the plants were treated with either $CdCl_2$ or $ZnSO_4$ (Fig. 7d). In the case of MTP4, the increase in protein abundance was higher in adult plants treated with $ZnSO_4$ (12-fold) compared to $CdCl_2$-treated plants (5.3-fold).

NtMTP1, *NtZIP1,* and *NtNRAMP1* expression in juvenile and adult *N. tabacum* plants under cadmium and zinc exposure

To further understand how cadmium and zinc enter and are sequestered in the cells in tobacco plants, we studied the expression of *NtMTP1, NtNRAMP1,* and *NtZIP1* in leaves at the transcript level. The transcript accumulation profiles for the *N. tabacum MTP1*, *NRAMP1,* and *ZIP1* genes after 5 days of $CdCl_2$ or $ZnSO_4$ treatments were obtained from leaves of juvenile plants (0.5-mM $CdCl_2$ or 1-mM $ZnSO_4$), and from leaves of adult plants (1-mM $CdCl_2$ or $ZnSO_4$) using semi quantitative RT-PCR. Transcript expression of *NtUBQ* was used as a constitutive control, and all data were normalized to the *NtUBQ* signal (Fig. 8a). In leaves of juvenile plants, *NtNRAMP1* responded positively to $CdCl_2$- (9.3-fold) and $ZnSO_4$ (10.6-fold)-treatments, in comparison to untreated conditions (Fig. 8a). *NtMTP1* expression was up-regulated by $CdCl_2$ (1.2-fold) and down-regulated by $ZnSO_4$ (64.5-fold; Fig. 8a). The transcript of the *NtZIP1* gene was not detected in leaves of juvenile plants under any condition (Fig. 8a, b); however, it was strongly induced in roots when the plants were treated with cadmium (Suppl. Fig S1a).

In leaves of adult plants, the transcript expression of the *NtMTP1* gene increased 1.48 and 1.46-fold when the plants were treated with $CdCl_2$ or $ZnSO_4$, respectively (Fig. 8b). The transcript abundance of *NtNRAMP1* was not affected by cadmium exposures compared to the untreated leaves; however, the expression of this gene was suppressed in the presence of $ZnSO_4$. *NtZIP1* gene expression was induced by 107-fold in leaves of $CdCl_2$-treated plants, while no expression was detected in $ZnSO_4$-treated plants (Fig. 8b). Finally, as in the leaves, NtZIP1 transcript accumulation in the roots of adult plants was mainly detected under $CdCl_2$-treatment (Suppl. Fig S1b).

Discussion

A plant metal hyperaccumulator can grow on metalliferous soils and accumulate high amounts of heavy metals in the aerial organs without suffering phytotoxic effects (Rascio and Navari-Izzo 2011). The main characteristic of these plants is that they can compartmentalize the metals that they take up into the vacuole of the cell (Clemens 2006). Most hyperaccumulating species are herbaceous with a small biomass making them unattractive for phytoremediation purposes. Tobacco plants make an ideal candidate for this purpose as in addition to their metal tolerance and ability to accumulate considerable amounts of heavy metals in their aerial tissue, they produce a large biomass. In this sense, we were interested in the mechanisms that tobacco plants use to grow on soils contaminated with heavy metals, particularly cadmium and zinc. In this study, we provide evidence for an important role of tonoplast transport mechanisms in the sequestration of Cd and Zn in tobacco leaf tissue, which would enable tobacco plants to grow without symptoms of toxicity to these two metals. We show that tobacco seeds could germinate in the presence of high levels of $CdCl_2$ and $ZnSO_4$, a result that demonstrates that both metals do

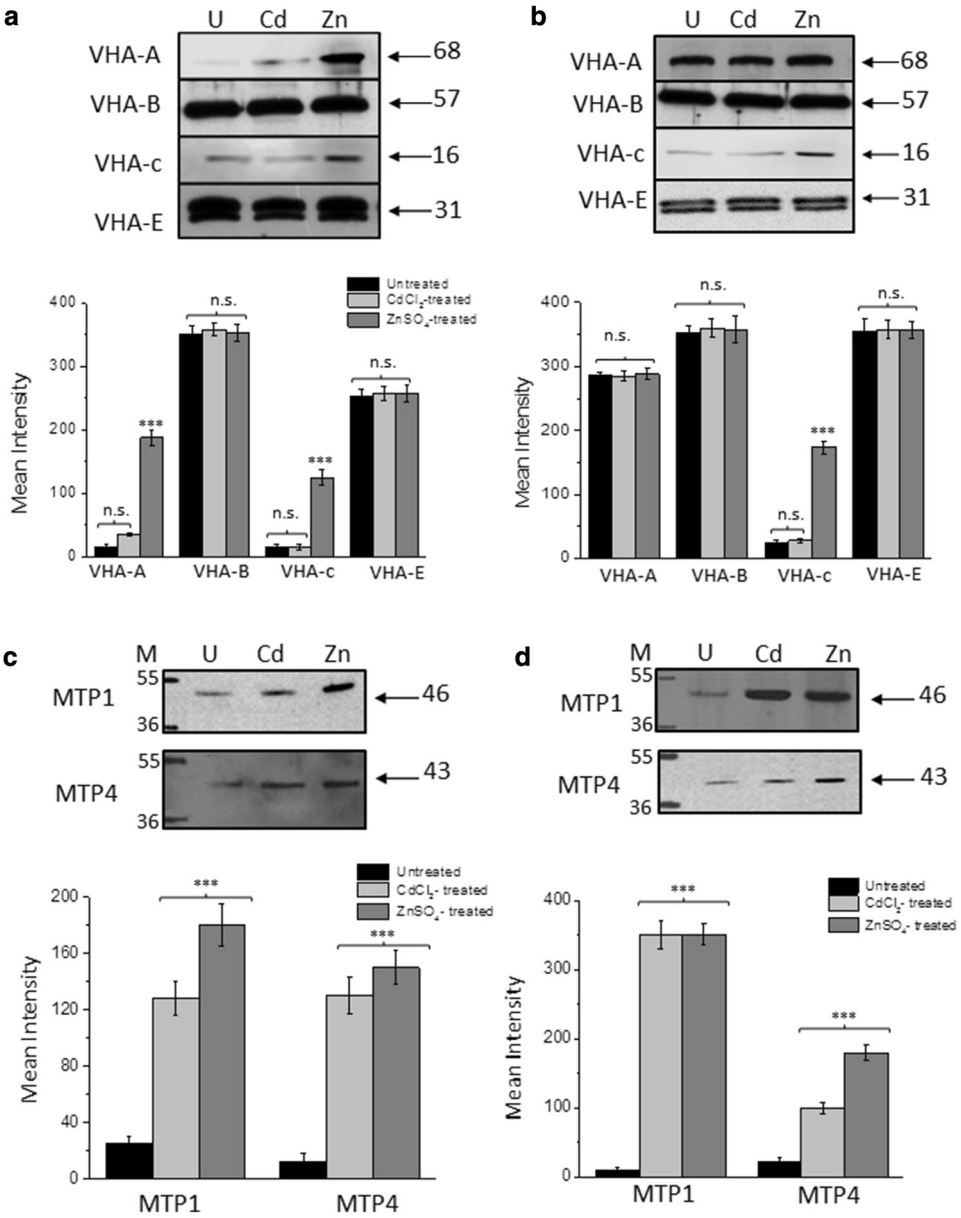

Fig. 7 Protein abundance of the V-ATPase, MTP1 and MTP4 in tobacco plants under $CdCl_2$ or $ZnSO_4$ treatment. Tonoplast fractions were isolated from leaves of juvenile (**a**, **c**) or adult (**b**, **d**) untreated (U) or exposed for 5 days to 0.5- (juvenile) or 1-mM (adult) $CdCl_2$ or 1-mM $ZnSO_4$ on discontinuous Suc density gradients at the 0–16% (w/v) Suc interface. Western-blot analysis was carried out as described in "Materials and methods" using antibodies directed against V-ATPase subunits (VHA-A, -B, -c, and -E). PS indicate Ponceau stain loading control. Blots are representative of five independent experiments (data correspond to the mean ± SD, $n = 5$, $^{***}P < 0.001$ compared to untreated plants; *n.s.* not statistically significant). Numbers are molecular weights (M) in kilodaltons

not affect the emergence of radicles and shoots. Even though root growth was negatively affected by high amounts of both metals, the more drastic effects were observed in the presence of 0.5-mM $CdCl_2$, showing the potential toxicity of this ion. Similar results have been reported for the roots of several wheat cultivars exposed to different concentrations of cadmium (Ahmad et al. 2012).

Tobacco plants grown in the presence of cadmium and zinc during their juvenile (6-week-old) and adult (10-week-old) stages showed little signs of metal toxicity as plants could grow under these conditions without presenting chlorosis or reduction of leaf size. However, a reduction in the length of the main root and a concomitant increase in the number of lateral roots were observed in the presence of 1-mM $ZnSO_4$ (Suppl. Fig. S2a). Changes in root morphology of plants grown in the presence of cadmium or zinc have previously been reported and have been called stress-induced morphogenic responses, which consist of a reduction in principal root growth with an increased density of lateral roots (Potters et al. 2009). Remans et al. (2012) reported that *A. thaliana* plants showed this morphogenic response when treated with micromolar concentration of cadmium or zinc. These results suggest that tobacco can grow and develop in the presence of heavy metals as previously reported (Rodríguez-Ortíz et al. 2006).

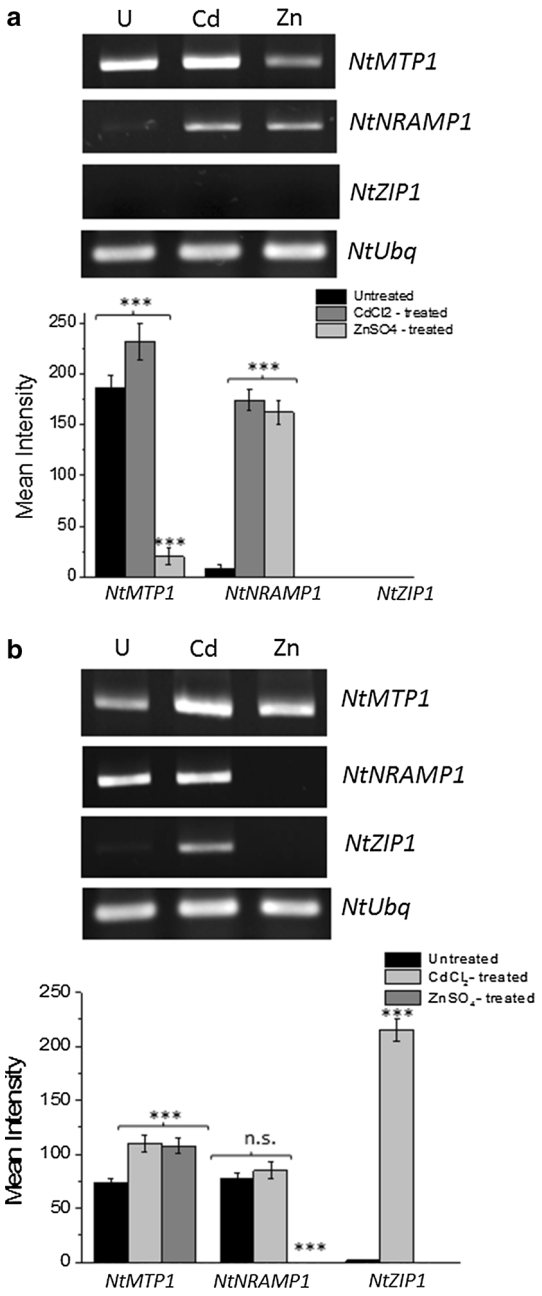

Fig. 8 Expression analysis of *NtMTP1*, *NtNRAMP1,* and *NtZIP1* in leaves of juvenile and adult tobacco plants under $CdCl_2$ or $ZnSO_4$ treatment. RNA was isolated from leaves of juvenile (**a**) or adult (**b**) untreated and 0.5- (juvenile) or 1-mM (adult) $CdCl_2$- or 1-mM $ZnSO_4$-treated plants for 5 days. RT-PCR was carried out as described in Materials and methods using gene-specific primers (Suppl. Table S1). Photographs are representative of four independent experiments. Transporters transcript accumulation was normalized by dividing mean relative values by the mean relative *Nt*UBQ values. Transcript abundance optical density is the average mean ± SD from four independent blots

It is well established that one of the mechanisms that hyperaccumulators use to withstand high levels of heavy metals is by sequestration of the metals in the large vacuole, preventing deleterious effects on cellular metabolism. Our measurements demonstrated that when juvenile and adult plants were treated with $CdCl_2$, an increased concentration of both cadmium and zinc was measured in the leaf tissue. A similar response was observed when plants were exposed to $ZnSO_4$ (Table 1). These results suggested that the presence of either metal induced the uptake of both cadmium and zinc, opening the possibility that this response may be accounted for by the activation of the same transporter. Like results observed for the hyperaccumulator *N. caerulescens*, tobacco could accumulate up to 30 g kg^{-1} of dry weight of zinc in the leaves grown in a hydroponic culture without any visible toxic effects. As mentioned before, the definition of a hyperaccumulator is a plant that absorbs heavy metals and accumulates them in the aerial organs, to a greater concentration than that in the soil in which it is growing (Rascio and Navari-Izzo 2011). Here, we show that tobacco plants can accumulate cadmium and zinc in the leaves of both juvenile and adult plants to concentrations higher that those applied in the hydroponic media, suggesting that tobacco plants can be considered as cadmium and zinc hyperaccumulators. The previous reports have suggested that tobacco trichome cells are the main storage tissue of zinc (Sarret et al. 2006); however, even though we observed zinc in the trichomes, we did not find that this was the main storage tissue for the metal in this study (Fig. 4). Upon cadmium treatment, we found that cadmium and zinc contents increased in leaves of both juvenile and adult plants (Table 1). Unfortunately, we do not have a fluorescent dye that would allow us to detect cadmium in tissues, but we could corroborate zinc accumulation under all conditions and in all tissues analyzed using the zinc-specific fluorescent sensor Zinpyr-1 (Fig. 4).

Sequestration of metals into the vacuole requires membrane transporters and the electrochemical gradient of protons generated by the V-ATPase and the V-PPase (Kabała et al. 2014). We tested these mechanisms by quantification of the H^+ transport and hydrolytic activities of the V-ATPase and the hydrolytic activity of Type I and Type II V-PPases on tonoplast vesicles isolated from leaves of juvenile and adult plants grown in the absence or presence of $CdCl_2$ or $ZnSO_4$. Interestingly, we found no change in the hydrolytic activity of either the V-ATPase or Type I V-PPase; however, when we measured the H^+ transport of the V-ATPase and the hydrolytic activity of type II V-PPase, we found a significant increase in activity in leaves of both juvenile and adult plants grown in the presence of $ZnSO_4$ (Figs. 5, 6), while only the hydrolytic activity of Type II V-PPase was found to increase in $CdCl_2$-treated plants (Suppl. Table S3). These changes suggest that zinc treatment may promote a more efficient proton transport by the proton-ATPase, while upon $CdCl_2$

and $ZnSO_4$ treatments the Type II V-PPase may be the mechanism responsible to generate the proton motive force for metal transporters into the vacuole.

The variability in proton transport by the V-ATPase has been reported to occur due to changes in the C-terminal of the VHA-a subunit of the V_o complex (Kawasaki et al. 2003) or through the reversible oxidation/reduction of the Cys 252 and Cys 532 residues of the VHA-A catalytic subunit (Fodor 2002). Here, we found that the abundance of both, VHA-A and VHA-c subunits, increased in adult plants when treated with $ZnSO_4$, which may suggest that these two subunits participate in the observed upregulation of the V-ATPase activity by changing the coupling ratio. It has been reported that in isolated *Daucus carota* tonoplast vesicles, the coupling ratio of the V-ATPase changed after salt treatment (Löw and Rausch 1996). There is evidence that zinc could directly interact with –SH groups, which may change protein conformation and activity (Fodor 2002; Kabała et al. 2013); whether this may be the regulatory mechanism observed in this study is a possibility that should be further investigated.

In juvenile plants, the abundance of the VHA-c subunit increased upon $ZnSO_4$ treatment with a concomitant increased V-ATPase proton transport, while the abundance of other subunits, including VHA-A, VHA-B, and VHA-E, was not affected by any of the treatments (Fig. 5). It has been shown that specific subunits of the V-ATPase participate more actively in the assembly of the whole complex to either make this enzyme more active or to assemble more functional units on the tonoplast for the cell to be able to provide the required proton motive force used by the transporters involved in the sequestration of excess ions into the vacuole (Seidel et al. 2013). It is also possible that the increased abundance of the VHA-c subunit may change the stoichiometry of the V-ATPase, as it has been reported that this subunit is involved in H^+ coupling (Ratajczak 2000).

The V-ATPase hydrolytic and proton transport activities did not change upon $CdCl_2$ treatment; however, the increase in the type II V-PPase suggested that this enzyme may be responsible for generating the driving force for cadmium accumulation.

The detailed analysis of Cd^{2+}/H^+ and Zn^{2+}/H^+ exchange activity in tonoplast vesicles indicated that even though activity of the metal transporters was already present in the untreated plants, the increased activity measured when plants were treated with either cadmium or zinc, as indicated by the increased V_{max}, may reflect changes at the protein level. However, it should be noted that in the Zn-treated plants, the pH gradient generated by activation of the V-ATPase before the addition of bafilomycin was significantly higher than that measured in the untreated plants. This difference could influence the rate of fluorescence recovery in the presence of Zn in these assays. Hence, the higher Zn^{2+}/H^+ exchange in tonoplast prepared from Zn-treated plants could result from both, the higher V-ATPase activity and the higher MTP1 and MTP4 abundance. In untreated adult plants, the kinetics of both cadmium and zinc influxes were complex, showing non-saturating curves, indicative of the activity of low affinity transporters. Similar complex kinetics for root Zn^{2+} influx was previously obtained in wheat (Hart et al. 1998) and *N. caerulescens* (Lasat et al. 1996).

Based on the kinetics analysis from our study, it appears that the transport of both metals may be facilitated by the activity of low affinity transporters. Micromolar K_m values were also reported for Cd or Zn transport in yeast expressing either cucumber *Cs*MTP1 and *Cs*MTP4 (K_m 5.7 and 12 μM for Cd and 0.5 and 2 μM for Zn; Migocka et al. 2015), or *T. goesingense* MTP1 (6.6 μM for Zn; Kim et al. 2004) and for roots of *N. caerulescens* and *N. arvense* (8 and 6 μM, respectively; Lasat et al. 1996); however, the values in these reports were all in the low micromolar range. The relatively higher K_m values measured in tobacco in this study may be influenced by the specific metal-binding capacity of buffers used in the analysis or alternatively from binding of metals to the membranes.

Studies focusing on the mechanisms used for plants to hyperaccumulate cadmium and zinc have revealed the participation of an array of genes that are constitutively highly expressed when compared to a non-hyperaccumulating related species (reviewed in Rascio and Navari-Izzo 2011). These genes have roles in metal homeostasis and detoxification, transport to the major storage organs or tissues, chelation, subcellular compartmentalization, or efflux from the roots.

To investigate which transporters could be involved in cadmium and zinc accumulation in tobacco, we used antibodies raised against the metal/H^+ exchangers MTP1 and MTP4 to determine the effect of heavy metal treatment on protein abundance. We found that in both juvenile and adult plants, the abundance of the metal transporters MTP1 and MTP4 increased in the leaves of plants treated with either $CdCl_2$ or $ZnSO_4$ (Fig. 7). MTP1 and MTP4 have been implicated in zinc homeostasis and cadmium sequestration in the vacuole as increases in protein abundance of both metal transporters were detected in cucumber roots in response to elevated Cd or Zn (Migocka et al. 2015). In addition, we found that the transcript level of *NtMTP1* increased in the leaves of both young and adult tobacco plants when treated with cadmium, while an up-regulation of *NtMTP1* expression in response to zinc was only observed in adult plants (Fig. 8a, b). The down-regulation of *NtMTP1* in juvenile plants treated with zinc may indicate that this transporter is post-transcriptionally regulated as the protein amounts increased under the same

treatment. It has been shown that *A. thaliana AtMTP1* transcript abundance is not affected by the presence of elevated zinc concentrations (Kobae et al. 2004); however, the expression of *B. juncea* MTP1 has been found to be upregulated by cadmium and zinc (Lang et al. 2011), suggesting a differential response between non-hyperaccumulator (*Arabidopsis*) and hyperaccumulator (*B. juncea*) plants. As in *B. juncea*, the increase in transcript levels of tobacco MTP1 may be linked to the plant's ability to hyperaccumulate heavy metals (Sen et al. 2013).

Analysis at the transcript level of the metal/cation transporter *NtNRAMP1* showed that its expression was induced by both, $CdCl_2$ and $ZnSO_4$, in juvenile plants (Fig. 8). In adult plants, *NtNRAMP1* expression was more conspicuous than in juvenile plants and was similar under $CdCl_2$-treatment; however, *NtNRAMP1* was undetectable in the $ZnSO_4$ treated plants. These data suggested that the heavy metal regulation of *NtRAMP*1 expression is age- and metal-dependent. There is evidence that *NtNRAMP1* in plants is a manganese/iron transporter (Cailliatte et al. 2010); however, other gene family members, *AtNRAMP1*, *AtNRAMP3,* and *AtNRAMP4,* mediate cadmium transport when expressed in yeast (Thomine et al. 2000). Our results indicate that *NtNRAMP1* transcript is induced in both Cd- or Zn-treated juvenile plants. Whether this transporter is responsible for the transport of both metal ions is a possibility that must be further investigated.

Analysis of the expression of the *NtZIP1* gene indicated that this transporter was not expressed in the leaves of juvenile plants under any condition, but the transcript was mainly detected in leaves of adult cadmium-treated plants (Fig. 8b). Likewise, *Nt*ZIP1 transcript accumulation was strongly induced under cadmium treatment in the roots, although in both juvenile and adult plants (Suppl. Fig. S1a, b), ZIP1 has been identified as a zinc transporter down-regulated under high zinc concentrations (Assunção et al. 2003) but has also been shown to transport a variety of cations, including cadmium, iron, manganese, and zinc (Guerinot 2000). The fact that this transporter was induced only by cadmium suggests that it may play a role in cadmium detoxification in tobacco.

It is difficult to get a complete picture of how zinc and cadmium homeostasis and sequestration may occur in tobacco, as the gene families for heavy metal transporters are large and many members remain uncharacterized as to their substrate or cellular localization (Milner and Kochian 2008; Gustin et al. 2011). However, our results show that there may be a role for *Nt*MTP1; *Nt*MTP4, *NtNRAMP,* and *NtZIP1* transporters in cadmium and zinc homeostasis and sequestration as they appear to be regulated by these heavy metals. Further work to confirm this role is needed, including the characterization of overexpressing or mutant plants.

Nicotiana tabacum has extensively been used as a tool to overexpress genes from a variety of organisms to study their role in heavy metal tolerance. Genes isolated from a range of metal sensitive and tolerant plant species encoding metal transporters, like *AhHMA4, BjYSL7* (Wang et al. 2013), *BjCET3,* and *BjCET4* (Lang et al. 2011*),* metal detoxification enzymes, like glutathione transferase, phytochelatin synthase (Pomponi et al. 2006; Liu et al. 2011), metallothionein (Zhou et al. 2014), cystathionine β-synthase (Singh et al. 2012), and proline and glycine betaine synthesis enzymes (Islam et al. 2009) have all been introduced in tobacco plants to define their role in heavy metal homeostasis and detoxification (Barabasz et al. 2010). Recently, the expression of *Arabidopsis* cation exchanger (*AtCAX*) genes resulted in increased cadmium accumulation in tobacco root vacuoles (Korenkov et al. 2007). We have unpublished data which showed that wild-type tobacco plants accumulated similar amounts of cadmium and zinc than transgenic plants overexpressing HA-sCAX1, which is an N-terminal truncated form of the CAX1 exchanger which has been shown to transport calcium, cadmium, and zinc (Korenkov et al. 2007). These results suggest that tobacco employs other cation transporters in addition to CAX for metal transport.

Conclusions

Here, we show that tobacco possesses a high tolerance to heavy metals. Seeds can germinate in the presence of both cadmium and zinc and the juvenile and adult plants can grow and develop in the presence of high concentrations of these two metals without presenting symptoms of toxicity. We demonstrate that tobacco plants possess transport mechanisms to accumulate heavy metals within the vacuoles and these show differential regulation depending on the tissue, age, and metal ion applied. In addition, we show that vacuolar heavy metal/H^+ exchangers, the vacuolar H^+-ATPase, and Type II V-PPase may participate in the tolerance of tobacco to zinc but not cadmium. It is well documented that changes in V-ATPase activity and alterations in gene expression of subunits of the vacuolar ATPase might be a prerequisite of the plant response to heavy metals (Sharma et al. 2016). Our results indicated that the V-ATPase proton pumping and hydrolytic activities was increased by zinc treatment, but not affected by cadmium and that the type II vacuolar H^+-pyrophosphatase may play a role in energization of the vacuolar membrane under heavy metal stress.

Author contribution statement RVE and BJB conceived and designed research. MFGM and RVE performed and analyzed the transport and physiological experiments,

JCAR preformed and analyzed transcript experiments, and PRS conducted and analyzed the confocal experiments. OPA and BJB revised the manuscript, contributed to the discussion, and participated in preparing the manuscript. RVE wrote the manuscript. All authors read and approved the manuscript.

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