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ORIGINAL PAPER

Response to cytosolic nickel of Slow Vacuolar channels in the hyperaccumulator plant *Alyssum bertolonii*

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Abstract We applied the patch-clamp technique to investigate the transport properties of the Slow Vacuolar (SV) channel identified in leaf vacuoles of *Alyssum bertolonii* Desv., a nickel hyperaccumulator plant growing in serpentine soil of the northern Apennines (Italy). SV currents recorded in vacuoles from adult plants collected in their natural habitat showed high sensitivity towards cytosolic nickel. Dose-response analyses indicated half-maximal current inhibition at submicromolar concentrations, i.e. up to three orders of magnitude lower than previously reported values from other plant species. The voltage-dependent increase of residual currents at saturating nickel concentrations could be interpreted as relief of channel block by nickel permeation at high positive membrane potentials. Including young plants of *A. bertolonii* into the study, we found that SV channels from these plants did not display elevated nickel sensitivity. This difference may be related to age-dependent changes in nickel hyperaccumulation of *A. bertolonii* leaf cells.

Keywords *Alyssum bertolonii* · Metal hyperaccumulation · Vacuole · Nickel · Slow Vacuolar channel · Phytoremediation

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Introduction

In 1948, Minguzzi and Vergnano (1948) gave the first description of metal accumulation in plants when they reported an extremely high concentration of nickel (Ni^{2+}), reaching up to 1% of dry matter weight, in the shoots of *Alyssum bertolonii* Desv., growing in Tuscan serpentine soil. The term "hyperaccumulator" was coined only about 30 years later to describe plants that accumulate more than 1,000 ppm of nickel (Brooks et al. 1977). The genus *Alyssum* comprises 48 species showing the ability of nickel hyperaccumulation (Brooks et al. 1979; Brooks and Radford 1978). Many of them are endemic to ultramafic serpentine soils, characterised by a low capacity of water retention, high concentrations of heavy metals (such as nickel, cobalt and cadmium), low nutrient levels and a high Mg/Ca ratio.

Naturally selected hyperaccumulator plants have received great interest for their potential use in phytoremediation approaches, the plant-based clean-up strategy to decontaminate soils and waters. In the last decade, the basic processes underlying the maintenance of plant metal homeostasis in general and the phenomenon of hyperaccumulation in particular have been elucidated (Clemens 2001). At the cellular level, a major role has been ascribed to vacuolar compartimentalisation of excess cytosolic metals. Likewise, hyperaccumulation is connected to the ability to transport large amounts of metals into leaf vacuoles (Kramer et al. 2000; Persans et al. 2001). Nickel accumulation in *A. bertolonii* leaves is probably based on a similar mechanism of vacuolar sequestration (Kupper et al. 2001). To date, little is known about the interactions between metals and plant nutrient transport systems. The Slow Vacuolar (SV) channel has been implicated in cellular homeostasis of potassium, an essential macronutrient involved in osmoregulation and growth.

The SV channel displays poor cation selectivity as it is permeable not only to small (monovalent and divalent) cations (Pottosin et al. 2001) but also to organic cations (e.g. TEA^+) under the action of large membrane potentials (Dobrovinskaya et al. 1999). A wide dimension of the narrowest pore constriction, between 5 and 8 Å, has been suggested (Dobrovinskaya et al. 1999; Gambale et al. 1996). The large size of the pore is not surprising as known calcium-permeable channels have pore sizes in this range (McCleskey and Almers 1985). Consistently, the SV channel pore is accessible to a variety of cytosolic metal ions, which affect the channel at concentrations depending on plant species and tissue. For example, current inhibition by zinc and/or nickel has been reported in sugar beet taproot (Gambale et al. 1996; Hedrich and Kurkdjian 1988; Paganetto et al. 2001), *Eichhornia crassipes* (Paganetto et al. 2001) and radish root (Carpaneto 2003). Here, we investigated the effect of cytosolic nickel on SV currents in leaf vacuoles of *Alyssum* species.

Materials and methods

Plant material and maintenance

Adult plants and seeds of *A. bertolonii* Desv. were collected at an ultramafic site near Falcinello (La Spezia, Italy) in the Apennine mountains. Plants were placed into plastic pots together with soil collected at the same site and kept in open air under variable weather conditions, as field-grown plants deteriorated when maintained for prolonged periods of time in a growth chamber.

Seeds of *Alyssum montanum* L. and *Alyssum corsicum* Duby were provided by Ute Krämer (University of Heidelberg, Germany). For germination, seeds were kept on wet sand, first for 4 days at 4°C in the dark, then at 22°C in a growth chamber with 12-h light period. Seedlings were transferred into plastic pots containing either commercial potting compost or Ni-rich soil taken at the site where *A. bertolonii* plants were collected. The nickel content of the serpentine soil was 2,050 mg kg^{-1} dry weight, as measured by atomic absorption spectroscopy (ARPAL, Genoa). Young plants were used for patch-clamp experiments at an age of 6–9 weeks. For all data sets recordings from at least two individuals were used.

Protoplast isolation

The same protocol was used for protoplast isolation from primary leaves of adult and young plants. The latter presented generally shorter and more reproducible incubation times in enzyme solution. The upper and lower epidermis bearing stellate trichomes were removed using fine sandpaper. Leaf pieces were placed in enzyme solution containing 0.8% (w/v) cellulase Onozuka R-10 (Yakult), 0.08% (w/v) pectolyase Y-23 (Seishin), 1 mM $CaCl_2$, 0.5% (w/v) polyvinylpyrrolidone (PVP-10), 0.5% (w/v) bovine serum albumin, 500 mM D-sorbitol, 5 mM MES, pH 5.5 and incubated in the dark at 30°C for about 2 h (1 h for young plants). Resulting protoplasts were centrifuged (100×*g*, 2 min, 5°C) and washed twice with standard patch-clamp solution.

Patch-clamp recordings

Patch-clamp experiments on isolated vacuoles were performed as described previously (Carpaneto et al. 2001). Standard internal and external ionic solutions contained (in mM): 150 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES-Tris, pH 7.0. The osmolarity was adjusted by the addition of D-sorbitol to 600 mOsm (for vacuoles from young plants) and to the respective osmotic pressure of the leaf (for adult plants; normally 350 mOsm, but varying seasonally). Desired free Ni^{2+} concentrations in the bath solution were adjusted by the addition of EGTA and $NiCl_2$, according to the calculation by maxchelator (http://www.stanford.edu/~cpatton/webmaxcE.htm).

Vacuoles were released from protoplasts by addition of 10 mM EGTA to an aliquot of protoplast solution. The recording chamber was initially perfused with bath solution containing 1 mM dithiothreitol (DTT), as reducing agents are known to preserve the functional state of SV channels (Carpaneto et al. 1999). Prior to addition of $NiCl_2$, DTT was removed from the bath solution leading to a moderate SV current decrease of about 20–30% for all *Alyssum* species (data not shown). Recordings in the whole-vacuole and excised cytosolic side-out patch configurations did not differ regarding current–voltage relationships and response to cytosolic Ni^{2+} and thus were considered equivalent.

Results and discussion

We applied the patch-clamp technique on vacuoles from leaf protoplasts to study slowly activating vacuolar (SV) currents of the nickel hyperaccumulator *A. bertolonii* and the non-hyperaccumulator relative *A. montanum* and their response to cytosolic nickel. Recordings in standard ionic solutions showed typical macroscopic SV currents (Fig. 1a, c, left panels). Currents from both plants were outward rectifying and required voltage pulses of several seconds for full activation. SV channels were closed at negative membrane potentials and activated at voltages more positive than +40 mV (Fig. 1b, d, open symbols). Cytosolic nickel (Ni^{2+}) blocked SV currents in sugar beet, radish and *E. crassipes* with constants of half-inhibition between 25

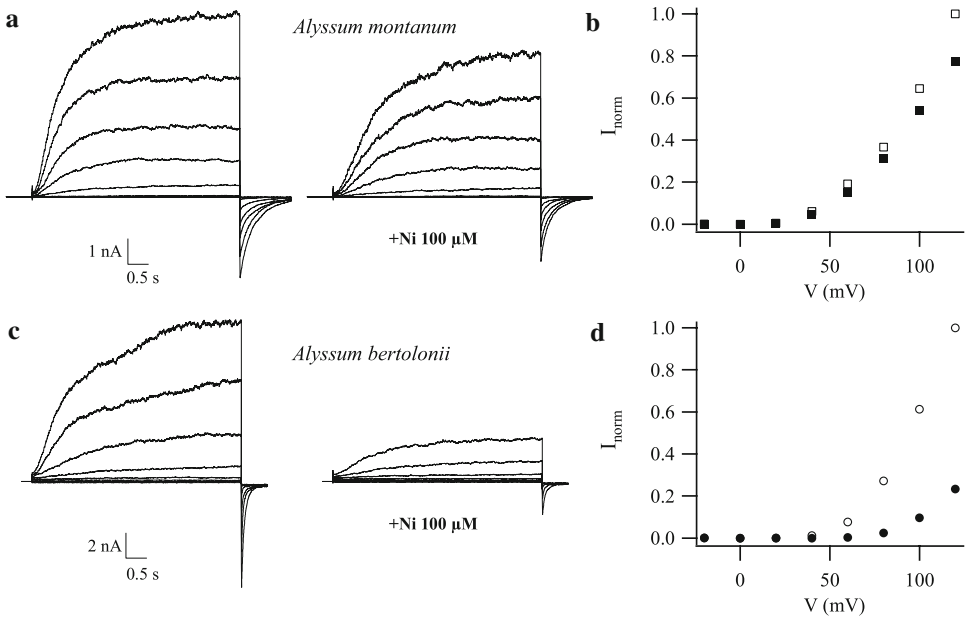

Fig. 1 **a** Macroscopic Slow Vacuolar currents of the non-hyperaccumulator plant *Alyssum montanum* before (*left panel*) and after addition of 100 µM $NiCl_2$ (*right panel*) to the bath solution. Currents were elicited by voltage pulses from −20 to +120 mV in steps of +20 mV. **b** Time-dependent currents from **a** were normalised to the control value at +120 mV and plotted against the applied membrane potential. **c** Macroscopic Slow Vacuolar currents of an adult *Alyssum bertolonii* plant before (*left panel*) and after addition of 100 µM $NiCl_2$ (*right panel*) to the bath solution. Voltage protocol was as in **a**. **d** Normalised time-dependent currents (from **c**) are plotted against the applied membrane potential

and 450 µM (Carpaneto 2003; Paganetto et al. 2001). When Ni^{2+} at a concentration of 100 µM was added to the bath solution, SV currents of the non-hyperaccumulator *A. montanum* decreased at all potentials between +60 and +120 mV by about 20% (Fig. 1a, b, closed squares) indicative of an inhibition constant comparable to those reported for other plants. Instead, the SV current response in vacuoles from adult field-grown *A. bertolonii* plants was much more pronounced, showing a reduction of at least 80% (Fig. 1c, d, closed circles). Therefore, we investigated the Ni^{2+} sensitivity of *A. bertolonii* SV currents in more detail.

Figure 2 shows SV current recordings from the same vacuole successively exposed to different nickel concentrations. Inhibition was already observed at 0.1 µM Ni^{2+} (Fig. 2b) and increased in a concentration-dependent manner. The effect was fully reversible (Fig. 2f). The results from all dose-response experiments in the concentration range from 0.1 to 100 µM Ni^{2+} are displayed in Fig. 3a. Normalised experimental data were fitted with the modified Hill equation

$$I_{\text{norm}} = (1 - I_{\text{res}})/\left(1 + \left(\left[Ni^{2+}\right]/K_{\text{h}}\right)^{n}\right) + I_{\text{res}} \quad (1)$$

where I_{res} is the fraction of the residual current at saturating nickel concentrations, $[Ni^{2+}]$ is the nickel concentration, K_{h} and n are the Hill constant and the Hill coefficient, respectively.

The global fit obtained with the restriction of an identical Hill coefficient for all data sets (taken at different membrane potentials) resulted in $n = 0.54$. Values less than 1 are found in case of negative cooperativity, in which ligand binding at one site interferes with binding to additional identical sites, or alternatively in case of two or more non-identical binding sites on the protein. According to Abeliovich (2005), the lower limit of n is given by the inverse of the subunit number. Thus, the Hill coefficient found in this study would be in agreement with the hypothesis of negative cooperativity, under the assumption that the *A. bertolonii* SV channel is a homodimer of a TPC1-like protein homologous to the one identified in *Arabidopsis thaliana* (Peiter et al. 2005). Instead, a simple screening mechanism of negative surface charges near the channel mouth can be excluded (Ravindran et al. 1991), as nickel concentrations in the micromolar range are too small to produce significant surface potential changes in the presence of 150 mM KCl.

The dissociation constant value K_{h} was about 0.25 µM for all potentials tested (Fig. 3b) indicating that Ni^{2+} interacts with the SV channel of adult *A. bertolonii* plants with high affinity. This value is up to three orders of magnitude lower than the ones found for other plant species. Voltage independence of nickel inhibition is in agreement with the study on radish vacuoles (Carpaneto 2003) and suggests that the nickel binding site is located outside the

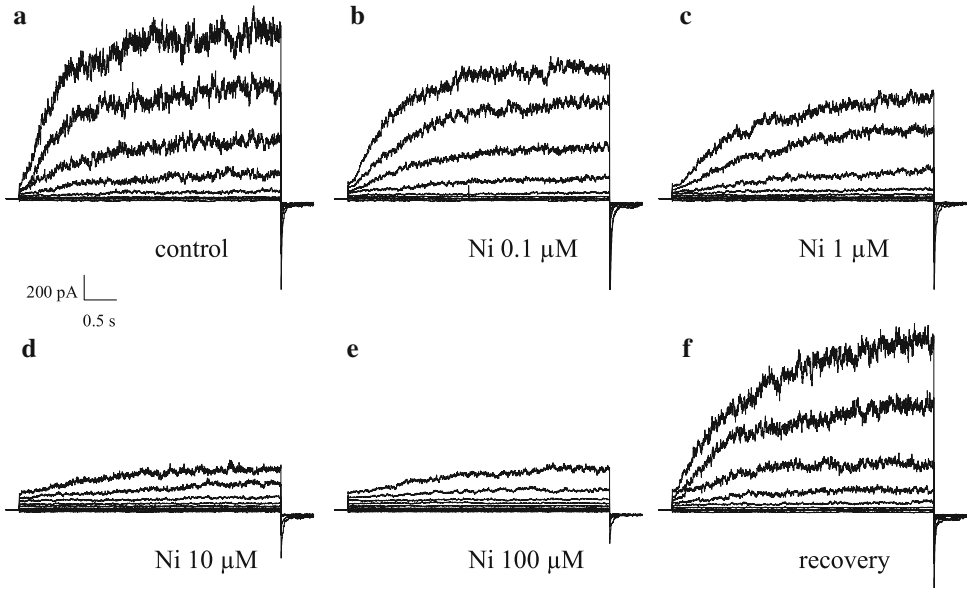

Fig. 2 SV currents of an adult *Alyssum bertolonii* plant recorded **a** in the absence of nickel (*control*), **b** in the presence of 0.1 µM $NiCl_2$, **c** 1 µM $NiCl_2$, **d** 10 µM $NiCl_2$, **e** 100 µM $NiCl_2$ in the bath solution, and **f** after washout (*recovery*). Currents were elicited by voltage pulses from −20 to +120 mV in steps of +20 mV

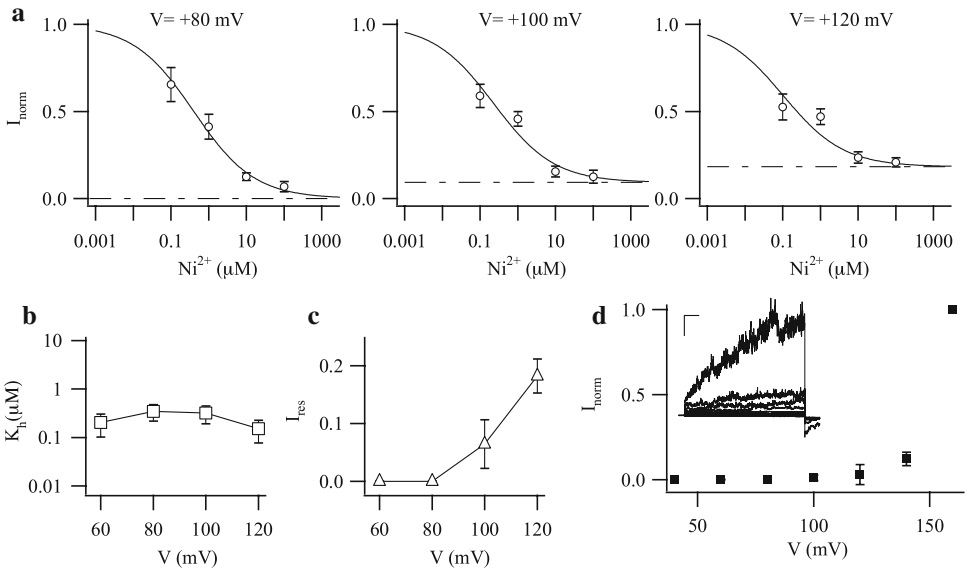

Fig. 3 **a** Semi-logarithmic plots of normalised currents $I_{norm} = I_{Ni}/I_{control}$ (mean ± SEM of at least three experiments) derived from recordings on vacuoles of adult *Alyssum bertolonii* plants (see also Fig. 2). Four different membrane potentials were considered: +60 mV (not shown), +80 mV (*left panel*), +100 mV (*middle panel*) and +120 mV (*right panel*). The four data sets were subjected to a weighted global fit with a Hill function (Eq. 1). The global Hill coefficient was 0.54. The fraction of the residual current at saturating nickel concentrations I_{res} (see also **c**) is indicated by a *broken line*. **b** Values of the Hill constant K_h (mean ± SEM), derived from the global Hill fit in **a**, are plotted against the applied membrane potential. **c** The fraction of the residual current at saturating nickel concentrations I_{res} (mean ± SEM), derived from the global Hill fit in **a**, is plotted against the applied membrane potential. **d** Current–voltage relationship of macroscopic SV currents (recorded from a large cytosolic-side out excised patch) after replacing 150 mM KCl with 75 mM $NiCl_2$. Currents were elicited by voltage pulses from +40 to +160 mV in steps of +20 mV. Data *points* represent the steady state current (mean ± SD; $n = 4$) normalised to the value at +160 mV. Typical current traces are shown in the *inset*; note the tail currents revealing the typical slow deactivation characteristics of the SV channel. *Scale bars* 0.5 s/100 pA

membrane electric field (Woodhull 1973). Interestingly however, the fraction of the residual current at saturating nickel concentrations, given by I_{res} values in Eq. 1, was voltage-dependent and increased with the applied tonoplast potential (Fig. 3c). This could be interpreted in a way that current inhibition may be relieved partly by the passage of Ni^{2+} through the SV channel pore at higher membrane potentials.

To test this hypothesis we completely exchanged the charge carrier potassium in the bath solution with nickel

(75 mM $NiCl_2$ to maintain the chloride concentration of the bath solution). Figure 3d shows a typical recording from an *A. bertolonii* vacuole (inset) and mean current–voltage relationships in the presence of 75 mM Ni^{2+}. Clearly, SV currents were neglectible in the voltage range between +40 and +100 mV, as expected from the dose-response analysis shown in Fig. 3a. Only under the action of higher membrane potentials (Fig. 3d), SV-type currents could be identified based on their activation and deactivation kinetics, suggesting that SV channels are permeable to Ni^{2+}. This, however, does not seem to be a specific property of *A. bertolonii* SV channels, as equivalent experiments with *A. montanum* vacuoles gave similar results (data not shown). It is well known that many transition metal ions are able to mimic physiological ions and pass through the pore of even highly selective cation channels (Bridges and Zalups 2005). Only few studies, however, have raised the question of Ni^{2+} permeation: the answer has been negative in case of animal calcium channels (Shibuya and Douglas 1992) and non-selective cation channels (Lotshaw and Sheehan 1999; Mayer and Westbrook 1987). To our knowledge, nickel permeation has only been reported for poorly selective channels with relatively wide pores like that formed by the peptide alamethicin (Fonteriz et al. 1991). We attribute nickel currents to the SV channel and discard the involvement of the calcium-insensitive vacuolar channel (CIVC) recently identified in *A. thaliana* vacuoles (Ranf et al. 2008). The activation of CIVC at highly positive membrane potentials produced small K^+ currents compared to the respective SV currents in the same vacuole. Therefore, Ni^{2+} currents recorded in our experiments would be compatible with CIVC activity only under the assumption of a higher permeability for nickel than for potassium.

Could the high nickel sensitivity of SV channels be related to the nickel hyperaccumulation in leaf vacuoles of *A. bertolonii* plants? If this were the case, the same response may be found in other nickel hyperaccumulator species. For this purpose, we isolated leaf vacuoles from young *A. corsicum* plants grown in commercial potting soil and tested the response of SV currents to 100 μM cytosolic nickel. The current reduction of about 35% in *A. corsicum* was not significantly different from *A. montanum* grown under identical conditions (Fig. 4a). Surprisingly, the same was true for young plants of *A. bertolonii* grown in Ni-rich soil identical to the one of adult plants (Fig. 4a) or grown in potting compost (data not shown). A typical SV current response of a Ni-grown *A. bertolonii* plant is shown in Fig. 4b. Taken together, these results pointed to a stage-dependent difference in the nickel sensitivity of SV currents in *A. bertolonii*. Vacuolar nickel accumulation in this hyperaccumulator plant is thought to occur predominantly in the epidermal cells of the leaf (Kupper et al. 2001).

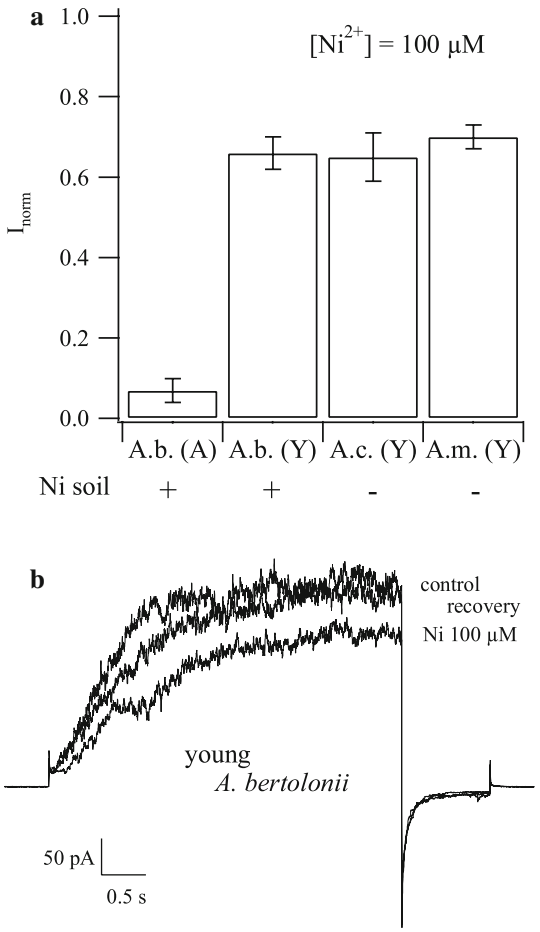

Fig. 4 **a** SV current responses ($I_{norm} = I_{Ni}/I_{control}$) of different *Alyssum* species to the presence of 100 μM $NiCl_2$ in the bath solution. Adult plants of *Alyssum bertolonii* A.b.(A) were taken from the field (Ni-rich serpentine soil; +), young plants A.b.(Y) were grown in the same Ni-rich soil in a growth chamber. Young plants of the nickel hyperaccumulator *Alyssum corsicum* A.c.(Y) and the non-hyperaccumulator *Alyssum montanum* A.m.(Y) were grown in commercial potting compost (−). Data are the mean ± SEM of 4 (A.b.) or 6 (A.c./A.m.) different experiments. **b** SV currents of a young *Alyssum bertolonii* plant grown in Ni-rich soil. Current traces were recorded in the absence of nickel (*control*), in the presence of 100 μM $NiCl_2$ in the bath solution, and after washout (*recovery*). Holding potential 0 mV, test potential +80 mV, tail potential −50 mV

Mesophyll cells are targeted to a much lower extent, presumably to protect photosynthesis against metal toxicity. Broadhurst et al. (2004) reported, however, that palisade mesophyll cells became increasingly important as a storage site when *A. murale* was grown at higher nickel concentrations. In this study, mesophyll protoplasts were used for vacuole isolation, while the epidermal cell layers had been removed. Thus, an explanation for the observed differences could be that mesophyll cells from adult plants were indeed involved in nickel accumulation while those from young plants were not.

At present one can only speculate about the physical nature (let alone physiological relevance) of the high nickel sensitivity of SV currents in adult plants. It seems rather unlikely that the SV channel (being an ion channel, not an active transporter) should participate in nickel accumulation in the vacuolar lumen against a huge concentration gradient. The transport proteins involved in vacuolar metal sequestration in plants belong to the cation diffusion facilitator (CDF) family. For example, zinc tolerance in the zinc hyperaccumulator plant *Arabidopsis halleri* has been correlated to constitutively high metal tolerance protein 1 (*MTP1*) transcript levels (Drager et al. 2004). In this context it is noteworthy that the constant of half-inhibition for SV currents of adult plants is comparable to apparent affinity constants of CDF proteins: reported values are 0.16 μM zinc for the yeast ZRC1 protein (MacDiarmid et al. 2002) and 0.30 μM zinc for the *A. thaliana* MTP1 protein (Kawachi et al. 2008). Possibly the change in Ni^{2+} affinity of the *A. bertolonii* SV channel is caused by a posttranslational modification of the TPC1 protein or by differential gene expression. For example, heterogeneity of the nickel response among animal T-type calcium channels arises from three isoforms with almost identical biophysical properties, but differential nickel sensitivities (Lee et al. 1999). Future molecular cloning studies on *A. bertolonii* will help to address these questions.

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