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SPECIAL FEATURE

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Inducing Ni sensitivity in the Ni hyperaccumulator plant *Alyssum inflatum* Nyárády (Brassicaceae) by transforming with *CAX1*, a vacuolar membrane calcium transporter

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Abstract The importance of calcium in nickel tolerance was studied in the nickel hyperaccumulator plant *Alyssum inflatum* by gene transformation of *CAX1*, a vacuolar membrane transporter that reduces cytosolic calcium. *CAX1* from *Arabidopsis thaliana* with a CaMV35S promoter accompanying a kanamycin resistance gene was transferred into *A. inflatum* using *Agrobacterium tumefaciens*. Transformed calli were subcultured three times on kanamycin-rich media and transformation was confirmed by PCR using a specific primer for *CAX1*. At least 10 callus lines were used as a pool of transformed material. Both transformed and untransformed calli were treated with varying concentrations of either calcium (1–15 mM) or nickel (0–500 μM) to compare their responses to those ions. Increased external calcium generally led to increased callus biomass, however, the increase was greater for untransformed callus. Further, increased external calcium led to increased callus calcium concentrations. Transformed callus was less nickel tolerant than untransformed callus: under increasing nickel concentrations callus relative growth rate was significantly less for transformed callus. Transformed callus also contained significantly less nickel than untransformed callus when exposed to the highest external nickel concentration (200 μM). We suggest that transformation with *CAX1* decreased cytosolic calcium and resulted in decreased nickel tolerance. This in turn suggests that, at low cytosolic calcium concentrations, other nickel tolerance mechanisms (e.g., complexation and vacuolar sequestration) are insufficient for nickel tolerance. We propose that high cytosolic calcium is an important mechanism that results in nickel tolerance by nickel hyperaccumulator plants.

Keywords Ca:Mg ratio · *CAX1* · Genetic transformation · Ni tolerance · Serpentine

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Introduction

Plants found on serpentine soils have contributed greatly to the development of ecological and evolutionary theory (Harrison and Rajakaruna 2011) and have provided model systems for the study of ecophysiology (Palm and Van Volkenburgh 2014), ecological genetics (von Wettberg and Wright 2011; Selby et al. 2014), and speciation (Kay et al. 2011). Serpentine soils are challenging habitats for most plants because they are often deficient in plant essential macronutrients, have a calcium-to-magnesium (Ca:Mg) quotient of less than 1 (often < 1:10; Rajakaruna et al. 2009), and have elevated levels of toxic heavy metals such as nickel (Ni), cadmium (Cd), cobalt (Co), and chromium (Cr) (Brady et al. 2005; Kazakou et al. 2008; O'Dell and Rajakaruna 2011). The physical characteristics of serpentine soils (which are often rocky and shallow), and particularly their generally low soil moisture retention capacity, also impose water stress on plants (Palm and Van Volkenburgh 2014). Due to these intense selective pressures, serpentine soils promote speciation and the evolution of edaphic endemism, contributing to unique floras with high rates of rarity,

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Table 1 Changes made to the MS (Murashige and Skoog) medium composition to avoid depletion of ammonium and potassium in the Ca treatments

Chemicals	Concentrations in MS medium (mM)	Concentrations in modified MS medium (mM)
NH_4NO_3	20.6	25.6
KNO_3	18.8	23.8
$CaCl_2$	3	3
KH_2PO_4	1.2	0
KCl	0	2
KH_2PO_4	0	1.2

Table 2 Nitrate changes in MS (Murashige and Skoog) medium for the treatments of Ca (mM)

Values for each treatment	Final Ca concentration in medium (treatments)				
	1	3	5	10	15
$CaCl_2$ concentration	1	3	3	3	3
Added $Ca(NO_3)_2$	0	0	2	7	12
Increase in nitrate concentration due to added $Ca(NO_3)_2$	0	0	4	14	24
Decrease in ammonium nitrate + potassium nitrate	0	0	2 + 2	7 + 7	12 + 12
Final ammonium nitrate	25.6	25.6	23.6	18.6	13.6
Final potassium nitrate	23.8	23.8	21.8	16.8	11.8

endemism, and disjunct distributions (Anacker 2011, 2014).

It is unclear whether a particular chemical or physical factor is largely responsible for serpentine tolerance (Brady et al. 2005); it is generally believed that serpentine tolerance in plants results from adaptations to a combination of chemical, physical, and biotic stressors (i.e. serpentine syndrome sensu Jenny 1980). However, the low soil Ca:Mg quotient (often < 1:10, see Bradshaw 2005; Palm et al. 2012) and high heavy metal concentrations, particularly Ni (Gabbrielli et al. 1989; Burrell et al. 2012; Doubková and Sudova 2014), have received much attention as key factors driving evolution of plant serpentine tolerance.

Plants growing on serpentine soils have a range of strategies to deal with the disproportionately low soil Ca:Mg ratio (Palm et al. 2012; Palm and Van Volkenburgh 2014). For example, some plants have a requirement for and tolerance of high Mg (Main 1981; Johnston and Proctor 1984; Asemaneh et al. 2007) while others have an enhanced ability for Ca uptake (Rajakaruna et al. 2003; O'Dell et al. 2006; Ghasemi and Ghaderian 2009; Veatch Blohm et al. 2013) or the ability to exclude Mg (O'Dell and Claassen 2006; Sambatti and Rice 2007), enabling them to survive the relatively high Mg concentrations typical of serpentine soils. The discovery of genes responsible for maintenance of plant Ca:Mg homeostasis (Li et al. 2001; Cheng et al. 2003; Turner et al. 2008, 2010; Tang et al. 2015) is now making it possible to explore the genetic basis for tolerance of low soil Ca:Mg, a key factor associated with serpentine tolerance. Recent work by Tang et al. (2015) documents a novel function of the CBL–CIPK signaling network in vacuolar sequestration of excessive Mg^{2+}, thereby helping plants to survive the high Mg^{2+} concentrations typical of serpentine soils. Cheng et al. (2003) showed that *CAX1* (a calcium-proton antiporter on the tonoplast) maintains Ca homeostasis in plant cells by pumping excess Ca from the cytoplasm into the vacuole. However, under the low soil Ca concentrations typical of serpentine soils, cytoplasmic Ca can become too low in the presence of *CAX1* activity. Bradshaw (2005) showed that *CAX1* mutants of *Arabidopsis thaliana* (lacking the allele) exhibit greater tolerance of serpentine soils due to their higher cytoplasmic Ca concentations. Whether *CAX1* mutations play a role in the adaptation of natural plant populations to serpentine soil, however, is largely unexplored.

Tolerance to heavy metals, especially Ni, has also been the focus of studies examining tolerance and adaptation to serpentine soils (Freeman et al. 2004; Ingle et al. 2005; Meindl et al. 2014; Ghasemi et al. 2015a, b). Although Ni is an essential micronutrient (Polacco et al. 2013), high levels of Ni, as found in serpentine soils, can be toxic to plants (Yusuf et al. 2011). Plants have varying strategies to deal with Ni, including exclusion or restriction of entry of Ni into the cytoplasm and chelation of Ni by phytochelatins, metallothioneins, nicotianamide, organic acids, and amino acids, followed by sequestration in the vacuole (Ahmad and Ashraf 2011; Yusuf et al. 2011; Amari et al. 2016). Some plants can hyperaccumulate Ni (defined as > 1000 μg/g dry weight leaf tissue; van der Ent et al. 2013) and the genetic basis (Pollard et al. 2002; Verbruggen et al. 2009) for and ecological and evolutionary significance (Cecchi et al. 2010; Boyd 2014; Cappa and Pilon-Smits 2014) of Ni hyperaccumulation has received much attention.

The importance of Ca in alleviating metal toxicity is also known (Aziz et al. 2015). Calcium is an essential nutrient for plant growth, development, and metabo-

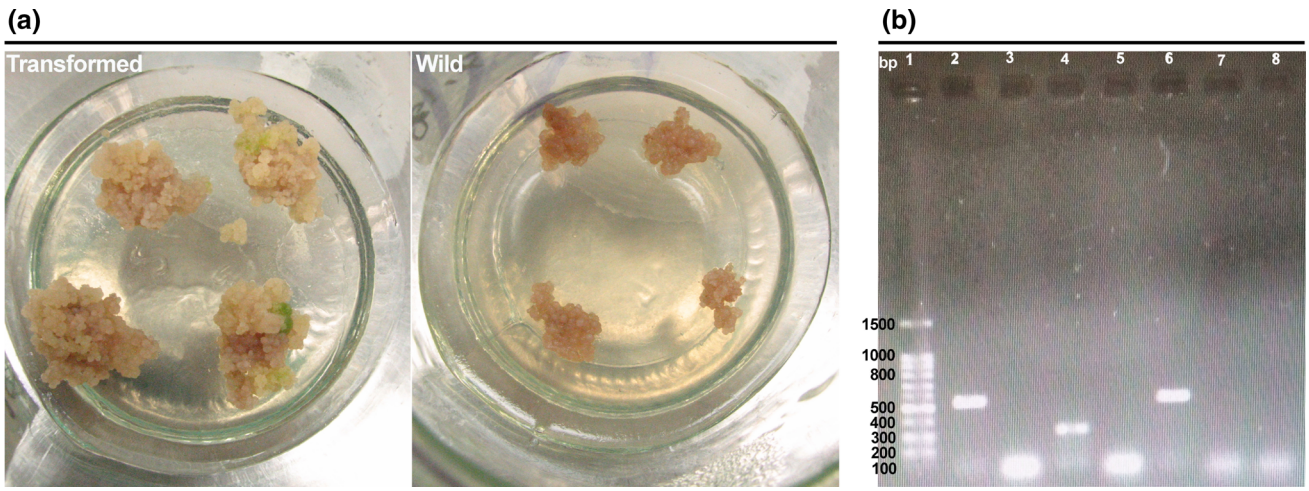

Fig. 1 **a** Transformed (left) and untransformed (right) calli of *Alyssum inflatum* on media containing 100 mg/L kanamycin. The untransformed callus has not grown after transfer onto the medium. **b** PCR detection of the multiplied fragment by using a specific primer for CAX1. Lanes (from L-R) are: 1) molecular weight marker of DNA, 2) callus of *Arabidopsis thaliana*, 3) untransformed callus of *Alyssum inflatum*, 4) transformed callus of *A. inflatum*, 5) *A. inflatum* plant, 6) *A. thaliana* plant, 7) *A. saxatile* plant, 8) negative control

lism, regulating the function of proteins and membrane transport systems as well as gene expression (Bush 1995; White and Broadley 2003; Hepler 2005). Calcium is also involved in the synthesis of glutathione, a precursor of phytochelatin, thereby contributing to the inactivation and detoxification of metal ions entering the cytoplasm (López Climent et al. 2014). An increase in Ca can therefore mitigate the deleterious effects of metals (Rengel 1992; Siddiqui et al. 2012; Eller and Brix 2016). The role of Ca in Ni tolerance has been studied in agricultural plants (Matraszek and Hawrylak Nowak 2010; Siddiqui et al. 2011; Mozafari et al. 2014) and in plants adapted to serpentine soils (Gabbrielli et al. 1989; Chaney et al. 2008). High cytosolic Ca contributes to alleviating Ni (and other metal) toxicity by increasing antioxidant enzyme activities and osmolytes such as proline (Siddiqui et al. 2011), reducing lipid peroxidation of cell membranes (Gong et al. 1997a, b; Jiang and Huang 2001; Hirschi 2004), and by heavy metal detoxification (Antosiewicz and Hennig 2004; Jáuregui Zùñiga et al. 2005). Magnesium, an important constituent for chlorophyll biosynthesis, also plays an essential role in decreasing heavy metal toxicity, both by reducing heavy metal uptake (Abul Kashem and Kawai 2007) and by enhancing antioxidant production (Chou et al. 2011).

Interactions between all three ions (Ca, Mg, and Ni) are complex. Magnesium may interfere with the uptake of Ca, Ca may reduce Mg toxicity, and both Mg and Ca may reduce Ni toxicity (Johnston and Proctor 1981; Gabbrielli and Pandolfini 1984; Heikal et al. 1989; Vergnano Gambi et al. 1992; Izosimova 2005; Chaney et al. 2008; Ghasemi and Ghaderian 2009). Plants adapted to serpentine soils provide ideal model systems to explore the interplay among these ions, particularly how serpentine plants are able to alleviate Ni toxicity under the low soil Ca:Mg ratios typical of serpentine soils. Molecular approaches can provide powerful tools to illustrate the underlying genetic mechanisms of serpentine tolerance (e.g., Turner et al. 2010; Burrell et al. 2012; Arnold et al. 2016; Porter et al. 2016).

Here, we examine the role of Ca in Ni tolerance using a Ni hyperaccumulator plant (*Alyssum inflatum*) that grows on serpentine soils in Iran. We transformed cells of *A. inflatum*, using *Agrobacterium tumefaciens* to insert the vacuolar membrane Ca^{2+}/H^{+} antiporter *CAX1* from *Arabidopsis thaliana*. We then treated cells with varying levels of Ca or Ni, measuring growth (as dry weight) and Ca and Ni concentrations in calli in response to those treatments. We sought to answer the following questions: (1) how do untransformed and transformed calli respond (in growth and callus Ca concentrations) to varying external Ca concentrations?, and (2) how do untransformed and transformed calli respond (in growth and callus Ni concentrations) to varying external Ni concentrations?

Materials and methods

Alyssum inflatum Nyárády (Brassicaceae) is a serpentine endemic perennial plant from western Iran belonging to section *Odontarrhena* (Ghasemi et al. 2009a, b; Ghasemi and Ghaderian 2009; Ghasemi et al. 2015a, b). Seeds of *A. inflatum* were harvested from serpentine soils in western Iran (N 35°, 13.625′ and E 46°, 27.184′) in 2008. Approximately 50,000 seeds were collected as a bulk sample from ~ 70 plants and mixed thoroughly. Seeds were stored at 4 °C for three months to break dormancy.

Seeds were surface-sterilized with 70% ethanol (2 min), 2.5% sodium hypochlorite (15 min) and rinsed six times (each time for 2–5 min) using distilled sterile water. Seeds were germinated and plants grown on modified Hoagland solution containing: 1 mM $Ca(NO_3)_2$, 0.5 mM KNO_3, 0.5 mM $MgSO_4$, 0.1 mM

KH_2PO_4, 10 μM H_3BO_3, 0.1 μM $ZnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM Na_2MoO_4, 2 μM $MnSO_4$, 1 μM NaCl, and 5 μM FeEDTA. Growth media were solidified using 0.85% agar–agar. Seeds and resulting seedlings were kept in a growth chamber with a 8:16 dark:light regime and temperature range of 24–26 °C.

Plasmid and bacteria

We used pBIN19, a binary disarmed vector carrying two kanamycin resistance genes for selecting transformed cells and agrobacterium, as well as the *CAX1* gene which was located in T-DNA. This plasmid was cloned in TOP10 *E. coli*. The plasmid was extracted from *E. coli* (mini-prep method; Tarczynski et al. 1994) and transferred into *Agrobacterium tumefaciens strain* GV3101 possessing a chromosomal rifampicin resistance gene and a gentamicin resistance gene on its helper plasmid. Transformation was performed according to An et al. (1988).

Plant cell transformation

Transformed *Agrobacterium* were cultured in liquid LB medium including 10 mg/L rifampicin, 10 mg/L kanamycin, and 100 mg/L acetocyringone (Ditt et al. 2001) for 18 h to reach OD600 of 0.8–1. The medium was centrifuged at 10000 rpm for 10 min, the supernatant discarded, and the pellet re-suspended in MS medium (Murashige and Skoog 1962; pH = 5.7, to OD_{600} = 1). Shoots of 35-day-old seedlings, which were used as explants, were wounded at their tips and internodes and then suspended/submerged in a solution containing the bacterium for 30 min. Explants were semi-dried using filter paper, and then put on MS medium with no added hormone or antibiotic for three days (8:16 h dark:light regime and constant 25 °C).

After three days, explants were submerged in liquid MS medium containing 150 mg/L cefotaxime for four min and then rinsed with liquid MS medium. Explants were semi-dried and sub-cultured on solidified MS medium including 300 mg/L carbenicillin and 100 mg/L kanamycin as selective antibiotics for three days at 24 °C and a 16:8 h light:dark regime.

Confirmation of transformation

Transformation was confirmed in two ways: (1) by growing calli on a selective medium, and (2) with PCR by using a *CAX1* specific primer. Calli were continually subcultured on a medium containing 100 mg/L kanamycin. As a control, some untransformed calli were also subcultured on kanamycin-supplemented media.

For PCR, the primers were designed using Primer Blast tool at NCBI. The selected primer was 5′ CCAAGCATAACGGCGAAAGG 3′ as the forward oligonucleotide and 5′ GACCACCCAATGTAGGACCG 3′ as the reverse one, based on the cDNA sequence (which was predicted to give a 343 bp product) of *CAX1* in *Arabidopsis thaliana*. PCR conditions were: first denaturing (94 °C, 4 min, 1 cycle), multiplication step including 30 cycles (each cycle including denaturing (94 °C, 1 min)), annealing (52 °C, 45 s), and extension (72 °C, 75 s), and final extension (72 °C, 5 min).

DNA extraction

Genomic DNA from *Arabidopsis thaliana*, *Alyssum inflatum*, *A. saxatile*, calli of *A. inflatum,* and transformed *A. inflatum* and *A. thaliana* was extracted according to Porebski et al. (1997). The congener *Alyssum saxatile,* and the model Brassicaceae species *A. thaliana*, obtained from Steinkraut Company, Germany and the laboratory of Ute Krämer, University of Bochum, Germany, respectively, were included as reference samples to confirm transformation of *A. inflatum* calli.

Effect of Ca on untransformed and transformed *A. inflatum* callus

Untransformed and transformed *A. inflatum* calli were grown on MS medium. The composition of the unmanipulated medium was: NH_4NO_3, 20.6 mM; H_3BO_3, 0.1 mM; $CaCl_2$, 2.99 mM; $CoCl_2$, 0.1 μM; $MgSO_4$, 1.5 mM; $CuSO_4$, 0.1 μM; KH_2PO_4, 1.25 mM; FeEDTA, 0.1 mM; KNO_3, 18.79 mM; $MnSO_4$, 10 μM; KI 5 μM; Na_2MoO_4, 1.2 μM; $ZnSO_4$, 30 μM. The Ca:Mg ratio of this medium is about 2. *Alyssum inflatum* calli were treated using solutions to vary concentrations of Ca, as $Ca(NO_3)_2$, in the MS medium to include experimental concentrations of 1, 3, 5, 10, and 15 mM. This range of Ca concentrations was selected based on previous research (Ghasemi et al. 2015a, b; Ghasemi and Ghaderian 2009). At higher concentrations (5, 10, and 15 mM) of $Ca(NO_3)_2$, the concentrations of nitrate were too high to allow normal plant growth. Therefore, modifications (shown in Tables 1 and 2) were made in the composition of the MS medium. First, concentrations of NH_4NO_3 and KNO_3 were both increased by 5 mM, to 25.6 and 23.8 mM, respectively (Table 1). This increase was done to prevent depletion of ammonium and potassium in media at higher Ca treatments. KH_2PO_4 was removed and K_2HPO_4 was added (1.2 mM) to compensate for the K depletion resulting from removing KNO_3 in some treatments. In addition, KCl was used to compensate for the Cl decrease in the 1 mM treatment (in which $CaCl_2$ was used at a low concentration). Second, for Ca treatments, as shown in Table 2, final concentrations of Ca were achieved by removing $CaCl_2$ or adding $Ca(NO_3)_2$. In the cases involving addition of $Ca(NO_3)_2$, equivalent nitrate was decreased by removing NH_4NO_3 and KNO_3 (Table 2).

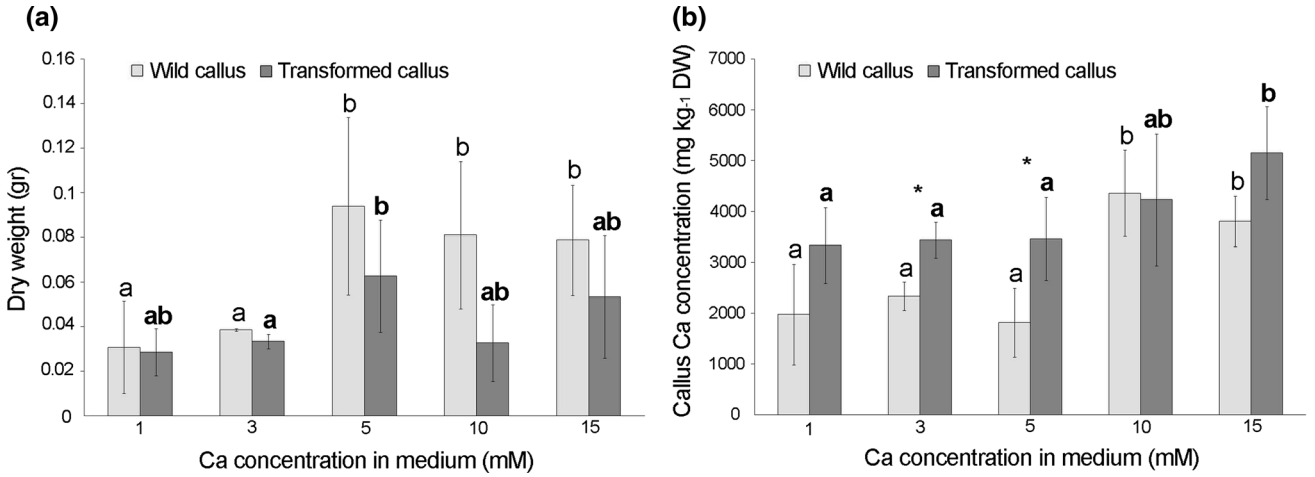

Fig. 2 Effect of Ca concentration in medium on growth of *Alyssum inflatum* untransformed (wild) and transformed callus. Values are means of 3 replicates ± SD. Different letters indicate statistically significant differences of Ca concentration within each type of callus (normal font for wild callus and bold font for transformed callus comparisons) according to Tukey's HSD ($P \leq 0.05$). **a** Results for callus dry weight data. No statistically significant difference was observed between untransformed and transformed calli at each Ca concentration (*t* test, $P \leq 0.05$). **b** Concentration of Ca (mg Ca kg^{-1} DW) in callus. *Indicates significant difference between untransformed and transformed calli at each Ca concentration in the medium (*t* test, $P \leq 0.05$)

This experiment was designed to document the response of untransformed and transformed *A. inflatum* calli to Ca concentration in the medium, using growth and Ca concentration of calli as response variables. To measure growth of calli, 0.25–0.35 g of callus was subcultured in containers and kept at 24 °C in a 8:16 h dark:light period. After 45 days, calli were dried at 70 °C for 24 h and dry mass values were recorded. Calli were desorbed using desorption solutions before digesting so that Ca measurements would reflect internal cellular concentrations rather than Ca adsorbed to cell wall components. Calli were desorbed first in an ice-cooled solution containing 1 mm MES (pH 5.7), and 5 mm $MgSO_4$ for 10 min and then with a solution containing 1 mm MES (pH 5.7), 5 mm $MgSO_4$, and 5 mm Na_2EDTA, for 5 min. Callus Ca concentrations were measured by digesting dried calli using 67% nitric acid at room temperature for 24 h and then heating solutions to 90 °C for 1 h. Suspensions were cooled to room temperature, 30% H_2O_2 was added, and then solutions were heated to 90 °C until clear. Finally, calli were rinsed in ultrapure water twice, each time for 1 min. All desorption steps were performed on ice. Calcium concentrations were measured by atomic absorption spectrophotometry (AAS, Philips, model: PU9100X, The Netherlands).

Effect of Ni on untransformed and transformed *A. inflatum* callus

The response of untransformed *A. inflatum* callus to Ni was determined by growing callus in media containing a range of Ni concentrations. Appropriate volumes of a $NiSO_4$ stock were added to generate medium Ni concentrations ranging from 0 to 500 μM (0, 25, 50, 100, 200, 300, 350, 400, 500 μM). Tolerance of Ni was documented by measuring callus dry weight and tissue Ni concentrations. Callus growth (as dry weight) and Ni concentrations were measured using the same methods as outlined above for Ca.

Statistical analyses

Analysis of Variance (ANOVA) was used to determine the effects of the various treatments and their interactions. For the experiments testing the response of untransformed and transformed calli to varying Ca and Ni concentrations, in which callus growth and callus Ca and Ni concentrations were measured, 2-way ANOVAs were used. Callus type and Ca concentration were the main effects and the interaction was included in the model. Multiple comparisons were performed by Tukey's HSD. Simple comparisons between two groups were performed by t-tests. All statistical analyses used the Statistical Package for the Social Sciences (SPSS) software (version 16).

Results

Transformation of *A. inflatum*

Transformed calli were able to grow on media containing 100 mg/L kanamycin (Fig. 1a—left) whereas untransformed calli did not grow in the presence of kanamycin (Fig. 1a—right). As shown in Fig. 1b, PCR revealed that the fragment was present in plants of *Arabidopsis thaliana*, in callus of *A. thaliana,* and in transformed callus of *Alyssum inflatum*. PCR did not

Table 3 Two-way ANOVA analyses of the effects of callus type (untransformed and transformed) and Ca concentrations in medium on callus growth and Ca concentration in calli of *Alyssum inflatum*

		Callus growth		Callus Ca concentration	
Source of changes	*df*	*F* value	*P* value	*F* value	*P* value
Callus type	1	6.11	0.015	4.67	0.008
Ca concentration	4	4.03	0.023	7.89	0.011
Callus type*Ca concentration	4	0.89	0.486	0.67	0.623

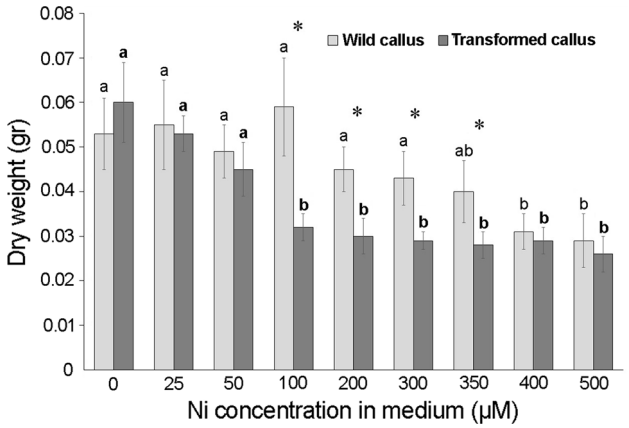

Fig. 3 Mean growth (dry weight) of untransformed (wild) and transformed *Alyssum inflatum* callus in response to varying Ni concentrations in the medium. Values are means of 3 replicates ± SD. Letters indicate statistically different means (normal font for wild callus and bold font for transformed callus comparisons) according to Tukey's HSD ($P \leq 0.05$). *Indicates significant difference between untransformed and transformed calli at each Ni concentration in the medium (*t* test, $P \leq 0.05$)

show the fragment in plants of *A. inflatum*, untransformed callus of *A. inflatum,* and plants of the congener, *A. saxatile* (Fig. 1b).

Effect of Ca on untransformed and transformed *A. inflatum* callus

Two-way ANOVA revealed significant effects of both callus type (untransformed vs. transformed) and Ca concentration in the medium on callus growth (Table 3). There was, however, no significant interaction between callus type and medium Ca concentration. As shown in Fig. 2a, growth by untransformed callus was generally greater than that of transformed callus, although growth did not differ statistically when compared at any particular medium Ca concentration. Calcium concentration in the medium also significantly affected callus dry weight, with a general trend of increasing dry weight with increases in medium Ca concentration (Fig. 2a). This trend was most pronounced for untransformed

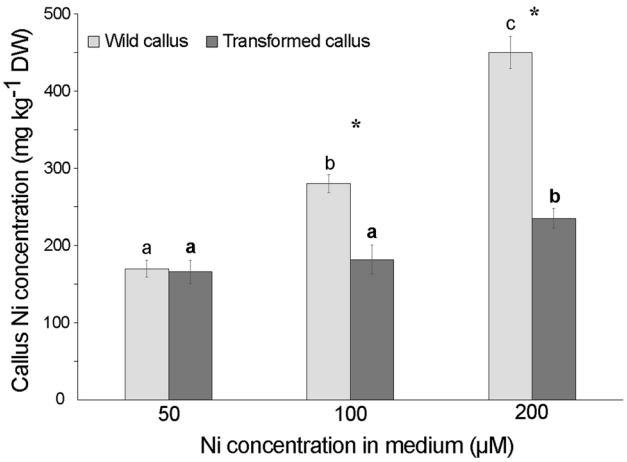

Fig. 4 Nickel concentrations in untransformed (wild) and transformed calli of *Alyssum inflatum* grown in media containing different Ni levels. Values are means of 3 replicates ± SD. Letters indicate statistically different Ni concentrations between the same type of callus (normal font for wild callus and bold font for transformed callus comparisons) treated with different Ni concentrations according to Tukey's HSD ($P \leq 0.05$). *Indicates statistically significant difference between Ni concentrations of untransformed and transformed calli at the same Ni concentration, according to a *t* test ($P \leq 0.05$)

callus, despite the lack of significance for the interaction term in the ANOVA (Table 3).

Two-way ANOVA also revealed significant effects of both callus type (untransformed vs. transformed) and Ca concentration in the medium on Ca concentration in calli (Table 3). In general, transformed callus contained higher levels of Ca, although statistical comparisons between the two callus types at each Ca concentration in the medium revealed statistically higher Ca in transformed calli only for the 3 and 5 mM Ca treatments (Fig. 2b). Not surprisingly, Ca concentration in the calli generally increased with Ca concentration in the medium (Fig. 2b). There was no significant interaction between callus type and Ca concentration in the medium (Table 3), indicating Ca concentrations in the two types of calli responded similarly to Ca in the medium.

Effect of Ni on untransformed and transformed *A. inflatum* callus

Untransformed callus was relatively Ni tolerant. As shown in Fig. 3, untransformed callus growth did not decline until Ni concentration reached 400 μM Ni in the medium. In contrast, transformed callus was comparatively sensitive to Ni and a significant decrease in growth was observed at 100 μM. Calli also differed in their Ni concentrations, depending on the Ni level in the medium. At low Ni concentration (sub-lethal level, 50 μM Ni in medium), there was no difference between Ni concentrations of untransformed and transformed calli (Fig. 4). At higher Ni concentrations in the medium (100

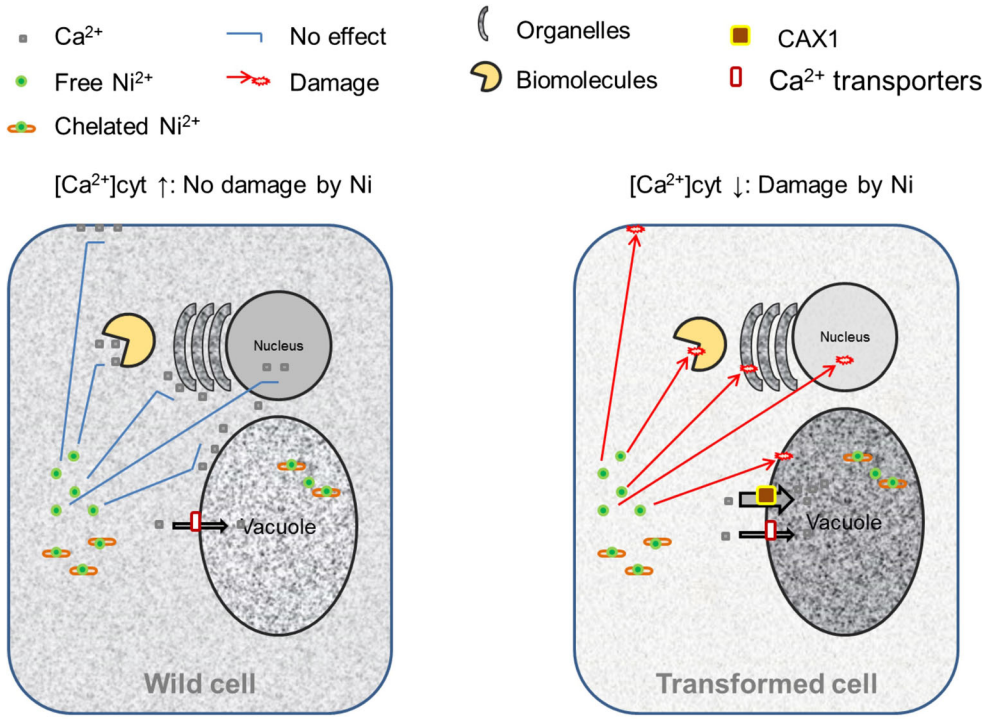

Fig. 5 Proposed model for the role of high cytosolic Ca in amelioration of Ni toxicity in the Ni hyperaccumulating plant *Alyssum inflatum*. In an untransformed cell, cytosolic Ca concentration is higher than in a transformed cell (which has a promoted *CAX1* expression). *CAX1* concentrates Ca in the vacuole, resulting in Ca removal from the cytosol. Ni ions that enter cells are partially chelated and finally sequestered in the central vacuole, but Ni that remains free could potentially harm membranes, biomolecules, organelles, and nucleic acids. High cytosolic Ca concentration protects untransformed cells from Ni toxicity whereas, in transformed cells, Ni becomes toxic due to low cytosolic Ca concentrations

and 200 µM), however, differences were significant, with less Ni in transformed calli. Nickel uptake in untransformed calli increased steadily with increased Ni in the medium, however, transformed calli only contained significantly increased Ni when Ni medium concentration was 200 µM (Fig. 4).

Discussion

The exceedingly low Ca:Mg quotients typical of serpentine soils, including in soils where *Alyssum inflatum* is found (Ghasemi et al. 2015a, b), require specialized physiological mechanisms to maintain adequate cytosolic Ca concentrations (Bradshaw 2005; Palm and Van Volkenburgh 2014). Such mechanisms may include a greater acquisition of Ca (Asemaneh et al. 2007), the exclusion of Mg (Madhok and Walker 1969; Sambatti and Rice 2007; but see Palm et al. 2012), and the restriction of Ca transport into the central vacuole, thereby maintaining high cytosolic Ca levels as suggested by Bradshaw (2005).

High cytosolic Ca can influence tolerance to heavy metals, including those that are found at high levels in serpentine soils (Yusuf et al. 2011; Gall and Rajakaruna 2013). Calcium-mediated metal tolerance appears to be influenced by cation/proton antiporters (*CAX*), a group of proteins that export cations from the cytosol to the vacuole to maintain cellular ion homeostasis (Pittman and Hirschi 2003; Cheng et al. 2005; Mei et al. 2009; Connorton et al. 2012; Punshon et al. 2012; Pittman and Hirschi 2016). Transgenic tobacco plants expressing *CAX1* display symptoms of Ca deficiency, including hypersensitivity to ion imbalances and cold shock, but increased Ca in the medium was able reverse these sensitivities (Hirschi 1999). Interestingly, Shigaki et al. (2005) show that a histidine to alanine mutation at position 338 within *Arabidopsis CAX1* can influence the transport function of *CAX1*, leading to an increase in Cd and Zn transport and a sharp decline in Ca transport. The mutants also were more tolerant of the heavy metals, providing insights into the role of *CAX1* in metal mobilization and tolerance. Baliardini et al. (2015) showed that *CAX1* expression in both roots and shoots was higher in the Cd hyperaccumulator *Arabidopsis halleri* than in two Cd-sensitive congeners, *A. lyrata* (L.) O'Kane & Al-Shehbaz and *A. thaliana*. Moreover, *CAX1* loss of function in *A. thaliana* led to higher Cd sensitivity at low concentration of Ca, higher sensitivity to methyl-viologen, and a greater accumulation of reactive oxygen species (ROS) following Cd treatment. The expression of an *Arabidopsis CAX1* in petunia plants also enhances Cd tolerance and accumulation (Wu et al. 2011). These studies confirm the roles of *CAX*

antiporters in an increasing range of cellular and physiological functions, including metal transport and tolerance, and especially of Cd (Hirschi et al. 2000; Mei et al. 2009; Pittman and Hirschi 2016).

However, there has been no evidence to date of *CAX*-mediated Ni tolerance. Unlike in previous studies of *CAX*-mediated metal tolerance, *CAX1* did not increase Ni tolerance in our experiment, suggesting that it does not act as a specific Ni transporter. To date, there is no evidence for a Ni^{2+}/H^{+} antiporter or a nucleotide-dependent Ni pump, suggesting either that the vacuole is not a major site for Ni accumulation (Brune et al. 1995; Gries and Wagner 1998) or that Ni exclusion is a mechanism of tolerance (Burrell et al. 2012). We propose that transformation of a Ni hyperaccumulator plant by *CAX1* leads to decreased cytosolic Ca in transformed cells (due to increased transport of Ca to the vacuole). Under decreased cytosolic Ca levels, other Ni tolerance mechanisms (Yusuf et al. 2011; Gall and Rajakaruna 2013) are likely inadequate for reducing Ni toxicity, especially in Ni hyperaccumulator plants. A minimum cytosolic Ca concentration may be necessary for Ni tolerance in Ni hyperaccumulators such as *A. inflatum*, even in the presence of constitutive Ni detoxifying mechanisms (Ghasemi et al. 2015a, b). This is especially critical in serpentine soils, where soil Ca is generally low compared to Mg and Ni (Bradshaw 2005; Palm et al. 2012).

Although the role of Ca in increasing metal (including Ni) tolerance has previously been documented (Chaney et al. 2008; Siddiqui et al. 2011; Aziz et al. 2015), the exact mechanisms involved in minimizing toxicity are not always elaborated. Further, there is limited work to date examining the role of *CAX1* in serpentine tolerance, including in the tolerance of Ni. A pioneering study by Bradshaw (2005) showed that *CAX1* mutants of *Arabidopsis thaliana* exhibit greater tolerance to Ca-deficient serpentine soils and he suggested this was due to higher cytoplasmic Ca (resulting from decreased transport to the vacuole). Our study demonstrates that modification of callus Ca levels is associated with change in tolerance to Ni, a metal that is found in high concentrations in serpentine soils. *Alyssum inflatum* calli transformed with *CAX1* showed an increase in Ca concentrations (Fig. 2) and a decreased tolerance to Ni compared with untransformed calli (Fig. 3). These results suggest that decreased cytosolic Ca, in response to transformation with *CAX1*, could lead to decreased Ni tolerance, suggesting that other Ni tolerance mechanisms, including complexation and vacuolar sequestration (Yusuf et al. 2011), are not sufficient for Ni tolerance at low cytosolic Ca concentrations.

We propose that serpentine tolerant and Ni-hyperaccumulating plants keep cytosolic Ca as high as possible to prevent Ni toxicity. Enhanced cytosolic Ca may be achieved via increasing Ca uptake and/or decreasing the activities of outward transporters (such as *CAX1*) which transport Ca into the apoplast or internal compartments such as vacuoles. A possible lack of *CAX1* activity in serpentine tolerant plants may allow them to maintain high cytosolic Ca despite low soil Ca and thereby tolerate the high cytosolic Ni concentrations found in Ni-hyperaccumulating plants. The lack of *CAX1* activity may also necessitate a lower requirement for Ca due to a higher sensitivity to increased Ca in serpentine-adapted plants (Ghasemi et al. 2015a, b); for example, *A. inflatum* is sensitive to both high temperature and nitrogen under higher soil Ca:Mg compared to *A. lanceolatum*, its non-serpentine congener. It is unknown if the tolerance threshold for cytosolic Ca, in general, is lower for serpentine-adapted plants than their serpentine-intolerant congeners or whether Ni tolerance in serpentine endemic plants can be achieved under lower cytosolic Ca than what would be needed for serpentine-intolerant or serpentine-tolerant but non-endemic congeners.

Our results imply that decreased cytosolic Ca resulting from transformation with *CAX1* leads to decreased Ni tolerance. This, in turn, suggests that other Ni tolerance mechanisms (including complexation and vacuolar sequestration) are not sufficient for Ni tolerance at low cytosolic Ca concentrations. As summarized in Fig. 5, we propose that high cytosolic Ca is essential for Ni tolerance in Ni hyperaccumulator plants and hypothesize that this may be a common pathway for metal tolerance in serpentine plants. However, there are several shortcomings in our approach, as well the need for additional investigation to confirm our results. Future studies that measure cytosolic and vacuolar Ca concentrations in wild-type and transformed plants will be needed to confirm our hypothesized mechanism. In addition, we limited our investigation of the role of Ca in Ni tolerance to *A. inflatum* calli: future studies should examine Ni tolerance using whole plants. Additionally, it is important to measure gene expression in both calli and transformed plants and measure cystosolic Ca in relation to Ni tolerance. It is also important to compare expression of *CAX1* mRNA and protein in *A. inflatum* (a serpentine endemic and Ni hyperaccumulator) with that in its closest non-serpentine relative (*A. lanceolatum* Baumg.) to determine whether *A. inflatum* lacks *CAX1* expression. If the serpentine endemic shows any *CAX1* expression, the *CAX1* gene can be cloned, sequenced, and examined for defects that would prevent normal *CAX1* function. *CAX1* genes cloned from any *Alyssum* taxon could also be directly tested for function in transgenic *Arabidopsis thaliana* or yeast. Finally, quantitative trait locus mapping in crosses between *A. inflatum* and *A. lanceolatum* could provide a comprehensive understanding of the genetic architecture of serpentine tolerance in *Alyssum*, identifying candidate genes (such as *CAX1*) for serpentine tolerance.

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References

Abul Kashem MDA, Kawai S (2007) Alleviation of cadmium phytotoxicity by magnesium in Japanese mustard spinach. J Soil Sci Plant Nutr 53:246–251

Ahmad MS, Ashraf M (2011) Essential roles and hazardous effects of nickel in plants. Rev Environ Contam Toxicol 214:125–167

Amari T, Lutts S, Taamali M, Lucchini G, Sacchi GA, Abdelly C, Ghnaya T (2016) Implication of citrate, malate and histidine in the accumulation and transport of nickel in *Mesembryanthemum cristallinum* and *Brassica juncea*. Ecotoxicol Environ Saf 126:122–128

An G, Ebert PR, Mitra A, Ha SB (1988) Binary Vectors. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer Academic Publishers, Great Britain, pp 1–19

Anacker BL (2011) Phylogenetic patterns of endemism and diversity. In: Harrison SP, Rajakaruna N (eds) Serpentine: the evolution and ecology of a model system. University of California Press, Berkeley, pp 49–79

Anacker BL (2014) The nature of serpentine endemism. Am J Bot 101:219–224

Antosiewicz DM, Hennig J (2004) Overexpression of LCT1 in tobacco enhances the protective action of calcium against cadmium toxicity. Environ Poll 129:237–245

Arnold BJ, Lahner B, DaCosta JM, Weisman CM, Hollister JD, Salt DE, Bomblies K, Yant L (2016) Borrowed alleles and convergence in serpentine adaptation. Proc Nat Acad Sci USA 113:8320–8325

Asemaneh T, Ghaderian SM, Baker AJM (2007) Responses to Mg/Ca balance in an Iranian serpentine endemic plant, *Cleome heratensis* (Capparaceae) and a related non-serpentine species, *C. foliosa*. Plant Soil 293:49–59

Aziz H, Sabir M, Ahmad HR, Aziz T, Rehman MZ, Hakeen KR, Ozturk M (2015) Alleviating effect of calcium on nickel toxicity in rice. Clean Soil Air Water 43:901–909

Baliardini C, Meyer C, Salis P, Saumitou-Laprade P, Verbruggen N (2015) Cation Exchanger1 cosegregates with cadmium tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in *Arabidopsis* spp. Plant Physiol 169:549–559

Boyd RS (2014) Ecology and evolution of metal-hyperaccumulator plants. In: Rajakaruna N, Boyd RS, Harris T (eds) Plant ecology and evolution in harsh environments. Nova Science Publishers, Hauppauge, pp 227–241

Bradshaw HD Jr (2005) Mutations in CAX1 produce phenotypes characteristic of plants tolerant to serpentine soils. New Phytol 167:81–88

Brady KU, Kruckeberg AR, Bradshaw HD Jr (2005) Evolutionary ecology of plant adaptation to serpentine soils. Annu Rev Ecol Evol Syst 36:243–266

Brune A, Urbach W, Dietz KJ (1995) Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmic compartmentation: a comparison of Cd, Mo, Ni and Zn stress. New Phytol 129:403–409

Burrell AM, Hawkins AK, Pepper AE (2012) Genetic analyses of nickel tolerance in a North American serpentine endemic plant, *Caulanthus amplexicaulus* var. *barbarae* (Brassicaceae). Am J Bot 99:1875–1883

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol 46:95–122

Cappa JJ, Pilon-Smits EA (2014) Evolutionary aspects of elemental hyperaccumulation. Planta 239:267–275

Cecchi L, Gabbrielli R, Arnetoli M, Gonelli C, Hasko A, Selvi F (2010) Evolutionary lineages of nickel hyperaccumulation in systematics in European Alysseae (Brassicaceae): evidence from nrDNA sequence data. Ann Bot 106:751–767

Chaney RL, Chen KY, Li YM, Angle JS, Baker AJM (2008) Effects of calcium on nickel tolerance and accumulation in *Alyssum* species and cabbage grown in nutrient solution. Plant Soil 311:131–140

Cheng NH, Pittman JK, Bronwyn JB, Shigaki T, Hirschi KD (2003) The *Arabidopsis cax1* mutant exhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transporters. Plant Cell 15:347–364

Cheng NH, Pittman JK, Shigaki T, Lachmansingh J, LeClere S, Lahner B, Salt DE, Hirschi KD (2005) Functional association of *Arabidopsis* CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol 138:2048–2060

Chou TS, Chao YY, Huang WD, Hong CY, Kao CH (2011) Effect of magnesium deficiency on antioxidant status and cadmium toxicity in rice seedlings. J Plant Physiol 168:1021–1030

Connorton JM, Webster RE, Cheng N, Pittman JK (2012) Knockout of multiple *Arabidopsis* cation/H^+ exchangers suggests isoform-specific roles in metal stress response, germination and seed mineral nutrition. PLoS ONE 7(10):e47455

Ditt RF, Nester EW, Comai L (2001) Plant gene expression response to *Agrobacterium tumefaciens*. Proc Nat Acad Sci USA 98:10954–10959

Doubková P, Sudová R (2014) Nickel tolerance of serpentine and non-serpentine *Knautia arvensis* plants as affected by arbuscular mycorrhizal symbiosis. Mycorrhiza 24:209–217

Eller F, Brix H (2016) Influence of low calcium availability on cadmium uptake and translocation in a fast-growing shrub and a metal-accumulating herb. AoB Plants 8:143

Freeman JL, Persans MW, Neiman K, Albrecht C, Peer W, Pickering IJ, Salt DE (2004) Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. Plant Cell 16:2176–2191

Gabbrielli R, Pandolfini T (1984) Effect of Mg^{2+} and Ca^{2+} on the response to nickel toxicity in a serpentine endemic and nickel accumulating species. Physiol Plant 62:540–544

Gabbrielli R, Grossi L, Vergnano O (1989) The effects of nickel, calcium and magnesium on the acid phosphatase activity of two *Alyssum* species. New Phytol 111:631–636

Gall JE, Rajakaruna N (2013) The physiology, functional genomics, and applied ecology of heavy metal-tolerant Brassicaceae. In: Lang M (ed) Brassicaceae: characterization, functional genomics and health benefits. Nova Science Publishers, New York, pp 121–148

Ghasemi R, Ghaderian SM (2009) Responses of two populations of an Iranian nickel-hyperaccumulating serpentine plant, *Alyssum inflatum* Nyar. to substrate Ca/Mg quotient and nickel. Environ Exp Bot 67:260–268

Ghasemi R, Ghaderian SM, Krämer U (2009a) Accumulation of nickel in trichomes of a nickel hyperaccumulator plant, *Alyssum inflatum*. Northeast Nat 16:81–92

Ghasemi R, Ghaderian SM, Krämer U (2009b) Interference of nickel with copper and iron homeostasis contributes to metal toxicity symptoms in the nickel hyperaccumulator plant *Alyssum inflatum*. New Phytol 184:566–580

Ghasemi R, Chavoshi ZZ, Boyd RS, Rajakaruna N (2015a) A preliminary study of the role of nickel in enhancing flowering of the nickel hyperaccumulating plant *Alyssum inflatum* Nyár. (Brassicaceae). S Afr J Bot 92:47–52

Ghasemi R, Chavoshi Z, Ghaderian SM (2015b) Stenocalcic properties in the serpentine-endemic plant *Alyssum inflatum* Nyárády. Aust J Bot 63:31–38

Gong M, Chen SN, Song YQ, Li ZG (1997a) Effect of calcium and calmodulin on intrinsic heat tolerance in relation to antioxidant systems in maize seedlings. Aust J Plant Physiol 24:371–379

Gong M, Li YJ, Dai X, Tian M, Li ZG (1997b) Involvement of calcium and calmodulin in the acquisition of HS induced thermotolerance in maize seedlings. J Plant Physiol 150:615–621

Gries GE, Wagner GJ (1998) Association of nickel versus transport of cadmium and calcium in tonoplast vesicles of oat roots. Planta 204:390–396

Harrison SP, Rajakaruna N (eds) (2011) Serpentine: the evolution and ecology of a model system. University of California Press, Berkeley, pp 49–79

Heikal MMD, Berry WL, Wallace A, Herman D (1989) Alleviation of nickel toxicity by calcium salinity. Soil Sci 147:413–415

Hepler PK (2005) Calcium: a central regulator of plant growth and development. Plant Cell 17:2142–2155
Hirschi KD (1999) Expression of *Arabidopsis CAX1* in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell 11:2113–2122
Hirschi KD (2004) The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol 136:2438–2442
Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ (2000) Expression of *Arabidopsis* CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol 124:125–133
Ingle RA, Mugford ST, Rees JD, Campbell MM, Smith JAC (2005) Constitutively high expression of the histidine biosynthetic pathway contributes to nickel tolerance in hyperaccumulator plants. Plant Cell 17:2089–2106
Izosimova A (2005) Modelling the interaction between calcium and nickel in the soil-plant system. Dissertation, Technical University of Braunschweig, Germany
Jáuregui Zùñiga D, Ferrer MA, Calderón AA, Muñoz R, Moreno A (2005) Heavy metal stress reduces the deposition of calcium oxalate crystals in leaves of *Phaseolus vulgaris*. J Plant Physiol 162:1183–1187
Jenny H (1980) The soil resource: origin and behavior. Springer, New York
Jiang Y, Huang B (2001) Effect of calcium on antioxidant activities and water relations associated with heat tolerance in two cool-season grasses. J Exp Bot 355:341–349
Johnston WR, Proctor J (1981) Growth of serpentine and non-serpentine races of *Festuca rubra* in solutions simulating the chemical conditions in a toxic serpentine soil. J Ecol 69:855–869
Johnston WR, Proctor J (1984) The effects of magnesium, nickel, calcium and micronutrients on the root surface phosphatase activity of a serpentine and nonserpentine clone of *Festuca rubra* L. New Phytol 96:95–101
Kay KM, Ward KL, Watt LR, Schemske DW (2011) Plant speciation. In: Harrison SP, Rajakaruna N (eds) Serpentine: the evolution and ecology of a model system. University of California Press, Berkeley, pp 71–95
Kazakou EP, Dimitrakopoulos G, Baker AJM, Reeves RD, Troumbis AY (2008) Hypotheses, mechanisms, and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. Biol Rev 83:495–508
Li L, Tutone AF, Drummond RSM, Gardner RC, Luan S (2001) A novel family of magnesium transport genes in *Arabidopsis*. Plant Cell 13:2761–2775
López Climent MF, Arbona V, Pérez Clemente RM, Zandalinas SI, Gómez Cadenas A (2014) Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants. Plant Biol 16:79–87
Madhok OP, Walker RB (1969) Magnesium nutrition of two species of sunflower. Plant Physiol 44:1016–1022
Main JL (1981) Magnesium and calcium nutrition of a serpentine endemic grass. Am Midl Nat 105:196–199
Matraszek R, Hawrylak Nowak B (2010) Growth and mineral composition of nickel-stressed plants under conditions of supplementation with excessive amounts of calcium and iron. J Toxicol Environ Health A 73:1260–1273
Mei H, Cheng NH, Zhao J, Park S, Escareno RA, Pittman JK, Hirschi KD (2009) Root development under metal stress in *Arabidopsis thaliana* requires the H^+/cation antiporter CAX4. New Phytol 183:95–105
Meindl GA, Bain DJ, Ashman TL (2014) Nickel accumulation in leaves, floral organs and rewards varies by serpentine affinity. AoB Plants 6:plu036
Mozafari H, Asrar Z, Rezanejad F, Pourseyedi S, Yaghoobi MM (2014) Oxidative stress tolerance by calcium and histidine in two tomato cultivars under nickel stress. J Stress Physiol Biochem 10:102–124
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
O'Dell RE, Claassen VP (2006) Serpentine and nonserpentine *Achillea millefolium* accessions differ in serpentine substrate tolerance and response to organic and inorganic amendments. Plant Soil 279:253–269
O'Dell RE, Rajakaruna N (2011) Intraspecific variation, adaptation, and evolution. In: Harrison SP, Rajakaruna N (eds) Serpentine: the evolution and ecology of a model system. University of California Press, Berkeley, pp 97–137
O'Dell RE, James JJ, Richards JH (2006) Congeneric serpentine and nonserpentine shrubs differ more in leaf Ca: Mg than tolerance of low N, low P, or heavy metals. Plant Soil 280:49–64
Palm ER, Van Volkenburgh E (2014) Physiological adaptations of plants to serpentine soil. In: Rajakaruna N, Boyd RS, Harris T (eds) Plant ecology and evolution in harsh environments. Nova Science Publishers, Hauppauge, pp 129–148
Palm E, Brady K, Van Volkenburgh EV (2012) Serpentine tolerance in *Mimulus guttatus* does not rely on exclusion of magnesium. Funct Plant Biol 39:679–688
Pittman JK, Hirschi K (2003) Don't shoot the (second) messenger: endomembrane transporters and binding proteins modulate cytosolic Ca^{2+} levels. Curr Opin Plant Biol 6:257–262
Pittman JK, Hirschi KD (2016) CAX-ing a wide net: cation/H + transporters in metal remediation and abiotic stress signaling. Plant Biol 18:741–749
Polacco JC, Mazzafera P, Tezotto T (2013) Nickel and urease in plants: still many knowledge gaps. Plant Sci 199–200:79–90
Pollard AJ, Powell KD, Harper FA, Smith JAC (2002) The genetic basis of metal hyperaccumulation in plants. Critic Rev Plant Sci 21:539–566
Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep 15:8–15
Porter SS, Chang PL, Conow CA, Dunham JP, Friesen ML (2016) Association mapping reveals novel serpentine adaptation gene clusters in a population of symbiotic *Mesorhizobium*. ISME J 11:248–262
Punshon T, Hirschi K, Yang J, Lanzirotti A, Lai B, Guerinot ML (2012) The role of CAX1 and CAX3 in elemental distribution and abundance in *Arabidopsis* seed. Plant Physiol 158:352–362
Rajakaruna N, Yaeesh Siddiqi M, Whitton J, Bohm BA, Glass ADM (2003) Differential responses to Na^+/K^+ and Ca^{2+}/Mg^{2+} in two edaphic races of the *Lasthenia californica* (Asteraceae) complex: a case for parallel evolution of physiological traits. New Phytol 157:93–103
Rajakaruna N, Harris TB, Alexander EB (2009) Serpentine geoecology of eastern North America: a review. Rhodora 111:21–108
Rengel Z (1992) Role of calcium in aluminum toxicity. New Phytol 121:499–513
Sambatti JBM, Rice KJ (2007) Functional ecology of ecotypic differentiation in the Californian serpentine sunflower (*Helianthus exilis*). New Phytol 175:107–119
Selby JP, Jeong AL, Toll K, Wright KM, Lowry DB (2014) Methods and discoveries in the pursuit of understanding the genetic basis of adaptation to harsh environments in *Mimulus*. In: Rajakaruna N, Boyd RS, Harris T (eds) Plant ecology and evolution in harsh environments. Nova Science Publishers, Hauppauge, pp 243–266
Shigaki T, Barkla BJ, Miranda-Vergara MC, Zhao J, Pantoja O, Hirschi KD (2005) Identification of a crucial histidine involved in metal transport activity in the *Arabidopsis* cation/H^+ exchanger CAX1. J Biol Chem 280:30136–30142
Siddiqui MH, Al-Whaibi MH, Basalah MO (2011) Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. Protoplasma 248:503–511
Siddiqui MH, Al Whaibi MH, Sakran AM, Basaleh MO, Ali HM (2012) Effect of calcium and potassium on antioxidant system of *Vicia faba* L. under cadmium stress. Int J Mol Sci 13:6604–6619

Tang R-J, Zhao F-G, Garcia VJ, Kleist TJ, Yang L, Zhang H-X, Luan S (2015) Tonoplast CBL–CIPK calcium signaling network regulates magnesium homeostasis in *Arabidopsis*. Proc Nat Acad Sci USA 112:3134–3139

Tarczynski MC, Meyer WJ, Min JJ, Wood KA, Hellwig RJ (1994) Two-minute miniprep method for plasmid DNA isolation. Biotechniques 16:514–519

Turner TL, von Wettberg EJ, Nuzhdin SV (2008) Genomic analysis of differentiation between soil types reveals candidate genes for local adaptation in *Arabidopsis lyrata*. PLoS ONE 3(9):e3183

Turner TL, Bourne EC, von Wettberg EJ, Hu TT, Nuzhdin SV (2010) Population resequencing reveals local adaptation of *Arabidopsis lyrata* to serpentine soils. Nat Genet 42:260–263

Van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H (2013) Hyperaccumulators of metal and metalloid trace elements: facts and fiction. Plant Soil 362:319–334

Veatch Blohm ME, Roche BM, Campbell M (2013) Evidence for cross-tolerance to nutrient deficiency in three disjunct populations of *Arabidopsis lyrata* ssp. *lyrata* in response to substrate calcium to magnesium ratio. PLoS ONE 8(5):e63117

Verbruggen N, Hermans C, Schat H (2009) Molecular mechanisms of metal hyperaccumulation in plants. New Phytol 181:759–776

Vergnano Gambi O, Gabbrielli R, Pandolfini T (1992) Some aspects of the metabolism of *Alyssum bertolonii* Desv. In: Baker AJM, Proctor J, Reeves RD (eds) The vegetation of ultramafic (serpentine) soils. Intercept, Andover, pp 319–332

Von Wettberg EJ, Wright JW (2011) Genomic approaches to understanding adaptation. In: Harrison SP, Rajakaruna N (eds) Serpentine: the evolution and ecology of a model system. University of California Press, Berkeley, pp 139–153

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92:487–511

Wu Q, Shigaki T, Williams KA, Han JS, Kim CK, Hirschi KD, Park S (2011) Expression of an *Arabidopsis* Ca^{2+}/H^{+} antiporter CAX1 variant in petunia enhances cadmium tolerance and accumulation. J Plant Physiol 168:167–173

Yusuf M, Fariduddin Q, Hayat S, Ahmad A (2011) Nickel: an overview of uptake, essentiality and toxicity in plants. B Environ Contam Tox 86:1–17