

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

**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 con-

tained 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

### 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 <sub>4</sub> NO <sub>3</sub>	20.6	25.6
KNO <sub>3</sub>	18.8	23.8
CaCl <sub>2</sub>	3	3
KH <sub>2</sub> PO <sub>4</sub>	1.2	0
KCl	0	2
KH <sub>2</sub> PO <sub>4</sub>	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 <sub>2</sub> concentration	1	3	3	3	3
Added Ca(NO <sub>3</sub> ) <sub>2</sub>	0	0	2	7	12
Increase in nitrate concentration due to added Ca(NO <sub>3</sub> ) <sub>2</sub>	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).

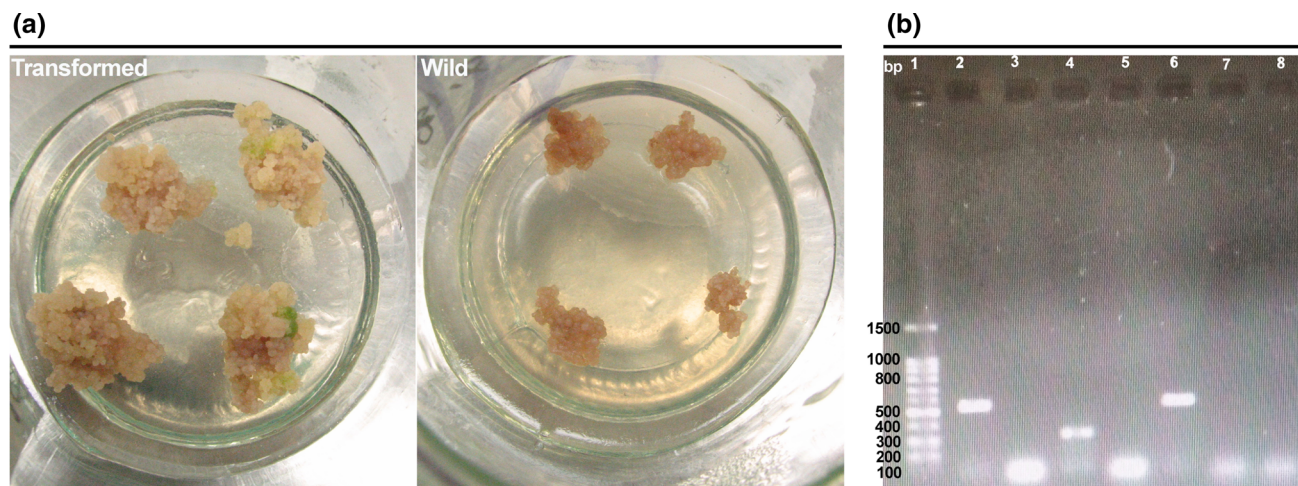
It is unclear whether a particular chemical or physical factor is largely responsible for serpentine tolerance (Brady et al. 2005); it is generally believed that serpentine tolerance in plants results from adaptations to a combination of chemical, physical, and biotic stressors (i.e. serpentine syndrome 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; Doubkova 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<sup>2+</sup>, thereby helping plants to survive the high Mg<sup>2+</sup> 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 concentrations. 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 phytochelatin, metallothioneins, nictianamide, 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 (Lopez 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; Jauregui Zúñiga 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  $\text{Ca}^{2+}/\text{H}^{+}$  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* Nyarady (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  $\text{Ca}(\text{NO}_3)_2$ , 0.5 mM  $\text{KNO}_3$ , 0.5 mM  $\text{MgSO}_4$ , 0.1 mM



KH<sub>2</sub>PO<sub>4</sub>, 10 µM H<sub>3</sub>BO<sub>3</sub>, 0.1 µM ZnSO<sub>4</sub>, 0.1 µM CuSO<sub>4</sub>, 0.1 µM Na<sub>2</sub>MoO<sub>4</sub>, 2 µM MnSO<sub>4</sub>, 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 OD<sub>600</sub> 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<sub>600</sub> = 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

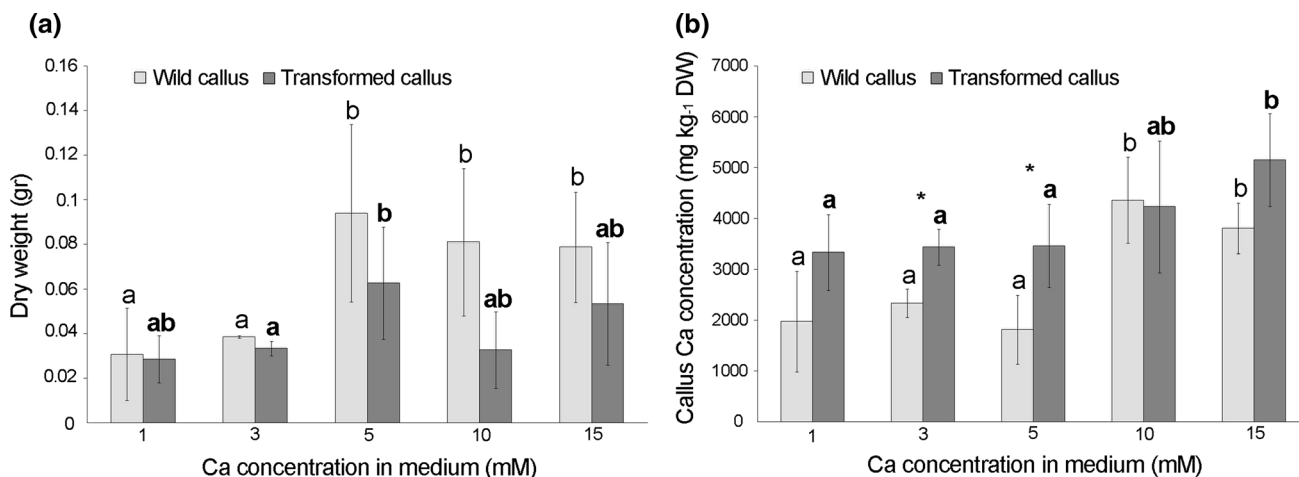
oligonucleotide and 5' GACCACCCAATGTAG-GACCG 3' as the reverse one, based on the cDNA sequence (which was predicted to give a 343 bp product) of *CAX1* in *Arabidopsis thaliana*. PCR conditions were: first denaturing (94 °C, 4 min, 1 cycle), multiplication step including 30 cycles (each cycle including 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<sub>4</sub>NO<sub>3</sub>, 20.6 mM; H<sub>3</sub>BO<sub>3</sub>, 0.1 mM; CaCl<sub>2</sub>, 2.99 mM; CoCl<sub>2</sub>, 0.1 µM; MgSO<sub>4</sub>, 1.5 mM; CuSO<sub>4</sub>, 0.1 µM; KH<sub>2</sub>PO<sub>4</sub>, 1.25 mM; FeEDTA, 0.1 mM; KNO<sub>3</sub>, 18.79 mM; MnSO<sub>4</sub>, 10 µM; KI 5 µM; Na<sub>2</sub>MoO<sub>4</sub>, 1.2 µM; ZnSO<sub>4</sub>, 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<sub>3</sub>)<sub>2</sub>, 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<sub>3</sub>)<sub>2</sub>, 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<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub> 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<sub>2</sub>PO<sub>4</sub> was removed and K<sub>2</sub>HPO<sub>4</sub> was added (1.2 mM) to compensate for the K depletion resulting from removing KNO<sub>3</sub> in some treatments. In addition, KCl was used to compensate for the Cl decrease in the 1 mM treatment (in which CaCl<sub>2</sub> was used at a low concentration). Second, for Ca treatments, as shown in Table 2, final concentrations of Ca were achieved by removing CaCl<sub>2</sub> or adding Ca(NO<sub>3</sub>)<sub>2</sub>. In the cases involving addition of Ca(NO<sub>3</sub>)<sub>2</sub>, equivalent nitrate was decreased by removing NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub> (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  $\pm$  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<sup>-1</sup> 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<sub>4</sub> for 10 min and then with a solution containing 1 mM MES (pH 5.7), 5 mM MgSO<sub>4</sub>, and 5 mM Na<sub>2</sub>EDTA, 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<sub>2</sub>O<sub>2</sub> 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<sub>4</sub> stock were added to generate medium Ni concentrations ranging from 0 to 500  $\mu$ M (0, 25, 50, 100,

200, 300, 350, 400, 500  $\mu$ M). 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).

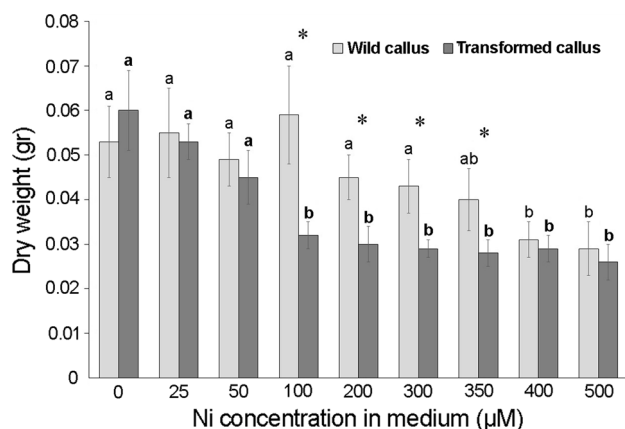
## Results

### 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*

Source of changes	df	Callus growth		Callus Ca concentration	
		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

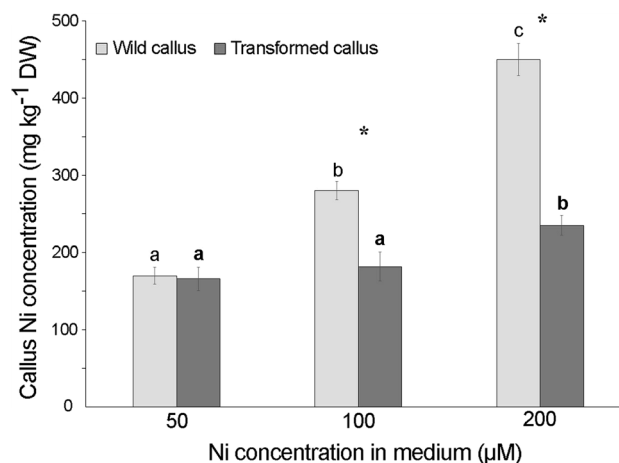


**Fig. 3** Mean growth (dry weight) of untransformed (wild) and transformed *Alyssum inflatum* callus in response to varying Ni concentrations in the medium. Values are means of 3 replicates  $\pm$  SD. Letters indicate statistically different means (normal font for wild callus and bold font for transformed 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



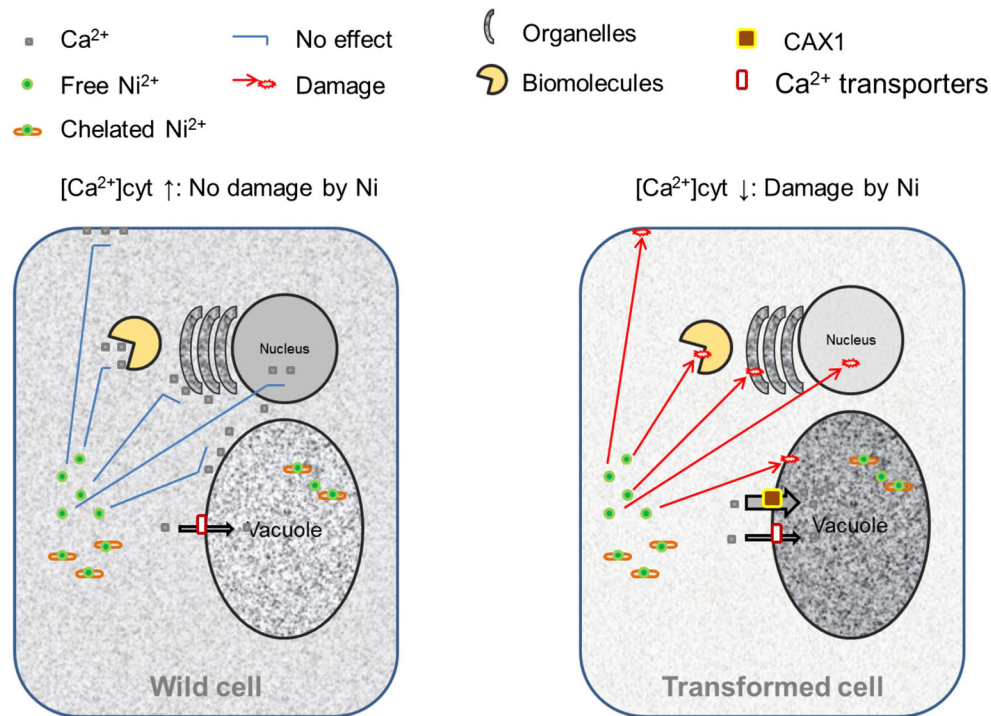
**Fig. 4** Nickel concentrations in untransformed (wild) and transformed calli of *Alyssum inflatum* grown in media containing different Ni levels. Values are means of 3 replicates  $\pm$  SD. Letters indicate statistically different Ni concentrations between the same type of callus (normal font for 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  $\mu$ M Ni in the medium. In contrast, transformed callus was comparatively sensitive to Ni and a significant decrease in growth was observed at 100  $\mu$ M. Calli also differed in their Ni concentrations, depending on the Ni level in the medium. At low Ni concentration (sub-lethal level, 50  $\mu$ M 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  $\mu\text{M}$ ), 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  $\mu\text{M}$  (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  $\text{Ni}^{2+}/\text{H}^{+}$  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-hyperaccumulator 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 cytosolic 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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