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Characterization of a cDNA from *Beta maritima* that confers nickel tolerance in yeast

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ABSTRACT

Nickel is an essential micronutrient due to its involvement in many enzymatic reactions as a cofactor. However, excess of this element is toxic to biological systems. Here, we constructed a cDNA library from *Beta maritima* and screened it in the yeast system to identify genes that confer resistance to toxic levels of nickel. A cDNA clone (*NIC6*), which encodes for a putative membrane protein with unknown function, was found to help yeast cells to tolerate toxic levels of nickel. A GFP fused form of Nic6 protein was localized to multivesicular structures in tobacco epidermal cells. Thus, our results suggest a possible role of Nic6 in nickel and intracellular ion homeostasis.

1. Introduction

At the end of the 1970's there was enough evidence showing that nickel is an essential element for diverse organisms including bacteria and animals (Eskew et al., 1984). For higher plants, such correlation was only confirmed at the end of the 1980s, and since then nickel has been considered as an essential element for higher plant growth (Eskew et al., 1984).

Nickel, mainly found in soil as in ionic form (Ni^{2+}) (Sajwan et al., 1996), enters to the plant cells mostly via root tissue, and then transported to the various plant organs (Sajwan et al., 1996). The uptake of nickel from soil is driven by active and passive transport, but the balance between these two systems was determined depending on to the soil nickel and pH levels (Peralta-Videa et al., 2002; Seregin and Kozhevnikova, 2006). High pH levels (pH > 8) and existence of different metal ions in soil were reported to have an inhibitory effect on nickel uptake (Yusuf et al., 2011), and this might select for active transport over passive diffusion. Even though main rout of nickel translocation in plants is from roots to leaves, direct entry of nickel through the leaf tissue is an alternative and highly dynamic strategy Sajwan and colleagues showed that, 37% of the nickel introduced via leaf tissue, is transported into different tissues (Sajwan et al., 1996).

The primary role of nickel is its ability to bind to some specific enzymes as a co-factor, and subsequently bring them to functionally active state (Watt and Ludden, 1999). The first identified nickel enzyme was Jack Bean urease which requires nickel as an essential element for its activity (Dixon et al., 1975). Urease converts urea to ammonia and carbamate by using water and while doing this it requires nickel in its active site. When plants are grown in soils containing urea as a nitrogen source, nickel becomes essential for the integrity of these plants; without nickel supply, urease cannot form its functionally active state, and this leads to the accumulation of urea as a highly toxic component (Eskew et al., 1984). Beside urease, enzymes such as hydrogenases, methyl coenzyme M reductase, CO dehydrogenase are defined as nickel enzymes with nickel biding sites, and all of these enzymes have bacterial origins (Walsh and Ormejohnson, 1987).

Until now, studies about nickel toxicity in plants have mostly focused on nickel hyperaccumulators that are capable of accumulating extremely high levels of nickel in their cell compartments. In nature, about 300 plant species have been identified as nickel hyperaccumulators (Boominathan and Doran, 2002). The strategies to produce plants that are resistant to toxic levels of nickel mainly depend on understanding the genetic and biochemical features of these hyperaccumulators.

Organisms have conserved mechanisms to detoxify heavy metals. Toxic metals can be pumped out of cells, their influx can be slowed down or they can be sequestered in special compartments such as vacuoles. Furthermore, increasing the antioxidant capacity of cells also helps the detoxification of heavy metals (Dietz et al., 1999).

The most effective way of detoxification involves specific transport proteins localized in the membranes. Recent studies have identified some genes encoding transport proteins from hyperaccumulators (Freeman et al., 2005; Mizuno et al., 2005) that are tested for their nickel transport activities in unicellular model organisms, like *Escherichia coli* and *Saccharomyces cerevisiae*. There are nickel transport proteins

Abbreviations: CDF, cation diffusion facilitator; CASP, casparian strips; MTP, metal tolerance protein; GFP, green fluorescence protein; ICP-MS, inductively coupled plasma mass spectrometry; CLSM, confocal laser scanning microscopy.

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identified in maize, tobacco and Arabidopsis (Arazi et al., 1999; Forzani et al., 2001; von Wiren et al., 2006). These transporters have also been tested for their abilities to transport other metal ions such as Zn, Cd, Mo, Fe, Pb, and have been shown to be capable of transporting some of these metals (Forzani et al., 2001; Mizuno et al., 2005; von Wiren et al., 2006).

In addition to the mechanisms mentioned above, secretory pathways, besides their involvement in crucial biological processes (protein folding, translocation, and endocytosis), are also shown to have important roles in heavy metal detoxification. The endoplasmic reticulum, Golgi, and small vesicles detoxify metals by removing them from the cytoplasm (Durr et al., 1998; Wu et al., 2002). For example, the Eca1 protein located in the endoplasmic reticulum leads to the uptake of manganese and confers manganese tolerance to the cells (Wu et al., 2002). *Arabidopsis thaliana* AtMTP11 (a member of Cation Diffusion Facilitator gene family) is another important example to highlight the importance of secretory pathway induced heavy metal detoxification system in plant cells (Peiter et al., 2007). AtMTP11 colocalizes with the Golgi-like compartments, and functions in removing manganese from cytosol into the endomembrane structures, and eventually pumps the element out of the cell via exocytosis (Peiter et al., 2007). Same researchers have also shown that *Populus trichocarpa* homolog of Arabidopsis Mtp11 protein, PtMtp11, function in the same manner (Peiter et al., 2007). Recently, a cDNA library study performed to identify *Beta maritima* manganese detoxification genes, added two more homologs to the *MTP11* genes: *BmMTP10* and *BmMTP11*. These two proteins have also been shown to localize and function in similar patterns to those Mtp11 proteins (Erbasol et al., 2013). Finally, in a very recently published study, researchers identified *OsMTP8.1*, which is the rice homolog of *AtMTP11*, and reported that *OsMTP8.1* confers manganese tolerance via vacuole sequestering of the same element (Chen et al., 2013).

Recent studies on manganese tolerance clearly show the robustness of cDNA library based studies to identify heavy metal detoxification genes in plant species (Chen et al., 2013; Erbasol et al., 2013; Papoyan and Kochian, 2004; Pence et al., 2000). Apart from that, these studies have also shown the evolutionary importance and convergence of the secretory pathway proteins' significant involvement on the heavy metal detoxification (Chen et al., 2013; Erbasol et al., 2013; Peiter et al., 2007). Even though all these different plants do not share the same environment, and adapted to different levels of soil manganese levels, they all do have similar mechanism, and even share highly identical protein sequences to cope with heavy metal stress (Chen et al., 2013; Erbasol et al., 2013; Peiter et al., 2007).

Nickel, although is an essential element for plant growth, our knowledge on the nickel transport proteins is poor. With this motivation, we wanted to identify nickel homeostasis genes, in a plant (*Beta vulgaris subspecies maritima*) that has recently been shown to share conserved mechanisms to detoxify manganese (Erbasol et al., 2013), and is readily adapted to abiotic stress environments such as saline, manganese (Doney, 1993; Sillanpää, 1982). Thus we constructed a cDNA library of *Beta maritima*, and screened this library in the yeast system. We have identified *NIC6* as a nickel tolerance gene, and a GFP fusion of Nic6 localized to multivesicular structures of tobacco cells and possibly acts in secretory pathway dependent nickel detoxification.

2. Materials and methods

2.1. Yeast growth and media

The yeast *S. cerevisiae* strain BY4741 (*MATa his3 leu2 met15 ura3*) was used in the experiments. Yeast cells were grown in either YPD media (with 2% glucose, 2% peptone, 1% yeast extract and 2% agar) or YNB (Yeast Nitrogen Base) minimal media including required amino acids and bases. For the solid media growth assays, wild type yeast cells were transformed with either empty vector pAG426GPD or *NIC6* cDNA containing pAG426GPD over-expression vector. Yeast cells (shaken at 200 rpm, at 30 °C), after overnight incubation, were diluted to $OD_{600} = 0.2, 0.02, 0.002, 0.0002$ with sterile water, and five microliters of these cultures was transferred to spots on solid YNB (–ura; 2% glucose) plates without $NiCl_2$ or with $NiCl_2$ (with 5 different nickel concentrations between 500 and 1700 μM). Cells were incubated for five days at 30 °C and plates were photographed.

2.2. Cloning and sequence analyses of NIC6 gene

Cloning of the *NIC6* cDNA was done using the Gateway Technology (Invitrogen, Karlsruhe, Germany). For expression purposes, the pAG426GPD (Addgene, USA) overexpression vector was used. Yeast transformation has been performed using the standard lithium acetate method (Kaiser et al., 1994). Blast searches were performed via the BLAST service of NCBI and for the multiple protein sequence alignments the T-coffee 6.85 (Notredame et al., 2000) software was used. The phylogenetic tree was calculated via MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and Newick format result of tree were given to TreeDyn 198.3 (Chevenet et al., 2006) for tree rendering. TMHMM Server v. 2.0 was used for prediction of membrane domains (Sonnhammer et al., 1998).

2.3. Intracellular nickel measurements

Intracellular nickel concentrations were measured as described by Mizuno (Mizuno et al., 2005). Pre-cultured yeast cells containing empty-vector or vector overexpressing *NIC6* cDNA were grown overnight. Diluted solutions of cells were prepared at a ratio of 1/500 in new YNB–ura medium that had been supplemented with 800 μM $NiCl_2$ and incubated for 30 h. Then, cells were washed two times with 25 mM EDTA and then were dried at 70 °C for 10 h. 5 milligrams of yeast cells was extracted using HCl, filtered, 1:10 dilutions of filtrate were measured by ICP-MS, and result was given as ppb.

2.4. Plant growth, RNA isolation, and real-time PCR analyses

B. maritima plants were grown in half-strength Hoagland Solution (Hoagland and Arnon, 1950) in a growth chamber under conditions of 12 h of dark and 12 h of light at 25 °C. Earlier studies have shown that nickel treatment between 50 μM and 200 μM to diverse plant species (such as wheat, soybean, Brassica juncea, and Triticum aestivum) resulted in significant toxic effects on plant growth (for a detailed review, see Yusuf et al., 2011) those resulted in reduced shoot growth/mass, lowered shoot length, decreased dry mass, reduced germination rates, chlorosis and necrosis phenotypes etc. We have applied 75 μM $NiCl_2$,which is not toxic level, was applied to the plants for seven days, leaf and root samples were collected every 24 h. Total RNA isolation was performed using the Invitrogen RNA Isolation Kit (Invitrogen, Germany). DNase-treated total RNAs were used for cDNA synthesis using a cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Maxima™ SYBR Green qPCR Master Mix was used (Fermentas, Germany) to perform the quantitative expression analyses of cDNAs using an IQ5 real-time PCR cycler system (Bio-rad, Germany). The Real-Time PCR primers used for amplification of *NIC6* cDNA were NIC6RTF 5′ AGCCTATCGTCC CACCAAAGTTGT-3′ and NIC6RTR 5′ ACATGGGTGTTCCCTTTCAGACCA-3′ which are concurrently used to amplify 130 bp of cDNA. Real-time PCR primers for beta actin cDNA were: BmActRTF: 5′-AGACCTTCAATGTGCC TGCT-3′ and BmActRTR 5′-TCAGTGAGATCACGACCAGC-3′, and amplified 187 bp of *B. maritima* beta actin cDNA. The conditions for PCR amplification were as follows: 95 °C 5 min, 40 cycles 94 °C for 25 s, 59 °C for 25 s, and 72 °C for 30 s.

2.5. Construction of pENTR4™ vector

The coding sequence for *NIC6* was amplified by PCR from pAG426GPD using forward-primer 5′-AGACCATGGCTTCGATGGAGACAGAAAAGGG-3′ and backward-primer 5′-AGACTCGAGTGGTATTTTGGGATGCGACGG-3′. The PCR primers used for amplification of Arabidopsis (*AT4G15610*) cDNA were AtNiGW6F 5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTATG GGTTATGAAACCAAATCGA-3′ and AtNiGW6RGFP 5′ GGGACCACTTTGTA CAAGAAAGCTGGGTCTCAACGGATTTTCTTGTAGAGG-3′.

Following a purification step the amplified gene fragment was digested with the restriction endonucleases *NcoI* and *XhoI* (restriction sites are underlined in primer sequences) and subsequently inserted in the corresponding restriction sites of pENTR4™ GATEWAY cloning vector® (Invitrogen).

2.6. Construction of pBatTL vector

For the construction of the GATEWAY®-compatible binary pBatTL plant expression vector the Venus fragment was amplified by PCR using forward-primer 5′-AGAAGATCTATGGTTAGCAAAGGAGAAGAAC-3′ and reverse-primer 5′-AGAAGATCTAAGATCCTCCTCAGAAATCAACTTTTGCTC TTTGTATAGTTCATCCATGCC-3′ for N-terminal Venus (restriction sites are underlined and the myc sequences are shown in italics). The corresponding fragment was then inserted into the *Bgl*II site of the Gateway (GW) vector for N-terminal fusions to obtain pBatTL-Venus-(GW). The *NIC6* construct was introduced into the pBatTL vector by recombination and verified by sequencing (Sanger, 1977) using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

2.7. Transient expression in plants

Transient expression of the fluorescence construct was carried out in intact plant leaves of 4-week-old *Nicotania benthamiana* by co-infiltration with *Agrobacterium tumefaciens* strain GV3101 containing the Venus-*NIC6* construct in combination with the C58C1 strain carrying the pCH32 helper plasmid which contains the RNA silencing suppressor protein p19 of tomato bushy stunt virus (Voinnet et al., 2003). Fluorescence was observed 3 days after infiltration by confocal laser scanning microscopy (CLSM) with a Leica DMIRE2 microscope (Leica Microsystems) using the excitation/emission wavelengths of 514/540–580 nm.

2.8. GFP fusion protein localization in yeast

Full-length coding sequences for *NIC6* were amplified using the following primers, NiGW6F 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTA TGTCATCGATGGAGACAGAAAAGG-3′ and NiGW6RGFP-5′-GGGGACCA CTTTGTACAAGAAAGCTGGGTCGTATTTTG GGATGCGACGGTAAAG-3′ that contained the Gateway-compatible attachment sites. Following the Gateway protocol (Invitrogen), this PCR fragment was cloned into destination vector PAG413GPD-ccdB-EGFP (Addgene). The resulting plasmid was transformed into yeast. Fluorescence was observed 3 days after infiltration by confocal laser scanning microscopy (CLSM) with a Leica DMIRE2 microscope (Leica Microsystems).

3. Results

3.1. Identification of NIC6 as a nickel tolerance gene

We screened cDNA library of *B. maritima* to identify plant genes that confer nickel tolerance to yeast. Transformed yeast cells were plated onto selective media containing 1500 μM of nickel, which is a toxic level for wild type cells, and only three colonies were able to grow. After streaking these colonies out onto new nickel plates we have decided to continue with one candidate which confirmed growth phenotype on toxic level of nickel media. Plasmids were recovered from these transformed cells, sequenced with vector-based primers and the cDNA, named as *NIC6* (ADA71986), was recovered. Spotting assay results confirmed that *NIC6* overexpressing cells were able to grow in the presence of 1700 μM nickel (Fig. 1). The *NIC6* open reading frame consists of 582 base pairs and encodes for a polypeptide, which consists of 194 amino acids. Domain analyses of this polypeptide suggested Nic6 as a membrane protein with four trans membrane domains. The Blastp result of Nic6 showed homology with a putative integral membrane protein. Alignment result and phylogenetic relations within these 6 homologous proteins are given in Figs. 2A and B. Out of these six homolog proteins, only Ab267825 (common name InPsr26p) has a known function, which plays role in programmed cell death in the *Ipomoea nil* plant (Shibuya et al., 2009). We also searched for similar proteins in *A. thaliana* to see if there are any homolog proteins with known functions. All of the identical proteins are likely to be integral membrane proteins but the functions of these proteins are unknown. One Arabidopsis homolog (*AT4G15610*) is also overexpressed in yeast cells to see whether this homolog also has a nickel detoxification role, but *AT4G15610* overexpressing yeast cells did not show any nickel tolerant phenotype (Data not shown).

3.2. Characterization of Nic6 activity

Genes playing role in heavy metal stress tolerance often provide cross resistance to many metal ions. In order to test whether Nic6 plays role in tolerance to other metals, we treated the transformants with toxic levels of cobalt, cadmium, and zinc. As seen in Figs. 3A, B, and C, *NIC6* overexpressing cells were not resistant to cobalt, cadmium, and zinc treatments. We also measured intracellular nickel concentration of yeast cells (either containing an empty-vector or *NIC6* overexpressing vector) treated with nickel to see if it functions as an efflux pump protein since it showed possible membrane localization. Statistical analysis (Two-tailed *t*-test, $df = 6$, $P = 0.3027$) showed no significant differences between the control and *NIC6* overexpressing cells (Fig. 4), and the reason for this result was further proven later on under cellular localization of Nic6 protein in yeast and tobacco cells.

3.3. Expression analysis of NIC6 in B. maritima

Next, we wanted to know whether *NIC6* expression is regulated via nickel in plant cells. Real Time PCR analyses of *NIC6* mRNA was performed on leaves and roots of *B. maritima* grown in half strength Hoagland solution (Hoagland and Arnon, 1950) containing 75 μM nickel for 3 days. Addition of 75 μM nickel to the plant growth medium did not alter *NIC6* transcript levels neither in leaf nor in root tissue (Figs. 5A and B). This suggests that *NIC6* regulation was not directly induced via nickel signalling.

3.4. Cellular localization of Nic6 protein in tobacco and yeast cells

Since Nic6 is a possible putative integral membrane protein, finally, we investigated the localization of the protein in plant and yeast cells. We monitored sub-cellular localization of Nic6 by expressing a Venus-Nic6 fusion protein construct in *Nicotiana benthamiana* epidermal cells. The entire coding region of *NIC6* was translationally fused to the carboxy-terminus of the fluorescent Venus protein and placed under the control of the double enhanced CaMV 35S promoter. Subsequently, the resulting construct was transformed into *N. benthamiana* leaves by agro infiltration in the presence of the potent RNA gene silencing suppressor protein p19 from tomato bushy stunt virus and confocal laser scanning microscopy was used to visualize the fluorescence signals. Fig. 6 representatively shows the outcome of three individual experiments. Fluorescence mediated by the Nic6-Venus fusion protein was specifically localized to the surface of multivesiculare vesicles (Figs. 6A–E). These structures are clearly localized within the cell

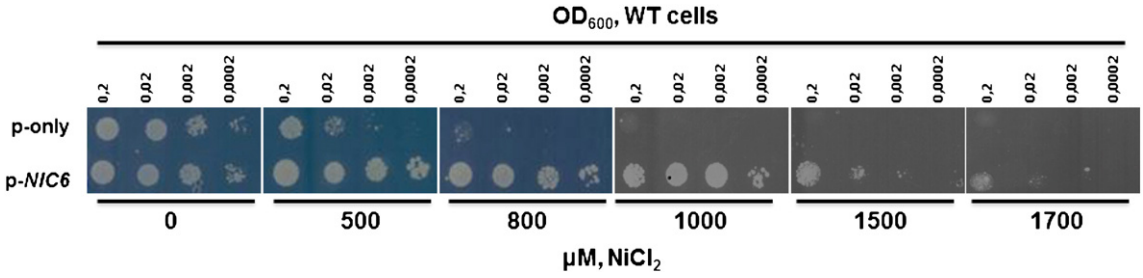

Fig. 1. *NIC6* confers nickel resistance up-to 1500 μM nickel levels when overexpressed in yeast cells. Four serial dilutions ($O.D._{600}$ = 0.2, 0.02, 0.002, 0.0002) were plated on minimal medium. Cells were incubated for 5 days at 30 °C.

cytoplasm as demonstrated by the co-expression of CaMV35S-mRFP constructs, which led to an overall red fluorescence of the cytoplasm (Figs. 6F–I). To distinguish the multivesiculare vesicles from chloroplasts, the auto-fluorescence of the chlorophyll is shown as blue fluorescence signal (Fig. 6J).

We have fused GFP protein to the *NIC6* cDNA, and transformed *NIC6-GFP* construct into the BY4741 yeast strain to monitor the yeast cell localization analysis of the protein. Fig. 6L shows the yeast cellular localization of the Nic6-GFP fusion protein. Construct accumulates as a punctuated pattern in the cytosol. As similar to the tobacco epidermal cell localization results, the yeast localization also might point to the endomembrane structures derived from secretory pathway members such as Golgi or endoplasmic reticulum.

4. Discussion

In this study, we screened a cDNA library of *B. maritima* to identify plant genes that confer nickel tolerance in yeast *S. cerevisiae* cells, and Nic6 was identified as a nickel tolerance protein. The only homologous protein (Fig. 2) with a known function is the *InPSR26* of the Japanese morning glory (*Ipomoea nil*), showed 53% sequence identity at the amino acid level (Shibuya et al., 2009). The *InPSR26* has a regulatory role in programmed cell death and autophagy (Shibuya et al., 2009), and we currently do not know whether *NIC6* gene product has any roles in autophagy pathway in plants.

The most effective way of detoxification involves specific transport proteins localized in the membranes. Recent studies have identified some genes encoding transport proteins from hyperaccumulators (Freeman et al., 2005; Mizuno et al., 2005) those are tested for their nickel transport activities in unicellular model organisms, like *E. coli* and *S. cerevisiae*. There are also nickel transport proteins identified in maize, tobacco and *Arabidopsis* (Arazi and Fromm, 1999; Forzani et al., 2001; von Wiren et al., 2006). These transporters have also been tested for their abilities to transport other metal ions such as Zn, Cd, Mo, Fe, or Pb and have been shown to be capable of transporting some of these metals (Forzani et al., 2001; Mizuno et al., 2005; von Wiren et al., 2006). Since *NIC6* encodes for a potential membrane protein, we reasoned that it might be playing role as an efflux pump, transports nickel

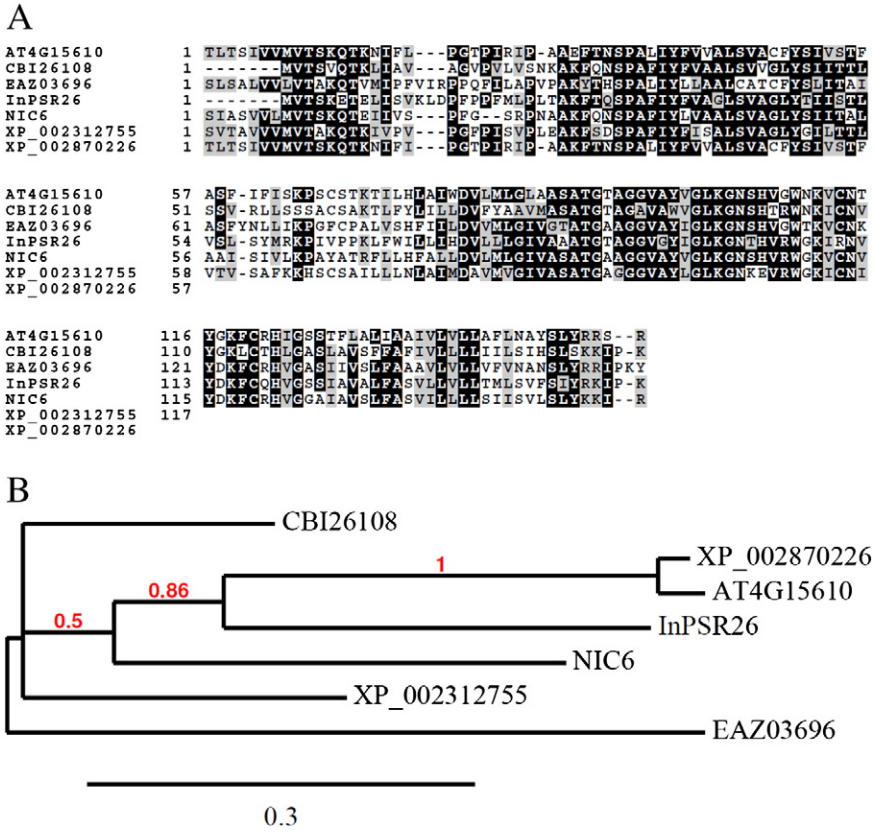

Fig. 2. Sequence analysis. (A) Multiple amino acid sequence alignments of the deduced Nic6 protein sequence with highly identical proteins including InPSR26. Same colours on columns indicate conserved amino acids between *Populus trichocarpa* (XP 002312755), *Vitis vinifera* (CBI26108), *Arabidopsis thaliana*. (AT4G15610), *Oryza sativa* (EAZ03696), *Ipomoea nil* (InPSR26), *Arabidopsis lyrata* (XP 002870226) and *Beta maritima* (Nic6). (B) Phylogenetic tree of the Nic6 and homolog proteins.

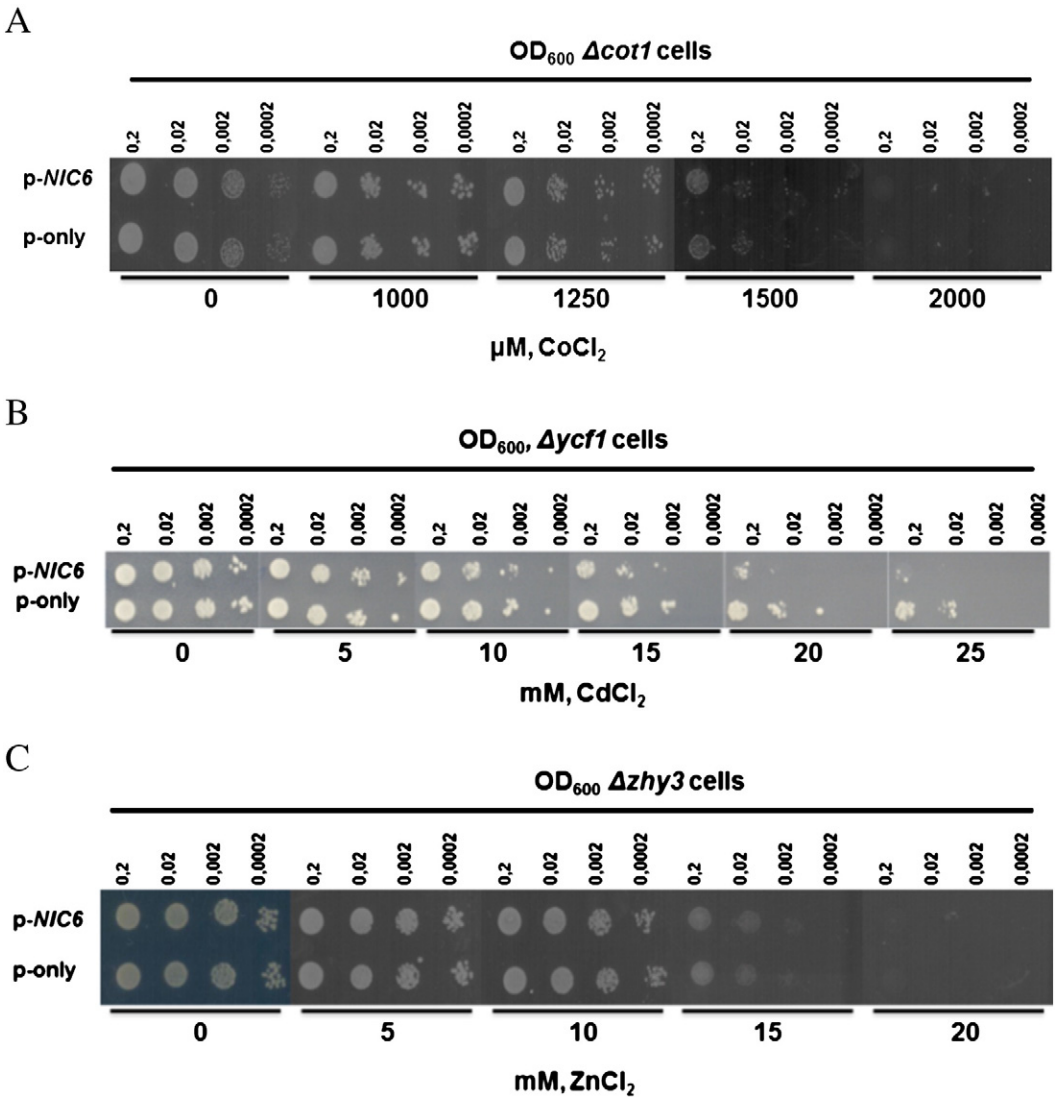

Fig. 3. Functional characterization of Nic6 (A), (B), (C) Growth of *NIC6* overexpressing yeast cells and empty vector containing yeast cells within the cobalt, cadmium and zinc containing YNB–ura plates, respectively (isogenic Δ*cot1*, Δ*ycf1*, and Δ*zhy3* mutant cells were used for cobalt, cadmium, and zinc stress experiments).

from cytoplasm to outside, and decreases intracellular nickel levels. However, nickel levels of both sham vector containing and *NIC6* overexpressing cells (Fig. 4) were not statistically different (Two-tailed *t*-test, df = 6, P = 0.3027). Delhaize and colleagues (Delhaize et al., 2007) also observed a similar result: intracellular Mn^{2+} concentration stayed at similar levels between yeast cells expressing *AtMTP11* and yeast cells with empty vector. This supports our results as, Nic6 does not export nickel to the outside of the cell but it enhances nickel tolerance via different strategies such as internal sequestration of heavy metals in the cell.

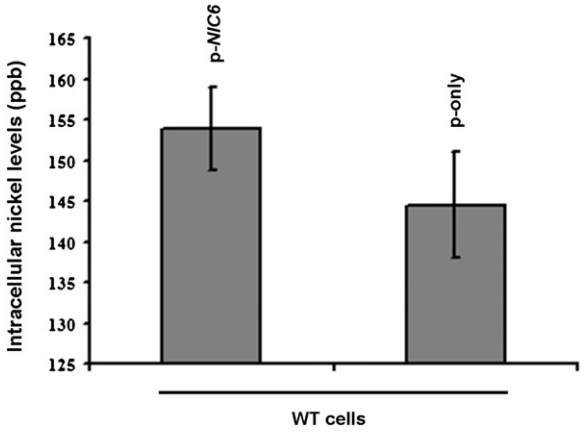

Fig. 4. Intracellular nickel concentrations of recombinant yeast cells showing that there is no significant difference between control and *NIC6* overexpressing cells within 95% confidence level (Two-tailed *t*-test, df = 6, P = 0.3027). Control cells contain empty pAG426GPD vector and NIC6 cells are overexpressing *NIC6* cDNA. Values are the mean of two independent experiments as 2 replicates each (SEM±, n = 4).

Detoxification proteins usually play role in detoxification of more than one substrate. In order to test if Nic6 function is specific for only nickel, *NIC6* overexpressing yeast cells were grown in cobalt, cadmium, and zinc media, but Nic6 conferred tolerance only against nickel toxicity (Fig. 3). Different metals need to be tested other than cobalt, cadmium, and zinc. We have also analysed Arabidopsis homolog of Nic6, At4g15610, to see nickel tolerance and found that Arabidopsis protein did not confer nickel tolerance in yeast. This is similar to the *Stylosanthes hamata* metal tolerance protein ShMtp1's case. This protein is a member of CDF transporter family, and detoxifies manganese in this plant (Delhaize et al., 2003). ShMtp1 shares high sequence similarity with Arabidopsis and rice CDF proteins, but only ShMtp1 protein has manganese detoxification activity which is parallel to our results with *NIC*6. Some of the metal transporter genes are transcriptionally activated upon treatment with its

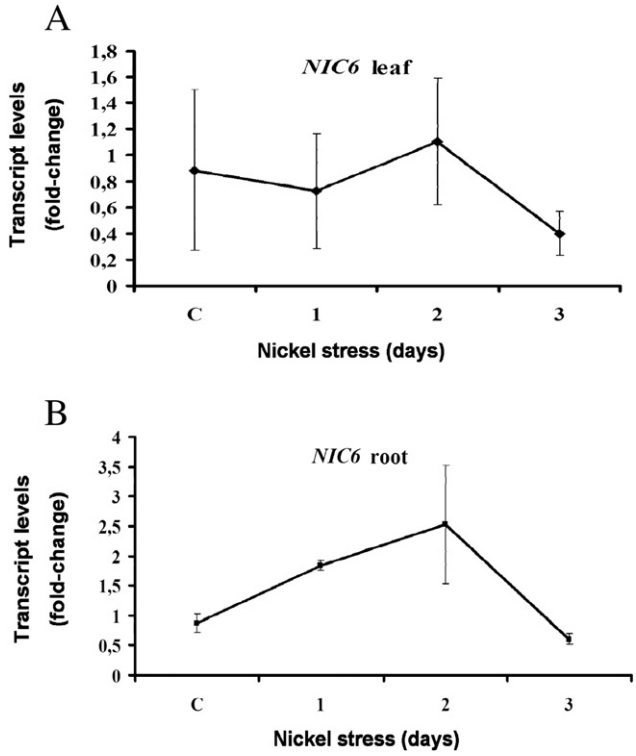

Fig. 5. Expression levels of *NIC6* mRNA after 75 μM nickel treatment of *Beta maritima*. (A) Expression of the *NIC6* cDNA in the leave from control (without Ni) to 3 days nickel treatment in the leave. (B) Expression of the *Nic6* cDNA in the root. Error bars represent the standard error of the mean (n = 4).

stressor. *AtHMA4*, a Zn-transporting ATPase, is up-regulated when Zn levels increased (Williams and Mills, 2005), and *IRT1*, Fe transporter, is up-regulated under low Fe conditions (Vert et al., 2001). Whereas, *MTP1/ZAT* transcript levels were not altered by metals (van der Zaal et al., 1999), and transcript levels of *AtMTP11*, cation diffusion facilitator (CDF) family protein which transports manganese, were not increased in COL 0 wild-type Arabidopsis plant under toxic manganese stress (Sanders et al., 2007). Our expression analysis also showed similar kind of expression patterns. In leaves (Fig. 5A), *NIC6* transcript levels were not induced via nickel stress within three days compared to the untreated control. But in root cells, more dynamic expression was seen due to the nickel treatment (Fig. 5B). But even though a more dynamic regulatory pattern was observed in the root cells compared to the leaf cells, expression analysis is not enough to prove a direct induction of *NIC6* transcription via nickel stress. It would have been more informative to perform the expression analysis in separate for young and old tissues. Although it is specifically related to the plant species (Yusuf et al., 2011), there has been some research showing that, nickel as being a highly mobile element, might be immediately transported to newly formed tissues (Riesen and Feller, 2005) Thus, nickel induced induction of *NIC6* transcripts, if there is, might have been more transparent in the young root and leaf tissues, compared to the older tissues. But still, using the control samples, which were also, as nickel treated samples, collected both from the young and old tissues rule out this masking effect.

Finally to gain a better understanding of the detailed localization of the Nic6 protein, we cloned cDNA to the upstream regions of GFP- and YFP-fusion proteins, and then transformed these constructs to the tobacco epidermal cells. From the results (Figs. 6A–J), it can be concluded that the Nic6 protein in plant and yeast localizes in multivesicular

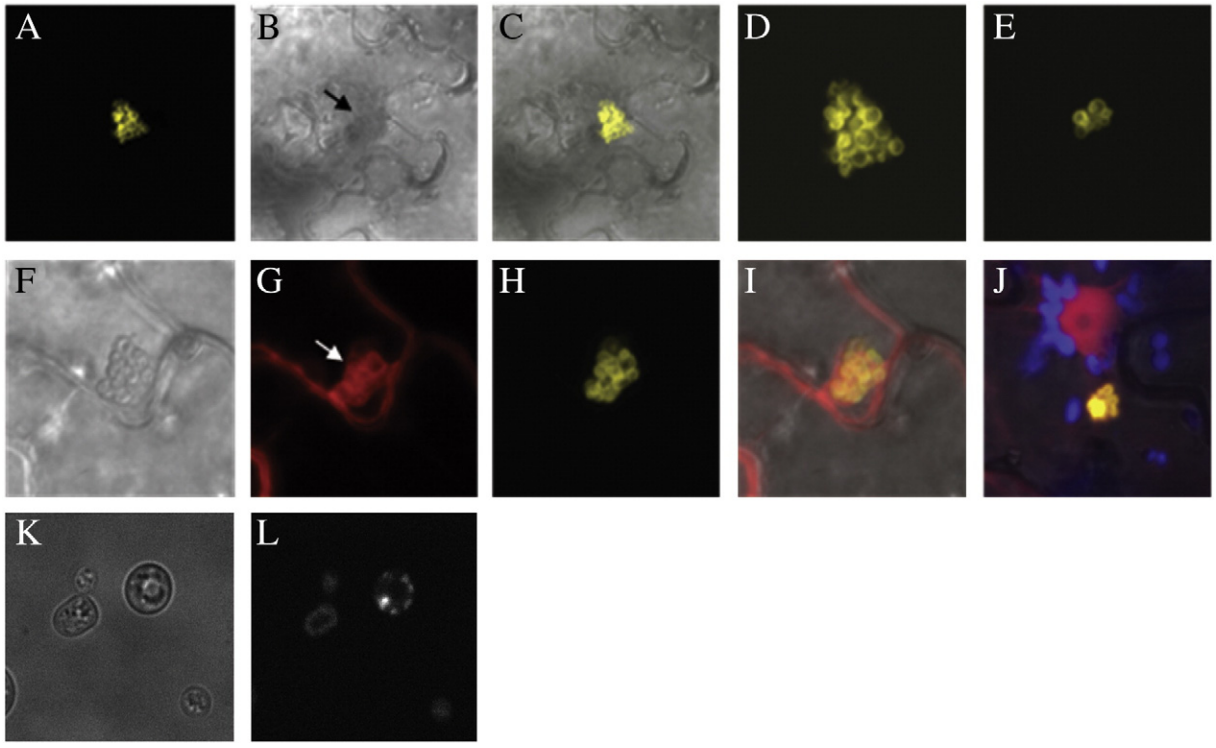

Fig. 6. Sub-cellular localization of Venus-Nic6 fusion protein in epidermal cells of *Nicotiana benthamiana* and yeast cells (A) Fluorescence of vesicular structures observed after expression of pBatTL::*Venus-nic6*. (B) Transmitted light image of a, the vesicular structure is indicated by an arrow. (C) Overlay of a and b. (D) Magnification of a. (E) Fluorescence of a small vesicular structures observed after expression of pBatTL::*Venus-nic6*. F–I Localization of the vesicular structure inside the cytoplasm of an epidermal cell. (F) Transmitted light image of the vesicular structure. (G) Fluorescence observed after expression of pBatTL::*mRFP*, localization of the vesicular structure shown in f is indicated by an arrow. (H) Venus-Fluorescence of vesicular structures. (I) Overlay of F, G and H. (J) Overlay of fluorescence patterns resulted from Venus-tagged *NIC6*, cytoplasmic mRFP and transmitted light image. The auto fluorescence of chloroplasts is shown in blue. (K) Transmitted light image of yeast cells. (L) Sub-cellular localization of Nic6-GFP fusion protein in yeast cells.

protein bodies, which may derive from endoplasmic reticulum, tonoplast, peroxisomes, or liposomes. Although there are limited findings in the literature, it is known that coated vesicles, endomembrane structures have been shown to transport metal ions such as Ca^{2+} and Mn^{2+} (Wu et al., 2002) and confer Mn^{2+} tolerance. During the transport of metals through these endomembrane structures, first, cells remove these toxic elements from cytoplasm where active metabolism takes place. And in some cases, with the transfer of these endomembrane systems through the plasma membrane, even the contents of these structures are removed from the cell itself. Arabidopsis homolog of Nic6, At4g15610, showed homology with Casparian strips (CASP) genes (Roppolo et al., 2011). CASP5-GFP localized large, irregular intracellular compartments in the cell. The authors speculated that CASP form polymeric scaffold within the membrane and serve as a platform to localize and immobilize cell wall biosynthetic enzymes and interacting proteins. Localization of Nic6 showed multivesicular structures closer to the plasma membrane similar to CASP5 protein localization. Nic6 might be serves as CASP like function and help nutrient and nickel uptake into root cells.

All in all, our analyses show that Nic6 confers nickel resistance to yeast cells, and localizes to the multivesicular bodies of tobacco epidermal cells and were not significantly induced via nickel treatment in *B. maritima* cells. Detailed detoxification mechanisms by which Nic6 operates need to be further investigated.

Conflict of interest

All authors declare no conflict of interest.

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References

Arazi, T., Fromm, H., 1999. Characterization of a novel family of calmodulin-binding plasma membrane channel-like proteins. Plant Biotechnology and in Vitro Biology in the 21st Century, 36 351–353.

Arazi, T., Sunkar, R., Kaplan, B., Fromm, H., 1999. A tobacco plasma membrane calmodulin-binding transporter confers Ni2+ tolerance and Pb2+ hypersensitivity in transgenic plants. Plant J. 20, 171–182.

Boominathan, R., Doran, P.M., 2002. Ni-induced oxidative stress in roots of the Ni hyperaccumulator, Alyssum bertolonii. New Phytol. 156, 205–215.

Chen, Z., et al., 2013. Mn tolerance in rice is mediated by MTP8.1, a member of the cation diffusion facilitator family. J. Exp. Bot 64, 4375–4387.

Chevenet, F., Brun, C., Banuls, A.L., Jacq, B., Christen, R., 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinform. 7.

Delhaize, E., Kataoka, T., Hebb, D.M., White, R.G., Ryan, P.R., 2003. Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. Plant Cell 15, 1131–1142.

Delhaize, E., et al., 2007. A role for the AtMTP11 gene of Arabidopsis in manganese transport and tolerance. Plant J. 51, 198–210.

Dietz, K.J, Baier, M., Krämer, U., 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: Prasad, M.N.V., Hagemeyer, J. (Eds.), Heavy metal stress in plants: from molecules to ecosystems. Springer, Verlag, Berlin, pp. 73–97.

Dixon, N.E., Gazzola, C., Blakeley, R.L., Zerner, B., 1975. Jack-Bean urease (Ec 3.5.1.5) – metalloenzyme – simple biological role for nickel. J. Am. Chem. Soc. 97, 4131–4133.

Doney, D.L., 1993. Broadening the genetic base of sugarbeet. J. Sugarbeet Research 30, 209–219.

Durr, G., et al., 1998. The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca2+ and Mn2+ required for glycosylation, sorting, and endoplasmic reticulum associated protein degradation. Mol. Biol. Cell 9, 1149–1162.

Erbasol, I., Bozdag, G.O., Koc, A., Pedas, P., Karakaya, H.C., 2013. Characterization of two genes encoding metal tolerance proteins from Beta vulgaris subspecies maritima that confers manganese tolerance in yeast. Biometals 26, 795–804.

Eskew, D.L., Welch, R.M., Norvell, W.A., 1984. Nickel in higher-plants — further evidence for an essential role. Plant Physiol. 76, 691–693.

Forzani, C., Loulergue, C., Lobreaux, S., Briat, J.F., Lebrun, M., 2001. Nickel resistance and chromatin condensation in Saccharomyces cerevisiae expressing a maize high mobility group I/Y protein. J. Biol. Chem. 276, 16731–16738.

Freeman, J.L., Persans, M.W., Nieman, K., Salt, D.E., 2005. Nickel and cobalt resistance engineered in *Escherichia coli* by overexpression of serine acetyltransferase from the nickel hyperaccumulator plant Thlaspi goesingense. Appl. Environ. Microbiol. 71, 8627–8633.

Hoagland, D.R., Arnon, D.I., 1950. The Water-culture Method for Growing Plants without Soil. Circ., Univ. of Calif. Agric. Exp. Station, Berkley 437.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Kaiser, C., Michaelis, S., Mitchell, A., 1994. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, New York.

Mizuno, T., Usui, K., Horie, K., Nosaka, S., Mizuno, N., Obata, H., 2005. Cloning of three ZIP/NRAMP transporter genes from a Ni hyperaccumulator plant Thlaspi japonicum and their Ni2+ — transport abilities. Plant Physiol. Biochem. 43, 793–801.

Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302, 205–217.

Papoyan, A., Kochian, L.V., 2004. Identification of Thlaspi caerulescens genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. Plant Physiol. 136, 3814–3823.

Peiter, E., et al., 2007. A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. Proc. Natl. Acad. Sci. U. S. A. 104, 8532–8537.

Pence, N.S., et al., 2000. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator Thlaspi caerulescens. Proc. Natl. Acad. Sci. U. S. A. 97, 4956–4960.

Peralta-Videa, J.R., Gardea-Torresdey, J.L., Gomez, E., Tiemann, K.J., Parsons, J.G., Carrillo, G., 2002. Effect of mixed cadmium, copper, nickel and zinc at different pHs upon alfalfa growth and heavy metal uptake. Environ. Pollut. 119, 291–301.

Riesen, O., Feller, U., 2005. Redistribution of nickel, cobalt, manganese, zinc, and cadmium via the phloem in young and maturing wheat. J. Plant Nutr. 28, 421–430.

Roppolo, D., et al., 2011. A novel protein family mediates Casparian strip formation in the endodermis. Nature 473, 380-U564.

Sajwan, K.S., Ornes, W.H., Youngblood, T.V., Alva, A.K., 1996. Uptake of soil applied cadmium, nickel and selenium by bush beans. Water Air Soil Pollut. 91, 209–217.

Sanders, D., et al., 2007. A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. Proc. Natl. Acad. Sci. U. S. A. 104, 8532–8537.

Sanger, F., 1977. Nucleotide-sequence of DNA of Ox174. Fed. Proc. 36, 266.

Seregin, I.V., Kozhevnikova, A.D., 2006. Physiological role of nickel and its toxic effects on higher plants. Russ. J. Plant Physiol. 53, 257–277.

Shibuya, K., Yamada, T., Suzuki, T., Shimizu, K., Ichimura, K., 2009. InPSR26, a putative membrane protein, regulates programmed cell death during petal senescence in Japanese morning glory. Plant Physiol. 149, 816–824.

Sillanpää, M., 1982. Micronutrients and the nutrient status of soils: a global study. Food and Agriculture Org. Rome (48).

Sonnhammer, E.L.L., Eddy, S.R., Birney, E., Bateman, A., Durbin, R., 1998. Pfam: multiple sequence alignments and HMM-profiles of protein domains. Nucleic Acids Res. 26, 320–322.

van der Zaal, B.J., et al., 1999. Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiol. 119, 1047–1055.

Vert, G., Briat, J.F., Curie, C., 2001. Arabidopsis IRT2 gene encodes a root-periphery iron transporter. Plant J. 26, 181–189.

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33, 949–956.

von Wiren, N., Schaaf, G., Honsbein, A., Meda, A.R., Kirchner, S., Wipf, D., 2006. AtIREG2 encodes a tonoplast transport protein involved in iron-dependent nickel detoxification in *Arabidopsis thaliana* roots. J. Biol. Chem. 281, 25532–25540.

Walsh, C.T., Ormejohnson, W.H., 1987. Nickel enzymes. Biochemistry 26, 4901–4906.

Watt, R.K., Ludden, P.W., 1999. Nickel-binding proteins. Cell. Mol. Life Sci. 56, 604–625.

Williams, L.E., Mills, R.F., 2005. P-1B-ATPases — an ancient family of transition metal pumps with diverse functions in plants. Trends Plant Sci. 10, 491–502.

Wu, Z.Y., et al., 2002. An endoplasmic reticulum-bound Ca2+/Mn2+ pump, ECA1, supports plant growth and confers tolerance to Mn2+ stress. Plant Physiol. 130, 128–137.

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