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Noccaea caerulescens populations adapted to grow in metalliferous and non-metalliferous soils: Ni tolerance, accumulation and expression analysis of genes involved in metal homeostasis

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ABSTRACT

Three populations of the Brassicaceae *Noccaea caerulescens*: (i) the metallicolous *Mt. Prinzera* population (Italy), which have adapted to grow on serpentinite, a soil naturally rich in Ni, Co, Cr; (ii) the metallicolous *La Calamine* population (Belgium), which have adapted to growth on soil highly contaminated by Cd, Zn and Pb, and (iii) the non-metallicolous population growing near the town of Rožnov pod Radhoštěm (Czech Republic), were grown in hydroponics and treated with different Ni concentrations (0, 10 and 100 μM $NiSO_4$). Ni tolerance and accumulation were analysed along with the expression of genes belonging to different families involved in plant metal homeostasis: *ZNT1*, *ZNT2*, *NRAMP3*, *NRAMP4* coding for non-ATP-hydrolysing plasma membrane and vacuolar metal transporters, *HMA3*, *HMA4* coding for ATP-hydrolising metal transporters, *NAS1*, *NAS3*, *NAS4* and *MT1B* involved in metal chelation. The three populations showed different levels of expression of some of the tested genes in condition of 0 μM Ni. In addition, the Ni hyperaccumulator *Mt. Prinzera* showed the highest Ni translocation capacity at 10 μM Ni and a specific up-regulation of *ZNT1*, *ZNT2*, *NAS3*, *NRAMP3* and *NRAMP4* genes. With the same Ni treatment, the *La Calamine* population induced *HMA4* and *MT1B* genes, while the Rožnov pod Radhoštěm population only displayed an over-expression of all the genes at 100 μM Ni, the condition in which this population suffers heavily from Ni stress. The expression of *ZNT1*, *ZNT2*, *NRAMP3*, *NRAMP4* was also tested on *N. caerulescens* plants growing in the natural environment on *Mt. Prinzera*. Higher transcript levels of *ZNT1* and *ZNT2* were associated with higher levels of plant's total Ni content. Overall these results suggested that some of the genes considered can have a role in determining metal tolerance and accumulation in metallicolous *N. caerulescens* populations, while they were not involved in metal homeostasis in the non-metallicolous population.

1. Introduction

Plants that accumulate exceptional concentrations of metals in their tissues, called hyperaccumulators, may provide valuable insights into fundamental mechanisms of metal-ion uptake, chelation, translocation and sequestration in plant cells (Baker et al., 2010). *Noccaea caerulescens* is a well-known metal hyperaccumulator of Zn, Cd and Ni. Belonging to the Brassicaceae family, it is widely found in western Europe on various metalliferous soil types, including mine wastes or smelter sites; it also occurs on non-metalliferous soils (Escarrè et al., 2000; Assunção et al., 2001).

Although the molecular mechanisms responsible for high-level metal tolerance and metal hyperaccumulation have not yet been completely identified, there is evidence to suggest an important role of transmembrane metal transporters and of metal chelators whose expression is altered in hyperaccumulator or metal-tolerant species (Clemens, 2001).

Until now, different classes of gene coding for proteins involved in metal uptake, chelation and sequestration have been shown to be differentially modulated between metal hyperaccumulators, tolerant and non-tolerant species. Genes responsible for Zn homeostasis and Cd stress response have been intensively studied in *N. caerulescens* and in the non-accumulator *Arabidopsis thaliana* (van de Mortel et al., 2006, 2008). Similarly, transcriptional differences were found between *N. caerulescens* and the related

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non-accumulator species *Thlaspi arvense* (Hammond et al., 2006) and between contrasting *N. caerulescens* populations, whether or not they are adapted to heavy-metal-contaminated soils (Plessl et al., 2010). In particular among *Noccaea* species, members of the ZIP family (zinc-regulated transporter, iron-regulated transporter protein), such as *ZNT1* and *ZNT2*, were found to be expressed differently in Cd- and Zn-tolerant and hyperaccumulator *N. caerulescens* populations from the related non-accumulator species *T. arvense* (Pence et al., 2000; Assunção et al., 2001).

Vacuolar membrane metal transporters are also up-regulated in hyperaccumulators; in particular *NRAMP3* and *NRAMP4*, which are involved in Fe homeostasis and belong to a family of efflux transporters that release metal ions from the vacuole into the cytoplasm (Thomine et al., 2000). These genes were expressed at high levels in *N. caerulescens* in Fe starvation conditions and in the presence of Cd and Ni. Their beneficial effect on metal tolerance is thought to be due to counteracting metal-induced Fe deficiency (van de Mortel et al., 2006; Oomen et al., 2009; Wei et al., 2009).

High expressions of genes for ATP-hydrolising metal transporters such as *HMA4* and *HMA3* were found in *N. caerulescens* compared to related non-tolerant, non-accumulator species (Papoyan and Kochian, 2004; van de Mortel et al., 2006; Ueno et al., 2011). In addition, *HMA3* and *HMA4* varied in copy number among populations of *N. caerulescens* adapted to grow on different soil types. Interestingly, both the copy number and the level of expression of these gene seem to be associated with *N. caerulescens* populations showing higher levels of Cd tolerance and accumulation (Ueno et al., 2011; Ó Lochlainn et al., 2011; Craciun et al., 2012).

The potential role for heavy metal chelators in hyperaccumulation process is still poorly outlined. The hyperaccumulators *Arabidopsis halleri* and *N. caerulescens* share elevated nicotianamine synthases (*NASs*) expression relative to non-accumulators among a core of alteration in metal homeostasis (Deinlein et al., 2012). NASs synthetise the nonproteinogenic aminoacid nicotianamine (NA) that can bind several transition metals with high affinity *in vitro*. NA seems to be involved in Fe, Cu, Zn and Mn homeostasis in non-accumulator species as *A. thaliana* (Curie et al., 2009), while in hyperaccumulators seems to contribute in metal loading/downloading and/or metal cell to cell transport thus increasing metal mobility and enhancing metal translocation (Weber et al., 2004; Deinlein et al., 2012).

Similarly metallothioneins showed differential levels of expression between *N. caerulescens* accessions: *MT2* and particularly *MT3* were highly expressed in shoots of superior Zn-accumulating *N. caerulescens* accessions compared to less accumulating and non-metallicolous ones even though they did not co-segregate with Zn accumulation (Roosens et al., 2004; Hassinen et al., 2009). They seem to contribute to metal adapted phenotype either through enhancing Zn or Cd tolerance or Cu homeostasis in a heavy metal stressing environment (Hassinen et al., 2009).

From all of these studies it is evident that enhanced transcription levels of metal transporters and metal chelators along with duplication events during microevolution could be involved in determining the hyperaccumulator phenotype. Thus gene expression analyses performed between hyperaccumulator and non-accumulator species and within *N. caerulescens* populations whether or not they are adapted to growth on metalliferous soils could allow for a better understanding of the plant hyperaccumulation process.

For this reason, we analysed Ni tolerance and accumulation capacities in three geographically distant *N. caerulescens* populations: (i) the *Mt. Prinzera* (MP) population growing on serpentinite, a soil naturally rich in Ni, Co, and Cr (Italy); (ii) the *La Calamine* (LC) population growing on a soil highly contaminated by Zn, Cd and Pb (Belgium); (iii) the Rožnov pod Radhoštěm (RpR) population growing on non-metalliferous soil (Czech Republic). The expression pattern of genes involved in metal transport and chelation were analysed in shoots of plants grown in hydroponics with different levels of Ni. Genes specifically up-regulated in the MP population grown in hydroponics, were also analysed at transcriptional level in plants sampled in their natural environment.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *N. caerulescens* plants were collected at the MP site (44.65096° N–10.08369° E), a serpentinite rock in the Northern Apennines (Italy). Seeds of the LC (Belgium) and RpR (Czech Republic) populations were kindly donated by Dr Mark Aarts. Site and accession characteristics are given in Visioli et al. (2012, 2013) (MP, RpR) and Assunção et al. (2003) (LC). Seeds were sterilised in 50% (v/v) commercial bleach for 10 min and then rinsed in sterile water. Seeds were then kept in the dark at 4 °C for three days before germination and growth was carried out on plates containing one-strength MS medium (Murashige and Skoog, 1962) and 1.5% agar on light racks in an environmentally controlled room (22° C; 16 h/8 h light/dark; 120 μmol m^{-2} s^{-1} photosynthetic photon flux, 75% HR). Two-week-old seedlings were transferred to 3-l polyethylene pots (5 seedlings per pot) filled with half-strength Hoagland's solution: 3 mM KNO_3, 2 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4{\cdot}7H_2O$, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4{\cdot}4H_2O$, 2 μM $ZnSO_4{\cdot}7H_2O$, 0.1 μM $CuSO_4{\cdot}5H_2O$, 0.1 μM $(NH_4)_6Mo7O_{24}{\cdot}4H_2O$, 20 μM Fe(Na)EDTA. 2-Morpholinoethan-sulphonic acid was added at 2 mM final concentration and KOH was added to buffer the solution at pH 5.5. The nutrient solution was replaced every week during the first two months and twice a week for the last month. After three months, five plants from each population were treated with 10 or 100 μM $NiSO_4$ for 28 days, during which the solution was replaced once a week (nutrient plus buffer and $NiSO_4$). After the treatments, three pools of treated and untreated plants (one pool containing one leaf from each of the five plants with the same treatment) were frozen in liquid nitrogen and stored at −80 °C for RNA extractions.

For the gene expression analysis of plants growing in the natural environment, three pools of plants (one pool containing one leaf from three different plants) were obtained for two different subsites on MP (MP1: 44.65138° N–10.08330° E and MP2: 44.65096° N–10.08369° E). The same plants were also analysed for the Ni concentration in their shoots and roots (Visioli et al., 2012). Leaves were washed with distilled water, immediately frozen in liquid nitrogen and then utilised for RNA extraction.

2.2. Mineral analysis

Roots and the remaining shoots of plants growing in hydroponics were dried out for three days at 70 °C. 100 mg of each dried sample was dissolved in 10 ml of 37% (v/v) HCl in 0.5 one glass cylinder at 160 °C for 20 min. The Ni concentration was determined using flame atomic absorption spectrometry (AAS) (Perkin Elmer 1100B). The data on Ni concentration in leaves and roots of MP plants growing in the natural environment were previously reported (Visioli et al., 2012). ANOVAs after log-transformation of the response variable and Tukey's Post Hoc tests were used to test for differences in the Ni concentration of plants. The response variables for Ni in plants were: total Ni in roots, total Ni in shoots and Ni translocation factor (shoot/root ratio).