Variation in HMA4 gene copy number and expression among Noccaea caerulescens populations presenting different levels of Cd tolerance and accumulation

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Received 23 January 2012; Revised 7 March 2012; Accepted 9 March 2012

Abstract

There is huge variability among populations of the hyperaccumulator Noccaea caerulescens (formerly Thlaspi caerulescens) in their capacity to tolerate and accumulate cadmium. To gain new insights into the mechanisms underlying this variability, we estimated cadmium fluxes and further characterized the N. caerulescens heavy metal ATPase 4 (NcHMA4) gene in three populations (two calamine, Saint-Félix-de-Pallières, France and Prayon, Belgium; one serpentine, Puente Basadre, Spain) presenting contrasting levels of tolerance and accumulation. Cadmium uptake and translocation varied among populations in the same way as accumulation; the population with the highest cadmium concentration in shoots (Saint Félix-de-Pallières) presented the highest capacity for uptake and translocation. We demonstrated that the four NcHMA4 copies identified in a previous study are not fixed at the species level, and that the copy truncated in the C-terminal part encodes a functional protein. NcHMA4 expression and gene copy number was lower in the serpentine population, which was the least efficient in cadmium translocation compared to the calamine populations. NcHMA4 expression was associated with the vascular tissue in all organs, with a maximum at the crown. Overall, our results indicate that differences in cadmium translocation ability of the studied populations appear to be controlled, at least partially, by NcHMA4, while the overexpression of NcHMA4 in the two calamine populations may result from convergent evolution.

Key words: cadmium, gene duplication, HMA4, hyperaccumulation, Noccaea caerulescens, translocation.

Introduction

Cadmium is one of the most toxic metals, that has by far a greater bioavailability than lead, arsenic, or mercury. It is considered as non-essential, except in marine diatoms where it can replace zinc in a specific isoform of carbonic anhydrase (Lane et al., 2005; Xu et al., 2008). Cd uptake in plants is achieved via systems for the acquisition of essential micronutrients, mainly Zn, iron, and calcium. Despite Cd being toxic, a number of plant species have evolved high tolerance, principally through exclusion (Clemens, 2006; Verbruggen et al., 2009a; Küpper and Kochian, 2010). In addition, some rare plants display an exceptional capacity to accumulate Cd in their above-ground biomass. To date,
only nine species have been recognized as Cd hyperaccumulators: Noccaea caerulescens (Baker et al., 1994; Lombi et al., 2000), Arabidopsis halleri (Bert et al., 2002, 2003; Zhao et al., 2006), Noccaea praecoax (Vogel-Mikut et al., 2005, 2008), Sedum alfredii (Yang et al., 2004; Deng et al., 2007), Arabis paniculata (Tang et al., 2009a), Pieris diversicata (Tang et al., 2009b), Viola baoghensis (Liu et al., 2004; Wu et al., 2010), Phytolacca americana (Liu et al., 2009), and Potentilla griffithii (Wang et al., 2009). The first two species have been studied intensively in recent years, and are considered to be model plants for the study of metal hypertolerance and hyperaccumulation.

N. caerulescens (J. & C. Presl) F.K. Mey (Brassicaceae; formerly Thlaspi caerulescens; see Koch and Mummennhoff, 2001) is an annual, biannual, or perennial weed that is able to survive on unusual substrates highly contaminated by metals, such as mine wastes, smelter sites, or ultramafic soils (Reeves et al., 2001). In addition to a high degree of metal tolerance, the species is well known for its capacity to hyperaccumulate Zn, nickel, and Cd (Baker et al., 2000; Verbruggen et al., 2009a). Interestingly, a significant growth-promoting effect by Cd was observed in two populations of N. caerulescens in the south of France [St-Félix-de-Pallières (SF population) and Ganges (Ga population); Roosens et al., 2003; Liu et al., 2008]. Cd may play a physiological role in these populations by enhancing the activities of some enzymes (Liu et al., 2008); however, no biological function has yet been identified. Another interesting feature of this species is the pronounced differences among populations in their ability to tolerate and accumulate Cd in their shoots, as well as in the shoot/root Cd concentration ratio (Escarré et al., 2000; Lombi et al., 2000; Assunção et al., 2003; Roosens et al., 2003; Deniau et al., 2006; Xing et al., 2008). It has been proposed that differences among populations occur at the levels of Cd uptake from soil into root cells, translocation from roots to shoots and detoxification in the shoots (Verbruggen et al., 2009b; Krämer 2010). The mechanisms involved in high-affinity Cd uptake by some populations are still unknown. Physiological experiments have suggested that the iron-regulated transporter 1 (IRT1) might be involved in the enhanced Cd uptake of the extraodinary Cd accumulator population Ga (Lombi et al., 2002). However, when expressed in yeast, NcITR1 from Ga did not exhibit Cd transport activity (Plaza et al., 2007).

The difference in Cd accumulation capacity between Ga and a lower-accumulating population Prayon (Pr, in Belgium) has been partly explained by the heavy metal ATPase 3 (HMA3) tonoplast transporter (Ueno et al., 2011). This transporter was demonstrated to play a major role in the sequestration of Cd into vacuoles of all leaves, and hence in the detoxification of this metal in N. caerulescens. HMA3 expression in the Ga population was 7-fold higher than in Pr, and this difference was shown to be partly due to gene copy number expansion (Ueno et al., 2011). Xing et al. (2008) investigated the variability in the efficiency of root-to-shoot translocation of Cd in N. caerulescens. They focused on the Ga and Pr populations, and on the role of the heavy metal ATPase 4 (HMA4), which is the prime candidate gene for root-to-shoot Cd and Zn translocation in hyperaccumulators. They observed significant differences in metal uptake and translocation between these populations. However, at a low Cd concentration (5 μM) differences in root NcHMA4 expression were not found.

The key role of HMA4 in root-to-shoot Cd and Zn translocation was first established in Arabidopsis thaliana (Hussain et al., 2004; Verret et al., 2004; Mills et al., 2005; Wong and Cobbett, 2009). At a subcellular level HMA4 was shown to localize in the plasma membrane of A. thaliana and of the hyperaccumulator A. halleri (Verret et al., 2004; Courbot et al., 2007) and, at the tissue level, in the pericycle layer of the root vasculature (Verret et al., 2004; Hanikenne et al., 2008). Striking constitutive overexpression of HMA4 was observed both in N. caerulescens and A. halleri compared to non-tolerant, non-accumulator relatives (Bernard et al., 2004; Papoyan and Kochian, 2004; Talke et al., 2006; van de Mortel et al., 2006). Furthermore, HMA4 co-localized with the major quantitative trait loci for Zn and Cd tolerance, as well as Zn and Cd accumulation, in A. halleri (Courbot et al., 2007; Willems et al., 2007; Frérot et al., 2010; Willems et al., 2010). A clear demonstration of the role of HMA4 in Cd and Zn tolerance and accumulation was provided by Hanikenne et al. (2008) by means of RNA interference. HMA4 was shown to be active as an ATPase with turnover rate increasing after the addition of Zn2+ and Cd2+ (Parameswaran et al., 2007). Recently, a N. caerulescens BAC genomic library was generated to investigate HMA4 duplication (Ó Lochlainn et al., 2011). In the Ga individual used for the constructions, two long (NcHMA4-1 and -2) and two short copies (NcHMA4-3 and -4) were identified (according to data S2, S5, and S7 from Ó Lochlainn et al., 2011). The short copies showed a truncation of 157 amino acids in the C-terminal part. The C-terminus of HMA4 (after the last transmembrane domain) does not include catalytic or transmembrane domains; however, when expressed alone in yeast, it confers tolerance to Cd and Zn throughout metal binding (Bernard et al., 2004; Courbot et al., 2007; Bakgaard et al., 2010). In planta, the C-terminal part of HMA4 seems to be important (Mills et al., 2010), but the role of this region remains unclear. Sequence identities among the NcHMA4, AhHMA4, and AthHMA4 copies have suggested relatively recent duplication events in N. caerulescens (Ó Lochlainn et al., 2011).

The aim of the present study was to investigate the possible role of HMA4 in the variability of Cd tolerance
and accumulation of \textit{N. caerulescens}. We characterized the change in \textit{NcHMA4} gene copy number and expression for three highly different \textit{N. caerulescens} populations from calamine and serpentinite sites (Roosens et al., 2003). In addition, we also demonstrate the functionality of the \textit{NcHMA4} copy truncated in the C-terminal region, and we provide the first report about the tissue specificity of \textit{NcHMA4} expression.

**Materials and methods**

\textit{Plant material and culture conditions}

Seeds of \textit{N. caerulescens} were obtained from populations growing in SF, France (Zn/Cd/Pb mine spoil site, 25 km from Ga), Puente Basadre (PB), Spain (Ni-enriched serpentinite soil), and Pr, Belgium (Zn/Cd/Pb smelter site). The relationship between Cd accumulation and tolerance and the capacity for metal accumulation of the three populations, and the chemical characteristics of the soils in which they grew, were described by Roosens et al. (2003). Population SF is considered to be the most Cd-tolerant, exhibiting the highest Cd concentration in shoots. PB is the least tolerant population, and displays slightly lower shoot Cd concentrations. The last population (Pr) is intermediate between populations SF and PB for Cd tolerance, but has 3.5-fold lower Cd concentration in shoots as compared to SF. Interestingly, this population showed a shoot:root biomass ratio that was 1.6-fold greater than that of populations SF and PB.

\textit{109Cd uptake and root-to-shoot translocation}

\textit{N. caerulescens} seeds were sown in sand, and were incubated for 2 days at 4 °C to synchronize germination. Ten days after germination, seedlings (= 9 or 8 individuals per population) were transferred to hydroponic culture in modified 0.1 strength Hoagland solution (Roosens et al., 2003). The Cd uptake in plants was monitored using \textit{109}Cd. The roots of the seedlings were arranged in a washing solution consisting of the diluted nutrient solution containing 1 mM CaCl\textsubscript{2}. The desorption period continued for 20 min. Plant organs (leaves, stems, and roots) were immersed in a washing solution diluted 10-fold with 0.17 mg 109Cd/l, 6.7 \times 10^{-2} mCi/ml. After a 12 h uptake period, plants were transferred twice to a washing solution consisting of the diluted nutrient solution containing 1 mM CaCl\textsubscript{2}. The desorption period continued for 20 min. Plant organs (leaves, stems, and roots) were immersed separately in a scintillation cocktail (Beckman coulter), and measured in a gamma counter to quantify the Cd uptake by roots and translocation to shoots. A short-term uptake period was selected to estimate uptake and translocation per se (Xing et al., 2008; Lu et al., 2010).

\textit{Variation in \textit{NcHMA4} gene copy number}

To investigate variation in \textit{NcHMA4} gene copy number of the three populations, real-time quantitative PCR (qPCR) on genomic DNA was performed using the LightCycler\textsuperscript{®} 480 SYBR Green I Master (Roche, Vilvoorde, Belgium). Genomic DNA from 24 plants was extracted using the GenJet\textsuperscript{TM} DNA purification kit (Fermentas, St. Leon-Rot, Germany). Primers were designed to amplify the four previously identified copies (qPCR\_HMA4\_1F and qPCR\_HMA4\_1R; see Supplementary Table S1). The \textit{epMotion} 5075 automated pipetting system (Eppendorf, Rotselea, Belgium) was handled for all pipetting, and qPCR reactions were performed using the LightCycler \textit{480} (Roche). Genomic DNA of \textit{A. thaliana}, which has a single copy of \textit{HMA4}, was used as the control. The data obtained were analyzed based on the C\textsubscript{t} value for \textit{SHR} (SHORT ROOT: At4g37650), which is a single-copy gene in \textit{A. thaliana}, and which was successfully used to investigate the gene copy number of \textit{NcHMA3} (Ueno et al., 2011). Each reaction was performed in triplicate. Amplification efficiency was calculated to be 100\% (\textit{NcHMA4}) and 99\% (\textit{SHR}). The presence of the short copies displaying a deletion in the ninth exon (according to O\’Lochlainn et al., 2011) was controlled using PCR on genomic DNA. We used a primer located upstream of the deletion as the forward primer (qPCR\_HMA4\_2F), in combination with a primer located downstream (qPCR\_HMA4\_2R). From this combination, we obtained one amplicon of 618 bp corresponding to the long copies (\textit{HMA4-1} and \textit{HMA4-2}) and one amplicon of 147 bp corresponding to the short copies (\textit{HMA4-3} and \textit{HMA4-4}). PCR products were confirmed by sequencing.

\textit{Expression of \textit{NcHMA4} in roots and shoots}

The expression level of \textit{NcHMA4} in roots and shoots of the three populations was quantified using real-time qPCR. After 6 weeks in nutrient solution (modified 0.1 strength Hoagland solution, according to Roosens et al., 2003) six plants of each population were transferred to a solution treated with 0 mM (as control) or 100 \mu M CdSO\textsubscript{4}. Roots and shoots were collected after 72 h of treatment, and the material belonging to each population was pooled. Total RNA was extracted from all pooled samples using the RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). About 5 mg of total RNA was used for the synthesis of cDNA in a final volume of 60 \mu l (RevertAid\textsuperscript{TM} H minus first stand cDNA synthesis kit, Fermentas). Real-time qPCR reactions were performed in a LightCycler\textsuperscript{®} Carousel-Based System (Roche), using the ready-to-use hot start reaction mix (Roche) and double dye probe (Eurogentec, Seraing, Belgium).

To amplify the long \textit{HMA4} copies only, we used a primer (qPCR \textit{HMA4F} F) located in the missing region of the short copies that produced an amplicon of 177 bp when used together with the primer qPCR\_HMA4\_R. To amplify the short copies we used the primers qPCR\_HMA4\_F and qPCR\_HMA4\_R. Specific amplification was obtained by reducing the extension time to 10 s, so that only the short copies were amplified, even if this primer combination allowed long copies to be obtained using longer extension times. The data were normalized to the expression levels of the homologues of \textit{At3g62290} and \textit{actin 2}. The 2^\text{-ACT} method (Livak and Schmittgen, 2001) was used to calculate the relative changes in \textit{NcHMA4} expression against the \textit{At3g62290} and \textit{actin 2} genes.

\textit{Yeast cultures, transformation, and growth assays}

The yeast (Saccharomyces cerevisiae) strain used for the heterologous expression of \textit{HMA4} was the Cd-sensitive \textit{ycf1} mutant (BY4741; Mat a; his3A1; leu2A0; met15A0; ura3A0; YDR135c:: kanMX4) obtained from Euroscarf (http://web.uni-frankfurt.de/fbi15/mikro/euroscarf/). The full-length \textit{HMA4} cDNA from SF was amplified by RT-PCR using the forward primer \textit{NcHMA4\_KpnAAStart} and the reverse primer \textit{NcHMA4\_NoSTOP\_Xho}. The long and short copies were separated on agarose gel according to their length, and extracted using the GeneJET\textsuperscript{™} Gel Extraction Kit (Fermentas). \textit{HMA4} cDNA, corresponding to the long and short copies, was cloned into the pYEC2/CT–GFP plasmid under the control of the galactose-inducible (\textit{GAL1}) promoter. In a previous study on \textit{AhHMA4}, a high-copy-number plasmid was used, and high \textit{AhHMA4} expression resulted in higher sensitivity probably due to accumulation in the endoplasmic reticulum (Courbot et al., 2007). In contrast, other studies of \textit{HMA4} in yeast using a centromeric plasmid showed that the expression of \textit{HMA4} can protect cells against Cd toxicity (Hanikenne et al., 2008). Yeast transformation was performed by standard lithium acetate method (Gietz et al., 1995). Transformed yeast were grown at 30 °C on a synthetic yeast drop-out medium without uracil (Synthetic Depleted, 6.7 g \textminus 1 yeast nitrogen base, 1.92 g \textminus 1 drop-out; Sigma-Aldrich, Bornem, Belgium), supplemented with 2% (w/v) galactose (induction medium). For metal tolerance assays, yeast cells were grown in liquid SD medium to an OD600 of 1 to perform...
further dilutions. The drop assays were performed on SD plates containing galactose (induction medium) or glucose (repressing medium; Supplementary Fig. S3) and 0 or 75 μM CdSO₄. Plates were incubated for 4 days at 30 °C. Drop tests were repeated at least three times with similar results.

**NcHMA4 RNA in situ hybridization**

Total RNA was prepared by LiCl precipitation (Sambrook and Russell, 2001), and 6 μg were used to synthesize first-strand cDNA with a biotinylated oligo-dT₂₅ primer and Superscript II™ reverse transcriptase (Invitrogen, Gent, Belgium). Second-strand synthesis was performed by strand displacement with Escherichia coli ligase (New England Biolabs, Ipswich, MA, USA), E. coli polymerase I (New England Biolabs), and RNase H (Invitrogen). The resulting double-stranded cDNA was purified using the Qiaquick PCR purification kit (Qiagen). The C-terminal region of NcHMA4 (from 2243 to 3498 bp according to the cDNA published by Bernard et al. 2004; accession number AJ567384; for primers see Supplementary Table S1) was subcloned in pTZ57R/T vectors (InsT/Aclone™ PCR product cloning kit, Fermentas) and used for the synthesis of sense and antisense riboprobes. To generate probes, cDNA inserts containing the flanking bacteriophage promoter sequence T₇ were amplified by PCR. Digoxigenin-11-UTP-labelled probes were then synthesized from purified PCR product templates by runoff transcription with T₇ or Sp₆ polymerase. In addition, we synthesized an antisense probe for NcEF1 as positive control. The negative controls were a sense probe for NcHMA4 and an antisense probe for the neomycin gene (not present in the plant genome).

**In situ hybridization** on paraffin sections from roots, hypocotyls, stems, petioles, and leaves of plants belonging to the populations SF, PB, and Pr were performed as described by de Almeida Engler et al. (2001). Before the experiment, plants were grown for 6 weeks in hydroponic culture and were exposed for 72 h to three CdSO₄ concentrations: 0, 10, and 100 μM. To objectively compare and discriminate the differences in expression between tissues, we prepared two types of slide for NcHMA4 and negative control hybridizations: (1) slides containing sections through only one type of tissue, derived from plants belonging the one population, and (2) slides containing sections of two to three different tissues. Because the sections were the same thickness, and all slides were exposed and developed at the same time, we assumed that the differences observed for the NcHMA4 expression were directly linked to the presence of different amounts of NcHMA4 mRNA in the different tissues of the three populations. Images were visualized using a Nikon Eclipse E800 microscope and a Nikon SMZ1000 zoom stereomicroscope (Nikon Instruments, Tokyo, Japan). Digital images were taken using a Nikon DXM1200 digital camera and the Nikon ACT-1 version 2.12 imaging software was used for image acquisition. Overlays of bright- and dark-field micrographs were generated to maximally visualize tissue anatomy and mRNA localization.

**Statistical analysis**

We performed non-parametric exact tests (STATXACT version 8, 2007; Cytel Studio, Cambridge, MA, USA) to analyse differences among populations. These tests make no assumptions about distributions, and are suitable for small and/or unbalanced samples. The Kruskall–Wallis exact test for k independent groups was used to analyse differences among populations for ¹⁰⁹Cd uptake and ¹⁰⁹Cd translocation from root-to-shoot. Monte Carlo approximations for P values were obtained using 10 000 permutations. To test for differences between pairs of populations, we used non-parametric post-hoc tests for multiple comparisons, according to Siegel and Castellan (1988). Correlation between metal uptake and metal translocation, and between translocation and shoot:root biomass ratio, was estimated using the Spearman coefficient.

**Results**

**¹⁰⁹Cd uptake and translocation to shoots**

The uptake of Cd by roots, and its translocation to shoots, was evaluated in the populations SF, Pr, and PB by ¹⁰⁹Cd radiotracer labelling experiment. SF has been described as the most Cd-tolerant, followed by Pr and PB (Roosens et al., 2003; Supplementary Fig. S1). The SF population was also the most able to concentrate Cd in its shoots, whereas Cd shoot concentrations in the PB population were slightly lower, and were 3.5-fold lower in Pr compared to SF (Roosens et al., 2003).

After 12 h of exposure to ¹⁰⁹Cd, the three populations showed different levels of ¹⁰⁹Cd uptake (P = 0.022, Fig. 1A) and root-to-shoot translocation (P < 0.001, Fig. 1B), and a high variability within populations for Cd uptake. Uptake, expressed on the basis of root fresh weight, was on average significantly higher for the SF population compared to Pr, whereas PB did not significantly differ from the two other populations. For all populations, root-to-shoot translocation of Cd was high compared to normal plants, even if it varied significantly among populations (Fig. 1B). On average, 93% of ¹⁰⁹Cd was transported in SF plants, 83% in PB plants, and 89% in Pr plants. Based on the total sample (n = 25), translocation efficiency appeared to significantly increase with metal uptake (r = 0.435, P = 0.0294, Supplementary Fig. S2A), whereas no significant correlation was found between translocation and shoot:root biomass ratio (r = 0.02, P = 0.9751, Supplementary Fig. S2B).

![Fig. 1.](https://academic.oup.com/jxb/article-abstract/63/11/4179/602189) Fig. 1. (A) Uptake of ¹⁰⁹Cd and (B) percentage of ¹⁰⁹Cd translocated to shoots among three *N. caerulescens* populations after an exposure of 12 h. Data are mean±SD (n = 9, 8, and 8 for SF, PB, and Pr respectively); different letters indicate significant differences at the 5% level. FW, fresh weight.
Variation in NcHMA4 gene copy number

The gene copy number of NcHMA4 was estimated in the SF, Pr, and PB populations using real-time qPCR. We also investigated the copy-number variation in the Ga population (purchased from Guy Delmot, Saint-Laurent-Le-Minier, France), which was previously used for the identification of the four NcHMA4 copies (Ó Lochlainn et al., 2011). After normalization with the SHR gene, which is a single-copy gene in A. thaliana, the NcHMA4 copy number was estimated to be four in Ga and SF populations and to be three and two in the Pr and PB populations, respectively (Fig. 2A). Variability in NcHMA4 copy number was not identified within populations (see SD in Fig. 2A). The presence of the short copies was controlled using PCR on genomic DNA. The two amplicons, resulting from the amplification of the short and long copies, were detected in all individuals from the Ga and SF populations (Fig. 2B). Individuals from Pr and PB populations had the only amplicon corresponding to the long copies.

Quantification of NcHMA4 expression in the root and shoot by real-time qPCR

Using the At3g62290 and actin 2 genes, which are expressed at similar levels in the three N. caerulescens populations (data not shown), as an internal control, we analysed the level of NcHMA4 expression in roots and shoots after 72 h treatment at 0 or 100 μM CdSO₄. In all populations, and in all conditions, expression of NcHMA4 was higher in the roots than in the shoots (Fig. 3). Expression in PB was clearly different from those of Pr and SF, whereby the transcript abundance was on average 5-fold lower in this population compared to the two other populations. The samples belonging to SF and Pr displayed similar NcHMA4 expression. The expression of NcHMA4 was slightly increased by Cd treatment in the Pr sample. Levels of expression of the long and the short copies were different: expression of the short copies was higher compared to those of the long copies in both treatments, and for shoots and roots (Fig. 3). To confirm the pattern observed in the Cd-treated samples, we
replicated this experiment. Expression of NcHMA4 was repeatedly lower in the PB population as compared to the other populations (in roots 1.66 compared to 4.48 and 10.50 for PB, Pr, and SF respectively; in shoots 0.32 compared to 1.79 and 5.96), and expression of the short copies was repeatedly higher than that of the long copies (in roots 6.08 compared to 4.42; in shoots 3.58 compared to 2.18).

Functional analysis of NcHMA4 in yeast

To investigate the function of the long and short copies, the full-length NcHMA4 sequences were cloned in the vector pYEC2/CT, and expressed in the Cd-sensitive ycf1 mutant. The expression of both NcHMA4 copies strongly enhanced Cd tolerance of the strains (Fig. 4).

Tissue specificity of NcHMA4

To reveal potential differences in the tissue expression of NcHMA4 we performed in situ hybridization on longitudinal and transverse sections through different organs of plants belonging to the SF, PB, and Pr populations exposed in hydroponic culture (72 h) to three CdSO4 concentrations: 0, 10, and 100 μM. We were unable to detect different patterns of expression among the three populations or between the Cd treatment conditions through the comparison of slides (data not shown). However, differences were observed among tissues for all measures, with the expression of NcHMA4 being higher in the vascular tissues (Fig. 5).

Based on the serial sections of roots, we observed that NcHMA4 expression was relatively strong in all cells of the root tips (Fig. 5A). In the elongation and hairy root region, its expression was mainly associated with the xylem parenchyma (with the poles of the protoxylem elements; Fig. 5B–5D), but it was also present in some epidermal cells. Towards the hypocotyl NcHMA4 expression extended to the protophloem and companion cells of the phloem sieve tubes, and also to the procambium cells. Above the hairy root region of the root NcHMA4 expression was contained inside the pericycle, with low expression in the pericycle itself. No expression was observed in the endodermis, cortical tissue, or absorbent hairs.

Among all tissues, the highest expression of NcHMA4 seemed to be present in the crown region (Fig. 5E–5G). In the central cylinder, high expression was observed in the interfascicular cells accompanying both the xylem and phloem vessels that form the vascular bundles (Fig. 5E). Strong NcHMA4 expression was also observed outside of the central cylinder in the parenchymatous cortex cells located between the epidermis and the central cylinder, in the subepidermal, and also in some of the epidermal cells (Fig. 5F and 5G). Interestingly, in these cells, ‘patches’ of NcHMA4 expression were observed on each side of the cell walls separating two adjacent cells (data not shown). These patches might be located at the level of the plasmodesmata.

In the stems, the petioles, the apical meristem, and the foliar buds (Fig. 5H–5J) NcHMA4 expression was observed only in the central cylinder associated with the vascular bundles. In leaves, NcHMA4 is highly expressed in the vasculature, but expression was also observed in the mesophyll cells (Fig. 5K–5M). Numerous patches of NcHMA4 expression, similar to those observed in the crown region, were observed throughout the mesophyll. No expression was observed in the guard cells or in the cells surrounding the stomatal pores, nor in the epidermis cells or in the trichomes (Fig. 5L). No signal was visible in any of the sections when sense NcHMA4 RNA and antisense neomycin RNA were used as probes (data not shown).

Discussion

N. caerulescens is known to display highly variable metal tolerance, accumulation, and fitness-related traits in response to metals (Meerts and Van Isacker, 1997; Zhao et al., 2002; Roosens et al., 2003; Jiménez-Ambriz et al., 2006; Dechamps et al., 2007). Here, we investigated the possible role of HMA4 in the variability of Cd tolerance and accumulation for three N. caerulescens populations.

Fig. 4. Comparison between the growth of yeast cells expressing the short and long NcHMA4 copies. Cd-sensitive ycf1 yeast cells were transformed with the pYEC2/CT–GFP vector (control cells) or with full-length NcHMA4 coding sequences. Cells grown to the log phase were diluted to an OD600 of 1, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ and 5 μM of each dilution were spotted onto medium containing galactose without or with added CdSO₄. The plates were incubated at 30 °C for 4 days before photography.
growing on calamine and serpentine soils. We demonstrated and quantified the differences in NcHMA4 gene copy number and expression for these populations. In addition, to improve our understanding of the mechanisms underlying the variability of Cd tolerance and accumulation, as well as the role of HMA4, we characterized variation in Cd root-to-shoot uptake and translocation for these populations.

Differences in shoot Cd concentration previously observed among these populations were probably due to changes in both metal uptake and translocation. The population exhibiting the highest accumulation (SF), showed the highest capacity for Cd uptake and translocation. In comparison, the other two populations, with lower Cd concentrations in the shoots, displayed either lower levels of uptake (Pr) or lower levels of translocation (PB). These results are congruent with those reported by Guimarães et al. (2009), who suggested that Cd hyperaccumulation in N. caerulescens is mainly determined by root processes. They showed that plants with N. caerulescens rootstocks and Thlaspi perfoliatum (a non-tolerant, non-accumulator relative) shoot scions accumulated the same level of Cd as N. caerulescens. Conversely, plants with T. perfoliatum rootstocks and N. caerulescens shoot scions accumulated similar levels of metal in shoots as T. perfoliatum. In our experiments, we also showed lower translocation in the least Cd-tolerant population (PB), indicating that root processes might also be involved in the variability of Cd tolerance. The higher fraction of Cd in roots, which are not dedicated to storage of Cd in N. caerulescens, might lead to higher metal toxicity. In A. thaliana, higher Cd accumulation in roots of hma4 mutants was associated with higher Cd sensitivity (Verret et al., 2004).

In addition to the variability observed for Cd fluxes, differences in NcHMA4 gene copy number and expression were found among the three populations. We demonstrated that the four NcHMA4 copies identified by Ó Lochlainn et al. (2011) are not fixed at the species level. The
importance of copy-number variation as a source of genetic and phenotypic variation has recently gained greater appreciation in a variety of organisms (Korbel et al., 2008; Cahan et al., 2009; Liao et al., 2009; Flagel and Wendel 2009; Qutob et al., 2009). Copy-number expansion has been observed for several genes involved in metal tolerance and homeostasis in A. halleri (HMA4, MTP1, ZIP3, ZIP9; Dräger et al., 2004; Mirouze et al., 2006; Talke et al., 2006; Shahzad et al., 2010) and N. caerulescens (IRT1, HMA3; Plaza et al., 2007; Ueno et al., 2011) compared to their relative A. thaliana. As described by Ó Lochlainn et al. (2011), we found four copies of NcHMA4 in the population Ga. We showed that the copy number was the same in the neighboring population SF (25 km away), and that at least one of the short copies identified by Ó Lochlainn et al. (2011) was present in this population. Conversely, the short copies were not identified in the population Pr and PB, and the HMA4 gene copy number seems to be smaller (three and two copies, respectively), suggesting different duplication events during microevolution. In A. halleri, the second duplication of AhHMA4 was estimated to occur after the speciation process (Roux et al., 2011).

Similar to other studies on NcHMA4 (Bernard et al., 2004; Papoyan and Kochian, 2004; van de Mortel et al., 2006; Xing et al., 2008), we found higher expression levels in the roots than in the shoots of all populations, and both in the presence and absence of Cd. We showed that in the three populations transcript abundance of NcHMA4 was particularly variable between the serpentine (PB) and the calamine populations (SF and Pr). Initially, our results appear to contradict those of Xing et al. (2008), who did not observe differences in NcHMA4 expression. However, they analysed NcHMA4 expression in two calamine accessions (and not in serpentine accessions). The low expression in PB could be related to low Cd or Zn contamination in serpentine soils, both being a substrate of NcHMA4. Variability of expression in these populations is at least partly explained by copy-number expansion. Indeed, weak expression was found in the serpentine population (PB), which also had a smaller number of NcHMA4 copies. Another interesting point is the similar overexpression of NcHMA4 in the calamine populations SF and Pr, which display different gene copy numbers. These results suggest on one hand different factors involved in the expression level of NcHMA4 and on the other hand possible convergent evolution in these populations. Empirical studies of the genetics of adaptation have shown that different populations within a species may evolve the same phenotype through different genetic changes (Arendt and Reznick 2007). Nevertheless, recent analyses of the pattern of genetic polymorphism in A. halleri support the evolution of metal hyperaccumulation well before human contamination (Roux et al., 2011). Overall, these populations are precious resources for further investigating the evolution of NcHMA4 through gene duplication and changes in regulation.

In this work, we showed that the population that translocates Cd less efficiently (PB) had lower NcHMA4 expression in the roots and shoots compared to SF and Pr, both of which exhibited similar NcHMA4 expression and capacity for translocation. Hence, the translocation of Cd seems to be correlated with the expression level of NcHMA4, supporting the idea that, in these populations, differences in translocation abilities are partly controlled by NcHMA4. In the three populations, NcHMA4 expression seemed to correlate more with Cd tolerance than Cd accumulation. The important contribution of HMA4 to Cd tolerance was also shown in A. halleri. The phenotypic variance explained by the HMA4 major quantitative trait loci was 2-fold higher for Cd tolerance than for Cd accumulation (42 and 21%, respectively; Courbot et al., 2007; Willems et al., 2010). Given these results, it would be interesting to validate the role of HMA4 in natural conditions.

Finally, the results of RNA in situ hybridization are consistent with NcHMA4 having a role in the translocation of Cd (and most probably of Zn) within the plant. NcHMA4 expression was associated with the vascular tissue in all organs, and the strongest expression was found in the crown zone (the zone of transition between roots and shoots). A specific mRNA accumulation was detected in the root pericycle and xylem parenchyma. In addition, the strong expression of NcHMA4 in the xylem parenchyma of the vascular tissue of leaves, and a diffuse expression in most of the mesophyll cells, indicates a possible role in metal distribution within the leaf blade, and the exclusion of metals from specific cell types. Expression was also observed in phloem tissues, and may indicate a role in the remobilization of Cd and Zn. Similar patterns of HMA4 expression were previously found in A. halleri and A. thaliana (Verret et al., 2004; Hanikenne et al., 2008).

Supplementary material
Supplementary data are available at JXB online.

Supplementary Table S1. List of primers used in this study

Supplementary Fig. S1. Relationship between (A) 109Cd translocation and 109Cd uptake (r = 0.435, P = 0.0294); (B) 109Cd translocation and shoot:root biomass ratio (r = 0.02, P = 0.9751) in three N. caerulescens populations (Saint-Félix-de-Pallières, Prayon and Puente Basadre), n = 25.

Supplementary Fig. S2. Phenotypes of plants belonging to the three investigated N. caerulescens populations after 1 month of exposure to 0, 3, and 30 μM of CdSO4 in hydroponic culture. Nutrient solution was supplemented with 100 μM ZnSO4.

Supplementary Fig. S3. Control growth of Cd-sensitive ycf1 yeast cells transformed with the empty vector pYEC2/CT−GFP or with the recombinant vector containing the short or the long NcHMA4 copy in the presence of glucose to suppress expression of transformed gene. Cells grown to the log phase were diluted to an OD600 of 1, 10−1, 10−2, 10−3, and 10−4 and 5 mL of each dilution was spotted on medium without or with added CdSO4. The plates were incubated at 30 °C for 4 days before photography.
Acknowledgements

This work was supported by the Belgian Science Policy (Interuniversity Attraction Pole Programme VI/33) and the Fonds National de la Recherche Scientifique (grant no. Fonds de la Recherche Fondamentale Collective 2.4.583.08). We thank J.P. Métaux (Université de Fribourg, Switzerland) to have welcomed N.R. to perform the radiolabelling experiment. We also thank anonymous reviewers for their helpful and constructive comments on the manuscript. We are grateful to the COST network 857 (European Cooperation in Science and Technology) for the mobility grant to N.R. in the laboratory of J.P. Métaux as well as for fruitful discussions during meetings.

References


