Expression of HMA4 cDNAs of the zinc hyperaccumulator Noccaea caerulescens from endogenous NcHMA4 promoters does not complement the zinc-deficiency phenotype of the Arabidopsis thaliana hma2hma4 double mutant

Mazhar Iqbal1 *, Ismat Nawaz1, Zeshan Hassan2, Henk W. J. Hakvoort1, Mattijs Bliek1, Mark G. M. Aarts2 and Henk Schat1

1 Department of Genetics, Faculty of Earth and Life Sciences, Vrije Universiteit, Amsterdam, Netherlands
2 Laboratory of Genetics, Wageningen University, Wageningen, Netherlands

*Correspondence: Mazhar Iqbal, Department of Genetics, Faculty of Earth and Life Sciences, Vrije Universiteit, Amsterdam, Netherlands
e-mail: mazz366@gmail.com

Expression of HMA4 cDNAs of the zinc hyperaccumulator Noccaea caerulescens (Nc) exhibits a very high constitutive expression of the heavy metal transporting ATPase, HMA4, as compared to the non-hyperaccumulator Arabidopsis thaliana (At), due to copy number expansion and altered cis-regulation. We screened a BAC library for HMA4 and found that HMA4 is triplicated in the genome of a N. caerulescens accession from a former Zn mine near La Calamine (LC), Belgium. We amplified multiple HMA4 promoter sequences from three calamine N. caerulescens accessions, and expressed AtHMA4 and different NcHMA4 cDNAs under At and Nc HMA4 promoters in the A. thaliana (Col) hma2hma4 double mutant. Transgenic lines expressing HMA4 under the At promoter were always fully complemented for root-to-shoot Zn translocation and developed normally at a 2 μM Zn supply, whereas the lines expressing HMA4 under Nc promoters usually showed only slightly enhanced root to shoot Zn translocation rates in comparison with the double mutant, probably owing to ectopic expression in the roots, respectively. When expression of the Zn deficiency responsive marker gene ZIP4 was tested, the transgenic lines expressing AtHMA4 under an NcHMA4-1-LC promoter showed on average a 7-fold higher expression in the leaves, in comparison with the double hma2hma4 mutant, showing that this construct aggravated, rather than alleviated the severity of foliar Zn deficiency in the mutant, possible owing to expression in the leaf mesophyll.

Keywords: Noccaea caerulescens, HMA4, promoter activity, Zn deficiency, Zn translocation, gene expression

INTRODUCTION

Zinc (Zn) is an essential element for all organisms. However, it is toxic when taken up in excess (Marschner, 1995; Kamal et al., 2004). Therefore, all organisms tightly regulate their cellular Zn status (Clemens, 2001). The network underlying Zn homeostasis in plants is incompletely known, but a number of Zn transporters of the ZIP/IRT, MTP, and HMA families have been shown to play essential roles in the acquisition, plant-internal transport of cation transporter ATPases. In Arabidopsis thaliana this subfamily is represented by eight members, of which HMA1 – HMA4 and HMA5 – HMA8 are transporting divalent and univalent heavy metal cations, respectively. All HMAs are effluxing heavy metal ions from the cytosol, either into the apoplast, the vacuole, or other organelles (Hussain et al., 2004; Andrés-Colás et al., 2006; Hussain et al., 2004; Ueno et al., 2011). In Arabidopsis thaliana, HMA2 and HMA4 are plasma membrane-localized, particularly expressed in the xylem parenchyma of the roots, and supposed to be involved in the loading of Zn and Cd into the xylem (Hussain et al., 2004; Wong and Cobbett, 2009). They seem to be partly redundant, since the single knock-out mutants, hma4 and hma2, have only a modest or no phenotype for Zn root-to-shoot transport respectively, whereas the double mutant has a very strong phenotype under normal Zn supply, including stunted growth, chlorosis and infertility (Hussain et al., 2004).

A minority of plant species, called metallophytes, are capable to grow and reproduce on strongly heavy-metal enriched “metaliferous” soils. These plants, or at least their metalliculous populations, exhibit extraordinary high levels of tolerance, also called hypertolerance (Clemens, 2006), to particular heavy metals (Antonovics et al., 1973; Ernst, 1974; Macnair, 1993). In so-called facultative metallophytes or pseudometallophytes, i.e., species occurring on non-metalliculous as well metalliculous soils, hypertolerance is largely metal-specific and confined to the metal or metals present at toxic concentrations in the soil at the site of population origin (Schat et al., 1996; Schat and Vooys, 1997). A small fraction of metallophytes, about 450 species worldwide, are classified as metal...
hyperaccumulator plants, accumulating particular heavy metals at extremely high concentrations in their foliage (Baker et al., 2006; van der Ent et al., 2013). Most of them hyperaccumulate nickel (Ni), but some of them mostly Zn, and/or cadmium (Cd), such as Noccaea caerulescens (formerly known as Thlaspi caerulescens) and Arabidopsis halleri (Cosio et al., 2014).

The mechanisms of hypertolerance and hyperaccumulation in metallophytes are far from completely understood. However, Cd and Zn hyperaccumulation and hypertolerance in Arabidopsis halleri have been shown to depend on a strongly enhanced expression of HMA4, which is in this case affected by tandem triplication and altered cis-regulation in comparison to A. thaliana (Courbot et al., 2007; Willem et al., 2007; Hanikenne et al., 2008). Recently, enhanced HMA4 expression, due to tandem quadruplication and altered cis-regulation, has also been demonstrated in Noccaea caerulescens, which is a remarkable case of parallel molecular evolution, since the hyperaccumulation trait must have been independently evolved in Noccaea and Arabidopsis (O’Lochlainn et al., 2011).

Although enhanced HMA4 expression is doubtlessly essential for the hyperaccumulation and foliar allocation of Zn and Cd in hyperaccumulators, as convincingly demonstrated by RNA-mediated silencing in A. halleri (Hanikenne et al., 2008), it does not seem to be sufficient to confer significant levels of Cd or Zn hyper-tolerance or hyperaccumulator-like foliar accumulation rates in a non-hyperaccumulator/non-metallophyte genetic background. Heterologous expression of AtHMA4 under the AHMA4 promoter, yielded enhanced Zn or Cd sensitivity, manifested as reduced shoot growth and chlorosis, but without considerably enhanced foliar metal accumulation in A. thaliana (Hanikenne et al., 2008) and tomato (Barbier et al., 2011). The reason for this is still elusive, and further characterization of the functioning of hyperaccumulator HMA4 genes in a non-hyperaccumulator genetic background is therefore required. Moreover, the three AHMA4 copies seem to show very similar expression patterns (Hanikenne et al., 2008), it cannot be excluded that there is some degree of functional differentiation among them. Therefore, in the present study we made an attempt to more precisely characterize the HMA4 cDNAs and HMA4 promoters from Noccaea caerulescens, through expression in the A. thaliana hma2hma4 double mutant. We were particularly interested in the potential of NHMA4 to revert the foliar Zn deficiency phenotype of the double mutant. We also phenotyped the transgenic lines for Cd tolerance and translocation. To better understand the function of HMA4 in hyperaccumulators and non-hyperaccumulators, we expressed the NHMA4 cDNAs under the AHMA4 promoter and AHMA4 under NHMA4 promoters, and compared the Zn and Cd translocation phenotypes of the transgenic lines with those of lines expressing AHMA4 under the native AHMA4 promoter. To detect potential differences in tissue or cell type specificity, we made the promoter:GUS constructs for the N. caerulescens HMA4 promoters and the A. thaliana one and compared their activities.

**MATERIALS AND METHODS**

**PLANT MATERIALS AND EXPERIMENTAL CONDITIONS**

Seeds of A. thaliana (Col) wild-type, the hma2hma4 double mutant and transgenic lines were sterilized in 96% ethanol, then 10% bleach, washed three times with sterilized water, suspended in 0.1% agarose and sown on 0.8% (w/v) gelrite plates (Duchefa, G1101.0250) containing half-strength Murashige and Skoog (MS) medium at pH 5.7–5.9 with 25 μM hygromycin for the transgenic lines and no antibiotics for wild-type on square petri plates that were vertically placed. Seeds were germinated at 22°C under a 10 h day/14 h photoperiod. After 2 weeks seedlings were transferred to hydroponics culture in 1-L polyethylene pots (three plants per pot, each plant of a different genotype) containing a modified half-strength Hoagland’s solution (Schat and Ten Bookum, 1992). Plants were grown in a climate room at 20±1°C day/night, light intensity 220 μmol m−2 s−1 at plant level, 10 h day−1, 75% RH. Nutrient solutions were renewed weekly. After 2 weeks in hydroponics, plants were exposed to five different concentrations of Cd (0.5, 12, 25 and 50 μM) and two concentrations of Zn (2 and 10 μM), supplied as CdSO4 or ZnSO4, ten plants per treatment.

Before exposure, roots were stained with active carbon powder (to facilitate the measurement of root length increment) and washed with demineralised water (Schat and Ten Bookum, 1992). After five days of exposure, root growth, i.e., the length of the longest unsustained root segment was measured.

Seeds of N. caerulescens, collected from the populations near La Calamine (LC), Belgium, and Saint Laurent de Miniers (this population is also known as Ganges, Ga) and Col du Mas de l’Aire (CMA), South-France, were sown on garden soil (Jongkind B.V., number 6, Aalsmeer, The Netherlands). Site and accession characteristics are given in Assunção et al. (2003; LC and Ga). CMA is another lead mine from the region around the village of Ga. Some accumulation and tolerance characteristics of the accesses are given in Peer et al. (2003) and Mottadai et al. (2012). Two-weeks-old seedlings were transferred to hydroponics in 1-L polyethylene pots containing a modified half-strength Hoagland’s solution (Schat and Ten Bookum, 1992). After 2 weeks leaves and roots were harvested, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

**DETERMINATION OF Cd AND Zn CONCENTRATIONS**

Cd and Zn concentrations were determined in roots and shoots (10 plants per population per concentration, pooled two by two, to make five samples) of wild-type, mutant and transgenic lines. Roots were carefully rinsed with ice-cold PhNO3 (5 mM) for 30 min and blotted with tissue paper. Cd and Zn were determined by digesting 50–100 mg of oven-dried plant material in 2 ml of a 1:4 (v/v) mixture of 37% (v/v) HCl and 65% (v/v) HNO3 in Teflon bombs for 7 h at 140°C, after which the volume was adjusted to 10 ml with demineralised water. Cd and Zn were determined on a flame atomic absorption spectrophotometer (Perkin Elmer AAS100).

**RNA AND DNA EXTRACTION AND 1st STRAND cDNA SYNTHESIS**

RNA was extracted from frozen root and shoot tissues using Trizol® (Invitrogen), following the manufacturer’s instructions and as described in Jack et al. (2007). Single-stranded cDNA was synthesized from total RNA (2.5 μg, boiled for 1 min) using 100 Units M-MLV Reverse Transcriptase (Invitrogen), 2 mM dNTPs, 100 mM DTT, 10× RT buffer and 10 μM oligo dT primer at 42°C for 1 h. DNA was isolated according to Rivera et al. (1999).
EXPRESSION ANALYSIS
cDNA was synthesized following the manufacturer’s protocol. Based on the sequence from the National Center for Biotechnology Information (NCBI) database1, intron spanning specific primers matching all of the HMA4 cDNAs were designed for real-time quantitative reverse transcriptase (RT) PCR (qRT-PCR; Table S1, Supplementary Material). Act2 was used as a positive internal control. The position of the intron was predicted by aligning coding sequences of NcHMA4 with the AhHMA4. Quantitative RT-PCR was performed using SensiMix™ SYBR No-ROX kit (Bioline) using the Bio-Rad MJ Research Opticon™ Real Time PCR detection system (Life Technologies; Invitrogen). SensiMix™ SYBR No-ROX kit includes the SYBR® Green I dye, dNTPs, stabilizers and enhancers. A dilution range in water of the cDNA samples was tested to identify the cDNA concentration that produced a Ct between 15 and 30 cycles. The final reaction conditions were, 10 μl SensiMix™ SYBR No-ROX master mix, 0.75 μl forward primer (final concentration of 250 nM), 0.75 μl of reverse primer (final concentration of 250 nM) and cDNA in a total reaction volume of 20 μl. An initial step of 95°C for 10 min was used to activate the polymerase. Cycling conditions were: melting step at 95°C for 10 s and annealing-extension at 60°C for 20 s, with 40 cycles, at the end melting curve from 60 to 90°C, read every 0.5°C, hold 10 s. All qPCR reactions were performed in triplicate, and a maximum difference of one cycle between the Ct of the triplicate samples was considered acceptable. Negative controls were included for each primer pair to check for significant levels of any contaminants. Expression values were calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

DNA BLOT HYBRIDISATION
We screened a BAC library of LC for HMA4. BAC clone DNA was extracted from 5 ml of overnight culture grown in LB medium containing 25 μg/ml chloramphenicol, using the Miniprep-Plasmid DNA Purification kit (QIAGEN) but skipping the column purification step. For Southern analyses, 2 μg of BAC clone DNA from two clones was digested with restriction enzymes (EcoRV, XbaI, BamHI, HindIII, EcoRI and PstI) at 37°C DNA from two clones was digested with restriction enzymes (EcoRV, XbaI, BamHI, HindIII, EcoRI and PstI) at 37°C for 6–16 h). For DNA blot analysis, the root growth between the transgenic and untransformed plants. After 2 weeks, there was a clear difference in root growth. After 2 weeks, there was a clear difference in root growth between the transgenic and untransformed plants. The transgenic plants were transferred to a nutrient solution containing a modified half-strength Hoagland’s nutrient solution (see above). After 2 weeks in hydroponics, samples were taken from roots and leaves to extract RNA. RNA was isolated using Trizol. cDNA was synthesized using M-MLV from Invitrogen. Then the relative transcript levels were measured by RT-qPCR, using actin-2 as a positive internal control (see above). The primers are given in Table S1, Supplementary Material.

Seeds of the homozygous A. thaliana hma2hma4 double mutant (Col; Hussain et al., 2004) were kindly provided by Prof. Chris Cobbett, University of Melbourne. A.thaliana (wt) and A.tha2hma2 double mutants were transformed by A. tumefaciens containing the promoter and all other constructs, respectively, given in Table 1, using the flower dip technique (Clough and Bent, 1998). T0 seeds (three lanes with ± 1000 seeds each) were surface-sterilized and sown on 0.8% (w/v) gelrite plates containing half-strength MS medium at pH 5.7–5.9 with 50 μg/ml hygromycin for screening. The plates were kept vertically to record the root growth. After 2 weeks, there was a clear difference in root growth between the transgenic and untransformed plants. The transgenic plants were transferred to a nutrient solution containing a modified half-strength Hoagland’s nutrient solution (see above). After 2 weeks in hydroponics, samples were taken from roots and leaves to extract RNA. RNA was isolated using Trizol. cDNA was synthesized using M-MLV from Invitrogen. Then the relative transcript levels were measured by RT-qPCR, using actin-2 as a positive internal control (see above). The primers are given in Table S1, Supplementary Material.

Table 1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are given in Table S1, Supplementary Material. ACT2 was used as a positive internal control (see above). The primers are gi

www.frontiern.org October 2013 | Volume 4 | Article 404 | 3
Table 1 | Constructs used for A. thaliana transformation and phenotyping experiments. For HMA4, cDNA was used.

| Construct Description | Insert Size (kb) |
|-----------------------|------------------|
| pAtHMA4::GUS | 2.0 |
| pAtHMA4::NcHMA4-1-LC | 2.0 |
| pAtHMA4::NcHMA4-2-LC | 2.0 |
| pAtHMA4::NcHMA4-3-LC | 2.0 |
| pAtHMA4::NcHMA4-4-LC | 2.0 |
| pAtHMA4::NcHMA4-5-LC | 2.0 |
| pAtHMA4::NcHMA4-6-LC | 2.0 |
| pAtHMA4::NcHMA4-7-LC | 2.0 |
| pAtHMA4::NcHMA4-8-LC | 2.0 |
| pAtHMA4::NcHMA4-9-LC | 2.0 |

pNcHMA4-1-LC and pNcHMA4-2-LC were used in a LR reaction with the pKGWGG-RR vector (Limpens et al., 2004) through Gateway® LR Clonase® II Enzyme Mix (Invitrogen™) to create an expression clone. Sequence reactions were done using the big dye terminator protocol. These expression clones were inserted separately in Agrobacterium rhizogenes by electroporation, and then used for hairy root transformation of N. caerulescens.

**Hairy Root Transformations of N. caerulescens**

Roots of N. caerulescens were transformed with pNcHMA4-LC constructs via Agrobacterium rhizogenes mediated transformation. Single colonies of transformed A. rhizogenes (MSU 440) were cultured in 5 ml LB media with 100 μg/ml spectinomycin at 28°C overnight. Two hundred μl from the liquid culture were plated on LB-agar plates containing the same antibiotic and grown for 2 days at 28°C. Seeds of N. caerulescens accession LC were sown in four agar plates containing half-strength MS (pH 5.8), 25 seeds per plate. Seven-days-old plants had their roots removed just below the hypocotyl. One dot of Agrobacterium bacteria was put at the tip of each hypocotyl. For co-culturing, plates were incubated in a climate chamber, set at 250 μmol m⁻² s⁻¹ light at plant level, during a 16-h day period, 24°C, and 70% relative humidity. After 5 days, seedlings were transferred to new ½ MS-agar plates supplemented with 100 μg/ml tricarcillin to kill the bacteria. Roots were checked every three days using a stereomicroscope under UV, for expression of the DsRED protein indicating transformation. Non-transformed roots were removed. After 8 weeks, the plants showing the expression of HMA4 were selected, vouchered, and used for hairy root transformation. The MSR statistic was used for one-way ANOVA. The MSR statistic was used for posteriori comparisons of individual means (Sokal and Rohlf, 1981). To obtain homogeneity of variances, all the data were subjected to logarithmic transformation prior to analysis, except for the root elongation data.

**RESULTS**

**DNA-BLOT Analysis**

A genomic BAC library of N. Caerulescens, LC, with an average insert size of 135 Kb was constructed by EPICENTRE Biotechnologies. Eight 384 well plates were screened for HMA4. We found two clones containing HMA4 DNA blot analysis showed that most of the lanes contained three bands, suggesting that HMA4 is at least triplicated in LC (Figure 1), because we used restriction enzymes that do not cut within the probe exon.

**N. CAERULESCENS HMA4 CODING AND PROMOTER SEQUENCES**

We constructed phylogenetic trees for NcHMA4s and other known HMA4 coding sequences and promoters (Figures S1 and S2, Supplementary Material). A. halleri HMA4 cDNAs are about 88% identical with the A. thaliana HMA4 cDNA (Hanikenne et al., 2008). N. caerulescens HMA4 cDNAs are less similar to the AhHMA4 cDNAs, sharing 80 to 85% identity. All the NcHMA4s share about 98% identity. On an amino acid basis AhHMA4 and NcHMA4s are around 86%, or 71 to 76% identical with HMA4, respectively and NcHMA4s are 94 to 99% identical among each other (Table S3-A). When comparing promoter sequences, the NcHMA4 promoters are 36 to 39% identical with the AhHMA4 promoter, and 38 to 43% identical with the AtHMA4 promoters. When compared among each other, the NcHMA4 promoters share 36 to 96% identity (Table S3-B and Alignment S3, Supplementary Material).

**EXPRESSION OF HMA4 IN A. THALIANA AND N. CAERULESCENS**

Transcript levels of HMA4 were determined by qRT-PCR in A. thaliana and N. caerulescens shoots and roots. In A.
Iqbal et al. Expression of HMA4 under NcHMA4 promoter

**FIGURE 1** | Southern blot of two BAC clones of *N. caerulescens*, accession La Calamine. Each clone was restricted by six different restriction enzymes (EcoRV, XbaI, BamHI, HindIII, EcoRI and PstI). Hybridization was done with a 521 bp probe from the last exon of HMA4.

**FIGURE 2** | HMA4 expression in three *Noccaea caerulescens* accessions (fold *A. thaliana* in shoot) La Calamine (LC), Ganges (Ga) and Col du Mas de l’Aire (CMA). Significant differences (P < 0.05) between means are indicated, separately for root and shoot, by different superscripted letters.

**FIGURE 3** | *AtHMA4* (solid bars) and *NcHMA4-1-LC* (open bars) expression under *A. thaliana* and *N. caerulescens* promoters in the root of transgenic *A. thaliana* hma2hma4 double mutants, relative to the average expression in the root of *A. thaliana* wild-type which is considered as 1 (means of 5 plants ± SE). Significant differences (P < 0.05) between means are indicated by different superscripted letters.

**FIGURE 6** | GUS was also expressed under the pNcHMA4-1-LC and pNcHMA4-2-LC in *N. caerulescens*, using *A. rhizogenes* mediated root transformation. Staining was observed mainly in the stele, like pAtHMA4, and the root tip, though exclusively in the root cap (Figure 6).

*AtHMA4* was mainly expressed in the root, i.e., 20 times higher than in the shoot. In *N. caerulescens* LC and CMA, *HMA4* was higher expressed in the shoot than in the root, whereas in Ga it was higher expressed in the root.

(Figure 2). Assuming equal expression of Act2, the shoot and root *HMA4* transcript concentrations were about 400-fold and 23-fold higher, respectively, in *N. caerulescens* than they were in *A. thaliana*.

**TRANSGENIC HMA4 EXPRESSION USING *N. CAERULESCENS* AND *A. THALIANA* HMA4 PROMOTERS**

We expressed NcHMA4-1-LC and AtHMA4 cDNAs under different NcHMA4 promoters and the AtHMA4 promoter in the Athma2hma4 double mutant. There was no significant effect of the cDNA source on the expression levels in 4-weeks old primary transgenic plants (Figures 3 and 4). When expressed under Nc promoters, the average transgene expression levels in the roots were, surprisingly, lower than those under the endogenous AtHMA4 promoter, except for the pNcHMA4-1-LC. The latter and the At promoter both yielded expression levels close to the wild-type level in untransformed *A. thaliana* (Figure 3). In the shoot, however, all the Nc promoters tested were significantly (4- to 60-fold) more active than the At one (Figure 4). To compare the patterns of tissue-specificity, we also expressed, under the same promoters, GUS in wild-type *A. thaliana*. When expressed under the AtHMA4 promoter, GUS activity was consistently high in the root stele, but negligible in the root tip. When expressed under NcHMA4 promoters, however, GUS activity was often low or negligible in the root stele, but (extremely) high all over the apical 2-mm root segment (Figure 5). Moreover, the NcHMA4 promoters often produced considerable GUS activity in the leaves, occasionally all over the leaf blade, rather than confined to the veins, whereas the AtHMA4 promoter was barely active in the leaves (Figure 5).

GUS was also expressed under the pNcHMA4-1-LC and pNcHMA4-2-LC in *N. caerulescens*, using *A. rhizogenes* mediated root transformation. Staining was observed mainly in the stele, like pAtHMA4, and the root tip, though exclusively in the root cap (Figure 6).
**Zn Root-to-Shoot Translocation and Mutant Complementation**

We compared Zn root-to-shoot translocation among wild-type and hma2hma4 double mutant and transgenic plants. In a first experiment we used selected T1 transgenic plants (10 plants per concentration) derived from T0 plants expressing AtHMA4 approximately at wild-type levels in the roots, under the pNcHMA4-1-LC, pNcHMA4-2-LC, pNcHMA4-3-Ga and pNcHMA4-1-CMA promoters, respectively. After 3 weeks of growth in hydroponics at 2 μM Zn, all of the transgenic lines had foliar Zn concentrations that were slightly, but significantly higher than that of the double mutant, but much lower than that of wild-type plants (Figure 8A). The same pattern was observed at 10 μM Zn (data not shown). The root Zn concentrations were highest in the double mutant and lowest in the wild-type, except for the pNcHMA4-1-CMA::AtHMA4 line at 10 μM Zn (data not shown). All the transgenic lines and the double mutant showed symptoms of foliar Zn deficiency.

In a second experiment we compared selected T1 derived from T0 plants transformed with pAtHMA4::AtHMA4, pAtHMA4::NcHMA4-1-LC, pAtHMA4::NcHMA4-2-LC, pNcHMA4-1-LC::AtHMA4, pNcHMA4-1-LC::NcHMA4-2-LC, and pNcHMA4-1-LC::AtHMA4 using the wild-type as a reference. For this experiment we chose the T1 progeny derived from T0 plants expressing NcHMA4-2-LC 5- to 10-fold higher than wild-type in the roots. The two AtHMA4 expressing lines were derived from T0 plants with approximately wild-type expression levels in their roots. The lines with the constructs containing the AtHMA4 promoter had wild-type foliar Zn concentrations, or even higher in the case of the pAtHMA4::AtHMA4 line, which was derived from a T0 plant with a particularly high root expression level (six times At wild-type level, in comparison with 1.5 and 4 times for the T0 parents of the other lines, Figure 8B). The lines with the constructs containing the pNcHMA4-1-LC exhibited foliar Zn concentrations that were significantly lower than At wild-type level. The two lines derived from the T0 plants with the highest root expression levels (9 and 10 times At wild-type level for pNcHMA4-1-LC::NcHMA4-1-LC, pNcHMA4-1-LC::NcHMA4-2-LC) showed much higher foliar Zn concentrations, i.e., more than half of the wild-type level, than the one derived from the T0 with the lowest root expression level (1.5 times for pNcHMA4-1-LC::AtHMA4), i.e., about 25% of wild-type, comparable to the former experiment (Figure 8A). As expected, the pNcHMA4-1-LC::AtHMA4 line the root Zn concentration was higher than in At wild-type, but this was also the case for the pNcHMA4::NcHMA4-2-LC line (Figure 8B). All the lines transformed with the pNcHMA4-1-LC, as well as the double mutant, showed symptoms of foliar Zn deficiency, whereas all the lines transformed with the At promoter did not.

To estimate the degree of foliar Zn deficiency in the lines transformed with the pNcHMA4-1-LC::AtHMA4 construct, we measured the foliar expression level of a Zn deficiency sensitive marker gene, ZIP4 (Assunção et al., 2010), in an pNcHMA4-1-LC::AtHMA4 T1 line, the hma2hma4 double mutant, and the wild-type. ZIP4 was five fold higher expressed in the double mutant, and even 35-fold higher in the transgenic line, in comparison with wild-type, showing that expression of HMA4 under the pNcHMA4-1-LC...
FIGURE 5 | Arabidopsis (wt) plants expressing GUS under AtHMA4 and NcHMA4 promoters from the accessions LC, Ga and CMA. (Scale bar is 5 mm in (A) to (B), 2 mm in (A′) to (I′) and 30 μm in (A′′) to (I′′)).
aggravates, rather than alleviates the foliar Zn deficiency of the double mutant (Figure 9).

**Cd TOLERANCE AND ACCUMULATION**

Cd tolerance, estimated from the root growth response, was tested in the same set of T1 lines that was used in the second Zn translocation experiment. Although there was significant variation in root length increments among the lines, the lines by Cd interaction was insignificant, showing that the lines did not significantly differ in their response to Cd (Figure 10). The Cd concentrations in shoot and root at the 0.5- and 12-μM Cd exposure levels are given in Figures 11A,B. At the 0.5-μM exposure level the lines transformed with the pAtHMA4 exhibited wild-type-like or higher foliar Cd concentrations, whereas the lines transformed with pNcHMA4-1-LC exhibited significantly lower concentrations, in comparison with wild-type. In the roots the Cd concentrations were higher in wild-type than in the pNcHMA4-1-LC::NcHMA4-1-LC and pNcHMA4-1-LC::NcHMA4-2-LC lines. Overall, the patterns of variation in the Cd concentrations found at the 0.5-μM exposure level were essentially the same as found for Zn among the same set of lines (see above). At the 12-μM exposure level, however, there were no considerable differences in the root and shoot Cd concentrations between the lines and wild-type, or among the lines (Figures 11A,B).

**DISCUSSION**

Our results clearly confirm that HMA4 is strongly over-expressed, both in roots and in shoots, in the Zn/Cd/Ni hyperaccumulator
Iqbal et al. Expression of HMA4 under NcHMA4 promoter

FIGURE 8 | Shoot Zn concentration (μmol g⁻¹ DW) at 2 μM ZnSO₄ in the nutrient solution in two successive experiments performed with Arabidopsis wt, untransformed hma2hma4 double mutant, and five transgenic lines expressing AtHMA4 under different NcHMA4 promoters (A), or six lines expressing three different HMA4 cDNAs, each under the AtHMA4 and the NcHMA4-LC promoter (B). Significant differences (P < 0.05) between means are indicated, separately for both shoots and roots, by different superscripted letters.

FIGURE 9 | Average ZIP4 expression in leaves in transgenic lines expressing AtHMA4 under the pNcHMA4-1-LC, the hma2hma4 double mutant, and wild-type (fold expression relative to Arabidopsis wt). Significant differences (P < 0.05) between means are indicated by different superscripted letters.

FIGURE 10 | Effect of Cd on root growth in Arabidopsis wt and transgenic lines. Root length increments were measured after 4 days.

N. caerulescens, in comparison with the non-hyperaccumulator A. thaliana. Like in the other Zn/Cd hyperaccumulator model, A. halleri, this over-expression must be due to a combination of copy number expansion and altered cis-regulation of the individual copies (Hanikenne et al., 2008). The copy number seems to vary between accessions in N. caerulescens, ranging from 2 to 4 (Ó Lochlainn et al., 2011; Craciun et al., 2012). The three copies that we found in the LC library matches with the three copies established in the nearby accession from Prayon (Craciun et al., 2012).

The most striking result that we obtained is that expressing either AtHMA4 or NcHMA4 cDNAs under any N. caerulescens HMA4 promoter did not fully complement, except for two T₁ plants, the Zn-translocation-deficient A. thaliana hma2hma4 double mutant, whereas expression under the endogenous AtHMA4 promoter always did. This is not simply a matter of the degree
of expression of the transgene in the root. Admittedly, the HMA4 expression levels produced by Nc promoters in the double mutant background were surprisingly low, in some cases even significantly lower than the wild-type expression level in A. thaliana. However, when compared in lines derived from plants with equal transgene expression levels in the roots, pNcHMA4::HMA4 constructs completely restored the Zn root-to-shoot translocation to wild-type level or higher, whereas pNcHMA4-1-LC::AtHMA4 constructs only slightly enhanced the foliar Zn concentration. Even in pNcHMA4::HMA4 lines expressing the transgene about 10 times higher than wild-type in the root, the foliar Zn concentrations were not higher than 40% of the wild-type level, whereas a strongly expressing pNcHMA4-1-LC::AtHMA4 line showed enhanced Zn translocation, in comparison with wild-type (Figure 8B). Admittedly, there was considerable variation regarding the root and shoot Zn concentrations between the two successive experiments that we performed (Figure 8). We cannot explain this variation, however, it is remarkable that the Zn shoot concentrations and translocation rates of the lines with the Nc promoters are consistently intermediate between those in wild-type and in the hma2hma4 double mutant. The reasons for this are not entirely clear, but ectopic expression under the Nc promoters, in terms of tissue-specificity, seems to be the most plausible explanation. As indicated by the GUS assays, the Nc promoters are often mainly active in all the cells of the 2-mm apical root segment, rather than in the xylem parenchyma, whereas the At promoter is exclusively active in the xylem parenchyma (Figure 5), as is the Nc promoter tested in the A. rhizogenes-transformed N. caerulescens roots. One might argue that this could be owing to the short length of the Nc promoter sequences that we isolated, i.e., 2100–2600 bp, in comparison with the At promoter (4103 bp), which covered the whole upstream intergenic region. However, as shown by the GUS assay with Rhizogenes-transformed N. caerulescens roots, the 2135 bp of the pNcHMA4-1-LC does produce expression predominantly in the xylem parenchyma in N. caerulescens itself (Figure 6), suggesting that A. thaliana and N. caerulescens may have different transcriptional regulators for HMA4 expression, at least in part.

There seemed to be a degree of positive correlation between the transgene expression levels in the root and the foliar Zn concentrations among the lines transformed with the Nc promoters (Figure 8A). However, in spite of this, the transgene expression levels in the roots were also positively correlated with the severity of foliar Zn deficiency symptoms, at least among the plants transformed with the pNcHMA4-1-LC::AtHMA4 construct (Figure 7). The most likely explanation for this phenomenon is that the transgene expression levels in roots are more or less correlated with those in shoots (Figures 3 and 4). It is remarkable that expression of AtHMA4 under the pNcHMA4-1-LC improved, albeit slightly, the root-to-shoot Zn translocation (Figure 8B), but aggravated rather than alleviated the foliar Zn deficiency symptoms in the hma2hma4 double mutant, as demonstrated by the strongly enhanced ZIP4 expression level (Figure 9). The latter might be attributable to the relatively high level of HMA4 expression in the leaf mesophyll (Figure 4), which would be expected to enhance the efflux of Zn from photosynthetically active cells. This might in turn cause or enhance Zn deficiency in the absence of hyperaccumulator-like uptake and root-to-shoot translocation rates. The high expression of HMA4 outside the veins in the leaves of mutants transformed with pNcHMA4::HMA4 constructs might well reflect the natural expression pattern in N. caerulescens itself. As demonstrated by Klein et al. (2008), N. caerulescens cells in suspension culture accumulate less Zn or Cd than A. thaliana cells, presumably due to higher metal efflux rates. It is therefore conceivable that a high-level of HMA4 expression in the leaf mesophyll in hyperaccumulators could serve to promote the cell-to-cell transport of metals from the vasculature to their final destiny, the large epidermal cells (Küpper and Kochian, 2010).

In the Cd tolerance test, we used only wild-type as a control, since we had insufficient seeds of the hma2hma4 double mutant. However, it can be safely assumed that the double mutant, at least when in the wild-type background, does not have a detectable phenotype for Cd tolerance (Hussain et al., 2004; Wong and Caballero, 2009). In any case, our results clearly show that HMA4 expression, regardless of the promoter and cDNA source, does not confer Cd tolerance, in comparison to wild-type, to the hma2hma4 double mutant. This is not surprising, because at the highest toxic Cd exposure levels there were no considerable effects of the transgenes on the root or shoot Cd concentrations (Figure 11). At the non-toxic...
0.5 μM Cd exposure level, however, there were pronounced and significant effects of the transgenes on the root and shoot Cd concentrations. Overall, these effects were about the same as those on the root and shoot Zn concentrations (Figure 8). This, together with the extremely low foliar Zn concentration in the mutant at the 0.5 μM exposure level, confirms that HMA4 can translocate both Zn and Cd in A. thaliana (Cobbett, 2009). However, at higher exposure levels, the translocation of Cd, in contrast with that of Zn, is apparently no longer dependent on HMA4, but mediated by other systems with lower affinity.

Comparing our results with those obtained with HMA4 genes and promoters from other model Zn/Cd hyperaccumulator, A. halleri, reveals several differences. First, the AhHMA4 promoters exhibited the correct tissue-specific patterns of expression in A. thaliana roots than did the NcHMA4 promoters in the present study. Second, over-expression in transgenic A. thaliana under the different AhHMA4 promoters exhibited the correct tissue-specificity patterns, i.e., exclusively in the xylem parenchyma and the root cap (Hanikenne et al., 2008). The reason for the low and different HMA4 expression under NcHMA4 promoters that we observed in our study is elusive, but it is well conceivable that it has something to do with the bigger phylogenetic distance between N. caerulescens and A. thaliana, in comparison with that between the congenic A. thaliana and A. halleri.

Finally, the question of whether correct over-expression of HMA4 can confer hyperaccumulator-like metal translocation rates to non-hyperaccumulator host species remains unanswered. HMA4-over-expression in A. thaliana has been reported to enhance Cd or Zn translocation to the shoot, albeit to a marginal degree (Verret et al., 2004; Hanikenne et al., 2008). However, in the latter studies HMA4 has been expressed under the 35S CaMV promoter, which is bound to yield ectopic expression. Barabasz et al. (2012) expressed a HMA4 copy from A. halleri under the endogenous Ah promoter in tobacco, but also found no more than a marginal effect on Zn translocation. It is unknown, however, whether the transgene was correctly over-expressed in the present study. Unfortunately, the NcHMA4 promoters that we used in the present study did not produce the correct expression patterns in A. thaliana roots, which probably explains the inconsiderable effects of the HMA4 transgenes on Zn and Cd translocation. It is reasonable to expect that this may not apply to the A. halleri HMA4 promoters (see above). However, even in cases where hyperaccumulator promoters would yield a correct hyperaccumulator-like HMA4 expression pattern, it is doubtful whether this would lead to more than marginal increments of root-to-shoot metal translocation rates. There are good reasons to assume that hyperaccumulator-like translocation rates are not only dependent on HMA4-mediated xylem loading, but also on “upstream mechanisms,” which prevent the retention of metals in root cell vacuoles (Eichler et al., 2009; Denielen et al., 2012).

ACKNOWLEDGMENTS

We thank the “Higher Education Commission (HEC), Pakistan” for the financial support of the first author.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fpls.2013.00404/abstract
Expression of HMA4 under NcHMA4 promoter

Limpens, E., Ramos, J., Franken, C., Raz, K., Hurkman, A., Van der Ent, A., Vrensen, G. F., and Kochian, L. V. (2001). Analysis of relative gene expression using real-time quantitative PCR and the Ze-Delta Ze Delta CT method. Method. 29, 452–466. doi: 10.1016/S0022-2836(01)00458-X

Michaud, M. R. (1993). The genetics of metal tolerance in vascular plants. New Phytol. 124, 541–559. doi: 10.1111/j.1469-8137.1993.tb02786.x

Morsczen, H. (1995). Mineral Nutrition of Higher Plants. 2nd Edn. Academia Press, London.

Murlati, A., Gheradian, S., and Schat, H. (2012). A comparison of lead accumulation and tolerance among heavy metal hyperaccumulating and non-hyperaccumulating metallophytes. Plant Soil 352, 267–278. doi: 10.1007/s11104-011-0994-5

Nelissen, J., Bossuyt, H. F., Fey, R. G., Hammond, J. P. K., King, G. J., White, F. J., et al. (2011). Tandem quadruplication of HMA4 in the zinc (Zn) and cadmium (Cd) hyperaccumulator Nicotiana alata. Proc. Natl. Acad. Sci. U. S. A. 108, 17814–17819. doi: 10.1073/pnas.1109270108

Peppey, P., and Kochian, L. V. (2004). Identification of Thlaspi caerulescens lines that accumulate high levels of heavy metals and tolerance to heavy metal stress. Plant Physiol. 136, 2805–2812. doi: 10.1104/pp.104.052900

Peer, W. A., Mamoudian, M., Lub, B., Revers, R. D., Murphy, A. S., and Sah, D. E. (2005). Identifying model metal hyperaccumulating plants: genetic analysis of 20 Brassicaceae accessions from a worldwide distribution area. New Phytol. 169, 421–430. doi: 10.1111/j.1469-8137.2005.01622.x

Richau, K. H., Kozhevnikova, A. D., Seregin, I. V., Vooijs, R., van der Ent, A., and van der Graaf, J. A. H. (2009). Chelation by histidine inhibits the vascular sequestration of nickel in roots of the hyperaccumulator Thlaspi caerulescens. New Phytol. 185, 116–129. doi: 10.1111/j.1469-8137.2009.02826.x

Rivera, R., Edwards, K. J., Barker, J. H., Arnold, G. M., Ayed, G., Hodgkin, T., et al. (1999). Isolation and characterization of polymorphic microsatellite lines in Canavalia ensiformis L. Genetica 102, 669–675. doi: 10.1023/A:10008178

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Edn. New York: Cold Spring Harbor Laboratory Press.

Schat, H., and Ten Boekum, W. M. T. (1992). Genetic control of copper tolerance in Silene vulgaris. Heredity 68, 219–229. doi: 10.1046/j.1365-2540.1992.t35

Schat, H., and Vosio, R. (1997). Multiple tolerance and co-tolerance to heavy metals in Silene vulgaris: a co-segregation analysis. New Phytol. 139, 489–498. doi: 10.1046/j.1469-8137.1997.00796.x

Schiers, G., and Kupper, H. (2007). The basic science of zinc tolerance in the metallophyte Arabidopsis halleri: a summary of quantitative trait loci. Genomics 87, 679–677. doi: 10.1016/j.ygeno.2005.11.004

Silene vulgaris Schreb. (1996). Identical major gene loci for lead accumulation and tolerance in metallicolous and non-metallicolous populations of Silene vulgaris (Moench) Geyeri. Environ. Exp. Bot. 39, 86–91. doi: 10.1016/S0098-8472(96)00025-5

van der Ent, A., Baker, A. M., Reeves, R. D., Murphy, A. S., Mühlbacher, A., and Kochian, L. V. (2004). P-type ATPase expression in metallicolous and non-metallicolous populations of Silene vulgaris. Plant Soil 267, 267–276. doi: 10.1023/B:PLSO.0000020860.63825.fc

Wong, C. K. E., and Cobbett, C. S. (2009). HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in Arabidopsis thaliana. New Phytol. 181, 71–78. doi: 10.1111/j.1469-8137.2009.02088.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received 30 April 2013; accepted: 24 September 2013; published online: 26 October 2013.

CrossMark

Frontiers in Plant Science | Plant Physiology October 2013 | Volume 4 | Article 404 | 12