Journal of Experimental Botany, Vol. 61, No. 11, pp. 3057–3067, 2010
doi:10.1093/jxb/erq129 Advance Access publication 19 May, 2010
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Metal accumulation in tobacco expressing *Arabidopsis halleri* metal hyperaccumulation gene depends on external supply

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Received 22 February 2010; Revised 22 April 2010; Accepted 22 April 2010

Abstract

Engineering enhanced transport of zinc to the aerial parts of plants is a major goal in bio-fortification. In *Arabidopsis halleri*, high constitutive expression of the *AhHMA4* gene encoding a metal pump of the P_{1B}-ATPase family is necessary for both Zn hyperaccumulation and the full extent of Zn and Cd hypertolerance that are characteristic of this species. In this study, an *AhHMA4* cDNA was introduced into *N. tabacum* var. Xanthi for expression under the control of its endogenous *A. halleri* promoter known to confer high and cell-type specific expression levels in both *A. halleri* and the non-hyperaccumulator *A. thaliana*. The transgene was expressed at similar levels in both roots and shoots upon long-term exposure to low Zn, control, and increased Zn concentrations. A down-regulation of *AhHMA4* transcript levels was detected with 10 μM Zn resupply to tobacco plants cultivated in low Zn concentrations. In general, a transcriptional regulation of *AhHMA4* in tobacco contrasted with the constitutively high expression previously observed in *A. halleri*. Differences in root/shoot partitioning of Zn and Cd between transgenic lines and the wild type were strongly dependent on metal concentrations in the hydroponic medium. Under low Zn conditions, an increased Zn accumulation in the upper leaves in the *AhHMA4*-expressing lines was detected. Moreover, transgenic plants exposed to cadmium accumulated less metal than the wild type. Both modifications of zinc and cadmium accumulation are noteworthy outcomes from the biofortification perspective and healthy food production. Expression of *AhHMA4* may be useful in crops grown on soils poor in Zn.

Key words: AhHMA4, *Arabidopsis halleri*, cadmium, P_{1B}ATPase, tobacco, transformation, zinc.

Introduction

Current knowledge on the molecular and biochemical mechanisms of transition metal homeostasis in plants is commonly used in attempts to modify metal uptake, root-to-shoot translocation, and distribution at the cellular, tissue, and organ levels. Such alterations are a focus in both phytoremediation and biofortification, although the needs in these two applications are different. In phytoremediation (phytoextraction) the aim is to increase the uptake from contaminated sites, translocation to shoots, accumulation and the level of tolerance to metals of interest for soil clean-up (Kunze *et al.*, 2002; Krämer, 2005; Zhao and McGrath, 2009). Biofortification aims at an efficient micronutrient uptake mainly from poor soils, and an efficient translocation to the edible parts of crop plants (Krämer *et al.*, 2007; Palmgren *et al.*, 2008; Krämer, 2009). The common goal, however, for both biotechnological applications is to increase the transfer of metals from the roots to the shoots. Genetic engineering is a promising tool in both applications.

As a micronutrient necessary for maintaining basic physiological processes, zinc is essential in plants, animals, and humans. However, it is frequently deficient in the diet, resulting in poor health. Across the world, there are many soils that are Zn-deficient or with low Zn bioavailability. Consequently, crops cultivated there contain low Zn concentrations leading to Zn-deficiency-based malnutrition.

There are also areas with extremely high soil Zn levels. It is well known that an excess of Zn is toxic, and phytoremediation is considered the only way to clean up soils contaminated with metals (Pilon-Smits and Pilon, 2002; Verkleij *et al.*, 2009; Zhao and McGrath, 2009). Altogether, there is a strong need to elaborate the way of engineering of enhanced Zn uptake and accumulation in the shoots.

Knowledge on the mechanisms that control the root-to-shoot translocation of zinc and other transition metals is still rudimentary. Only recently, studies performed by several research groups reported the involvement of *HMA4*, which encodes a P_{1B}-ATPase, both from *A. thaliana* (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2004, 2005) and *A. halleri* (Hanikenne *et al.*, 2008) primarily in the transport of Zn, but also Cd, to the shoots. In *A. halleri*, a Zn/Cd-hyperaccumulating species, *AhHMA4* is required for highly efficient root-to-shoot transport and thus for shoot Zn hyperaccumulation (Hanikenne *et al.*, 2008). *HMA4* transcript levels are much higher in *A. halleri* than in *A. thaliana*, although *HMA4* expression localizes largely to the same cell types in both species. It was shown that the *A. halleri HMA4* promoter governs high expression levels of *AhHMA4* or a reporter gene in both the *A. halleri* and the *A. thaliana* genetic backgrounds (Hanikenne *et al.*, 2008). In the closely related non-accumulating *A. thaliana*, *At*HMA4 and the homologous *At*HMA2 control Zn/Cd translocation to the aerial parts of the plant, and their general role is to maintain Zn-dependent processes in the shoot (Hussain *et al.*, 2004; Wong and Cobbett, 2009). Interestingly, the overexpression of *AhHMA4* cDNA under the control of its native promoter in *A. thaliana* was sufficient to recapitulate the radial Zn distribution pattern typical of *A. halleri* roots, with increased Zn concentrations in the xylem vessels (Hanikenne *et al.*, 2008).

Here, tobacco was transformed with a construct containing the *AhHMA4* cDNA downstream of its native promoter from *A. halleri* in an attempt to engineer more efficient root-to-shoot Zn translocation and thus enhanced accumulation in the shoots of an agricultural plant. The use of the same construct for tobacco transformation as for the transformation of *A. thaliana* (Hanikenne *et al.*, 2008) allowed us to examine: whether the endogenous *A. halleri HMA4* promoter (i) is effectively conferring expression of *AhHMA4* in tobacco; and (ii) confers root and shoot expression patterns in tobacco similar to those in *Arabidopsis*. In addition, it was tested whether expression of *AhHMA4* in tobacco (iii) generates modifications in Zn partitioning as observed in *A. thaliana* and (iv) can alter metal tolerance. Tobacco was chosen as a model plant for the heterologous expression because it is easy to transform and regenerate.

Materials and methods

Transformation

AhHMA4-1, one of the three *AhHMA4* gene copies that are constitutively very highly expressed in the Zn/Cd hyperaccumulator *Arabidopsis halleri* was used in this study. The construct which was generated and used previously by Hanikenne *et al.* (2008) for the transformation of *A. thaliana* was used here for the transformation of tobacco (*Nicotiana tabacum* var. Xanthi).

The standard procedure (Murashige and Skoog, 1962; Horsch *et al.*, 1985) of *Agrobacterium*-mediated transformation of leaf discs of tobacco (seeds from the stock of the Institute of Biochemistry and Biophysics PAS, Warszawa, Poland) was applied as described in Antosiewicz and Hennig (2004). Transgenic plants were selected on medium supplemented with 25 μM hygromycin. T_1 seeds were collected from the T_0 generation that was confirmed for the presence of *AhHMA4* cDNA sequence by PCR and mRNA expression by RT-PCR. The primer sequences and the reaction conditions are given in the sub-section 'Determination of Cd-tolerance and accumulation'.

As a result of the transformation, 22 independent transgenic lines were selected. The segregation ratio of hygromycin-resistant to hygromycin-sensitive T_1 plants, determined for all of them, was found to be 3:1 (tolerant:sensitive) for 10 lines, indicating a single-locus insertion of the transgene.

Plant material and general growth conditions

Tobacco wild type (*Nicotiana tabacum* var. Xanthi) and independently transformed lines were included in the experiments. Six heterozygous T_1 lines with a 3:1 hyg^R:hyg^S segregation ratio were selected at random (nos 2, 10, 23, 27, 28, and 35). The expression of the transgene was determined, and three lines with differing levels of *AhHMA4* expression (low and high) were used to obtain homozygous T_2 plants (nos 2, 28, and 35; details in the Results, section 'Tobacco expressing $AhHMA4_{p1}$::*AhHMA4*'). Depending on the experiment, heterozygous and/or homozygous lines were used (details in the Results and in the figure legend).

All experiments were carried out in a growth chamber, at 23/16 °C day/night temperatures, 40–50% constant humidity, with a 16 h photoperiod at a quantum flux density (PAR) of 250 μmol m^{-2} s^{-1} using fluorescent Flora tubes. Seeds were surface-sterilized in 8% (v:v) sodium hypochloride for 2 min and germinated on Petri dishes positioned vertically, containing quarter-strength Knop's medium supplemented with 2% (w/v) sucrose and solidified with 1% (w/v) agar (Wojas *et al.*, 2008). To germinate seeds of the T_1 heterozygous generation, 10 μM hygromycin was added to the medium described above to select transgenic plants for the experiments. Seedlings were grown for 3 weeks on agar plates and subsequently transferred to 2.4 l pots (6 plants per pot) containing aerated quarter-strength Knop's solution with modified concentrations of cadmium and zinc, as indicated in the sub-section 'Experiments using a different Zn supplies'. The nutrient solution was changed every four days (unless indicated otherwise). The composition of the quarter-strength Knop's medium is as follows: 0.75 mM $Ca(NO_3)_2$, 0.375 mM KNO_3, 0.312 mM $MgSO_4$, 0.25 mM KH_2PO_4, microelements: 10 μM NaFeEDTA, 6.25 μM H_3BO_3, 0.5 μM $MnCl_2$, 0.5 μM $ZnSO_4$, 0.025 μM $CuSO_4$, 0.125 μM Na_2MoO_4, 1.25 μM KJ, 0.025 μM $CoCl_2$. This medium was used as a basic reference (control) medium in parallel to all Zn and Cd treatments. Since the experiments included testing the plant response to low Zn, the medium without Zn addition was determined by ICP-MS to contain on average 0.4±0.03 μM zinc (arithmetic mean ±SD; n=3). All applied zinc concentrations used in this study are shown as the final (total) concentration present in the medium.

Experiments using a different Zn supply

The response of tobacco seedlings to various zinc concentrations was tested in four types of experiment performed under hydroponic conditions. As a control and reference, quarter-strength Knop's medium containing a total of 0.9 μM $ZnCl_2$ was used in parallel to all treatments. Three-week-old seedlings were transferred from the agar plates (as described in the section, 'Plant

material and general growth conditions') to hydroponic solutions and subjected to the following treatments. (i) Long-term low Zn treatment: pre-culture in control medium for 1 week, then growth in medium containing 0.4 μM $ZnCl_2$ for 5 weeks. (ii) Low Zn followed by re-supply with moderately high Zn: pre-culture on control medium for 2 weeks, followed by 1 week of exposure to 0.4 μM Zn and 1 d exposure to 10 μM Zn. (iii) Moderately high Zn concentration: growth on the control medium supplemented with 10 μM Zn for 3 weeks. (iv) High Zn: pre-culture on control medium for 3.5 weeks followed by growth in a medium containing toxic Zn concentrations of 150 and 500 μM Zn for 8 d (during the exposure to high Zn the medium was changed every second day).

At the end of each experiment, plant health (the presence of necrosis, the colour of the leaf blades, etc.) was assessed visually and plant material was harvested for further analysis (Zn accumulation, gene expression).

Determination of Cd-tolerance and accumulation

Seedlings transferred from the agar plates were pre-cultured on control liquid medium for 3.5 weeks, then exposed to 0.25 and 4 μM $CdCl_2$ for between 1 d and 8 d as indicated. During the exposure period, the medium was changed every second day. At the end of the exposure period, roots and shoots were harvested for further analysis (Cd accumulation, gene expression).

Determination of AhHMA4 *and* NtIRT1 *expression*

Material (roots, and 4th and 5th leaves from the top) was harvested for the analysis of gene expression, frozen in liquid nitrogen and stored at –80 °C. The expression level of *AhHMA4* was evaluated in the roots of six heterozygous transgenic plant lines in the T_1 generation (nos 2, 10, 23, 27, 28, and 35) after cultivation in control liquid medium for 3 weeks. In more detailed studies, expression was then determined in the roots and leaves of two representative homozygous transgenic lines (nos 2 and 35) grown under the following conditions (details for Zn treatment described in the sub-section 'Experiments using a different Zn supplies'; for cadmium treatment in the sub-section 'Determination of Cd-tolerance and accumulation'): (i) under control conditions; (ii) under long-term Zn-deficiency; (iii) under Zn-deficiency/resupply experiment; (iv) in the presence of 10 μM Zn; (v) in the presence of 0.25 and 4 μM Cd (24 h exposure).

The expression level of *NtIRT1* was determined in the roots and leaves of homozygous transgenics (lines 2 and 35) and control plants in the experiments described above marked (i)–(iv).

Approximately 100 mg of frozen tissue of hydroponically grown plants was used for RNA extraction using the RNeasy Plant Mini-Kit (Qiagen), following the manufacturer's recommendations. The RT-PCR was performed as described by Wojas *et al.* (2007). The following primers were used for PCR (30 cycles): (i) for *AhHMA4*-1 (GenBank accession number EU 382073): forward: 5′-ACGGGGACAGTGAAACAAAG-3′; reverse: 5′-TGCATAACTCCTGCAACAGC-3′ (product 588 bp); (ii) for *NtIRT1* (GenBank accession no. AB263746): forward: 5′-GTTGTCGTCCAGTTGCTTGA-3′; reverse: 5′-AGAATG-CAACCACCAAGTCC-3′ (product 438 bp). (iii) for *Actin* (used as a control; *NtTac9*, GenBank accession no. X69885): forward: 5′-CCTCCCACATGCTATTCTCC-3′; reverse: 5′-AGAGCCTC-CAATCCAGACAC-3′ (product 523 bp).

Measurements of Zn and Cd accumulation

Zn and Cd concentrations were assessed: (i) in the whole roots and shoots of plants from all treatments described in the sub-section, 'Determination of *AhHMA4* and *NtIRT1* expression', except for long-term Zn-deficiency plants; (ii) in long-term Zn-deficiency plants in the whole roots as well as in separated parts: stems and three groups of leaves (out of a total number of leaves of 14 to 15), counting from the top; (*) upper five leaves; (**) four to five middle leaves; (***) five lower leaves. Plant parts were collected (as indicted above) at the end of each experiment. Roots were washed in water and then, to remove unbound and weakly bound metal from the apoplast, desorbed in 5 mM $CaCl_2$ at 4 °C for 15 min, and again washed in water. Plant samples were dried at 55 °C in an oven for 4 d, and dry biomass was determined. Dried plant material was mineralized in 65% HNO_3 and 39% H_2O_2 (9:1, v:v) in a closed system microwave mineralizer (Milestone Ethos 900, Milestone, Bergamo, Italy) (Antosiewicz, 2005; Wojas *et al.*, 2009). Cadmium and zinc concentrations were determined by flame atomic absorption spectrophotometry (TJA Solution Solar M, Thermo Electron Manufacturer Ltd., Cambridge, Great Britain). Certified reference material (Virginia tobacco leaves CTA-VTL-2; Commission for Trace Analysis of the Committee for Analytical Chemistry PAS and Institute of Nuclear Chemistry and Technology, Warsaw) was included in each analysis run.

Statistical analysis

All data are expressed as arithmetic means ±SD of replicate plants within an experiment. All data shown are from one experiment representative of a total of two to four independent biological experiments. Statistical significance was evaluated at the 0.05 probability level using Student's *t* tests. Analysis was performed with Excel 2003 for Windows.

Results

Tobacco expressing AhHMA4$_{p1}$::AhHMA4

Six-week old plants of six representative heterozygous plant lines of the T_1 generation exhibiting a 3:1 hyg^R:hyg^S segregation ratio and cultivated under control conditions were tested for the expression of *AhHMA4* in their roots. They all expressed the transgene, although at different levels (Fig. 1). Transgenic plants cultivated on control medium did not differ from the wild-type plants in their appearance and development (data not shown).

AhHMA4 *expression modifies Zn accumulation and decreases Zn-tolerance*

Exposure to control, moderately high and high zinc concentrations: Upon growth at 0.9 μM Zn (control conditions), shoot Zn concentrations were between 23% and 35% lower in transgenic tobacco lines than in wild-type plants, and there was a general trend for slightly lower root Zn concentrations, although this was only statistically significant in two lines (Fig. 2A, B). Upon cultivation at a moderately high, non-toxic Zn concentration of 10 μM,

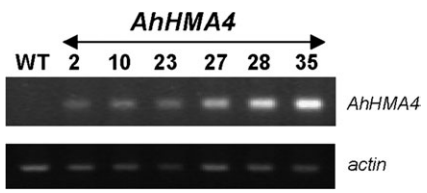

Fig. 1. RT-PCR analysis of transcript levels of *AhHMA4*. Representative results are shown for independent heterozygous transgenic lines of the T_1 generation of plants grown on control medium for 3 weeks. Bottom panels shows the PCR product for actin as a constitutively expressed control gene. WT, wild type.

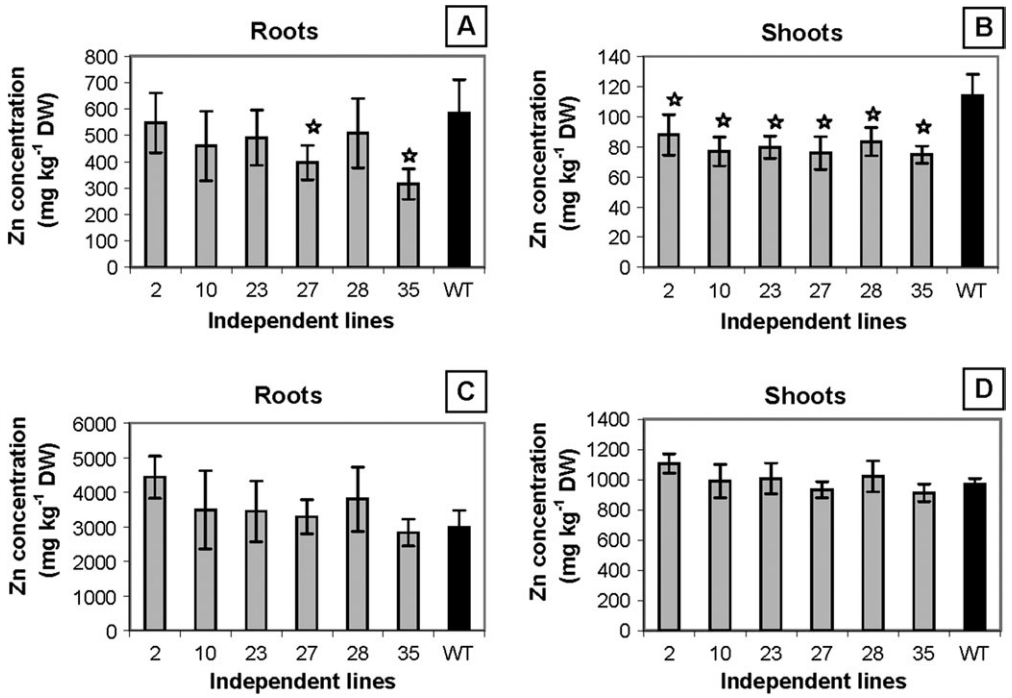

Fig. 2. Zinc accumulation in tobacco plants transformed with *AhHMA4*-transformed and wild-type tobacco lines cultivated in control Zn conditions and in a moderate, non-toxic excess of Zn. Values represent Zn concentrations in the roots (A, C) and the shoots (B, D) of 6-week-old tobacco plants grown hydroponically in quarter-strength Knop's medium containing 0.9 μM $ZnCl_2$ (A, B) or 10 μM $ZnCl_2$ (C, D) for 3 weeks. Lines 10, 23, and 27 are heterozygous (T_1); lines 2, 28, and 35 are homozygous (T_2). Values correspond to arithmetic means ±SD (n=5); values significantly different from WT are highlighted by an asterisk ($P \leq 0.05$) (evaluated by Student's t test).

Zn concentrations were equivalent in transgenic and wild-type plants in both shoots and roots (Fig. 2C, D). Expression of *AhHMA4* in tobacco did thus not result in enhanced shoot Zn accumulation under the conditions used. It is worth noting that Zn concentration in plants from heterozygous lines of the T_1 generation (nos 10, 23, and 27) was at the same level as in homozygous lines of the T_2 generation (nos 2, 28, and 35) (Fig. 2). The response of plants to very high, toxic Zn concentrations was tested only on homozygous lines. In plants exposed to high, toxic concentrations of 150 and 500 μM Zn, again no difference in Zn accumulation was observed between transformed and wild-type plants (Fig. 3). At the end of the period of cultivation at toxic Zn concentrations, transformant lines exhibited a larger number of necrotic spots within the leaf blades than the wild type (Fig. 4A–D). Thus, symptoms of Zn toxicity were more severe in *AhHMA4*-expressing plants.

Long-term low Zn treatment: Wild-type plants and transgenic lines grown for 5 weeks in medium containing 0.4 μM Zn displayed similar symptoms. They all had lower fresh biomass relative to the control plants cultivated in control medium (% of fresh biomass of plants of the same line grown in control conditions: 77.8±11.4% for the wild type; 83.9±15.8% for line no. 2; 84.3±13.2% for line no. 35), without significant differences between transgenics and the wild type (data not shown). In all plants grown on low-zinc medium, stems were shorter but the number of leaves (there were between 14 and 15) remained the same as in the plants grown in control conditions, whereby the three to four lowest leaves were yellow in colour (data not shown). In contrast to the wild-type plants, Zn concentration in the upper leaves of transgenic lines was significantly higher than in the middle and lower leaves (Fig. 5). In addition, Zn concentration in the upper leaves was higher than in the wild type (significantly higher in line no. 35). However, the comparison of the total Zn concentration demonstrates only its tendency to be higher in transgenic plants. In tobacco, expression of *AhHMA4* specifically enhanced Zn accumulation in the youngest, rapidly growing leaves.

AhHMA4 *expression in tobacco decreases Cd accumulation and Cd tolerance*

Upon exposure to a low Cd concentration of 0.25 μM, root Cd concentrations in *AhHMA4*-expressing lines were, on average, only 50% of those in control plants (Fig. 6A). Shoot Cd concentrations were similar in wild type and transgenic lines (Fig. 6B) resulting in an average of 1.8-fold higher shoot:root Cd concentration ratios in the transgenic lines, both when heterozygous (nos 10, 23, and 27) and homozygous (nos 2, 28, and 35), (Fig. 6C). Under this condition, none of the plants exhibited any visual symptoms of cadmium toxicity (data not shown). When plants were exposed to higher Cd concentrations of 4 μM, the differences in Cd partitioning between *AhHMA4*-expressing and

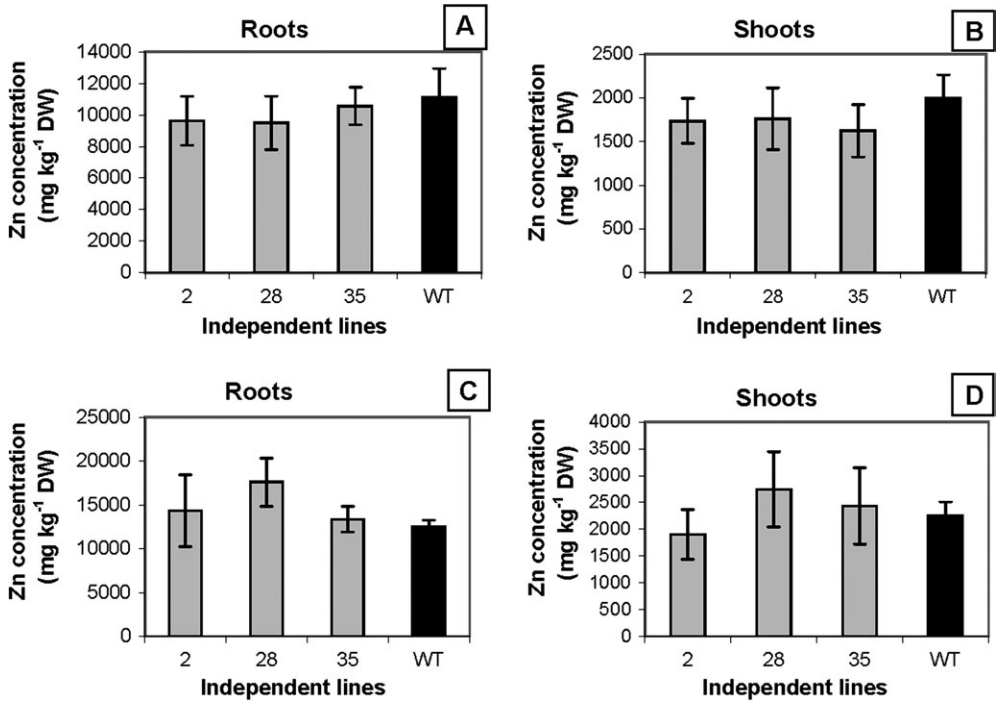

Fig. 3. Zinc accumulation in wild-type and homozygous *AhHMA4*-transformed tobacco lines upon growth in high Zn concentrations. Values are concentrations in roots (A, C) and shoots (B, D) of 6-week-old tobacco plants transformed with *AhHMA4* (homozygous lines, T_2 generation) and of wild-type (WT) tobacco (var. Xanthi) grown hydroponically in quarter-strength Knop's medium for 3.5 weeks and subsequently exposed to 150 μM $ZnCl_2$ (A, B) or 500 μM $ZnCl_2$ (C, D) for 8 d. Values correspond to arithmetic means ±SD (n=5); no statistically significant differences were detected between WT and transgenic lines (evaluated by Student's t test).

wild-type plants were quantitatively much less pronounced although qualitatively similar as observed upon exposure to 0.25 μM Cd (compare Fig. 6D–F with A–C).

Expression of *AhHMA4* in tobacco did not increase Cd tolerance. The number of necrotic spots on the leaves of transgenic plants exposed to 4 μM Cd was similar or slightly higher when compared with the wild type (Fig. 4E, F).

The AhHMA4$_{p1}$*::*AhHMA4 *construct is expressed under different Zn and Cd conditions*

Heterologous introduction of *AhHMA4* under its endogenous promoter into the tobacco genome raises some fundamental questions: (i) is it expressed? (ii) is it regulated in response to metal supply? To test this, the expression level of *AhHMA4* was assessed in two plant lines (nos 2 and 35) grown hydroponically in the presence of a range of zinc and cadmium concentrations.

As shown in Fig. 7, *AhHMA4* was expressed at similar levels after 4–5 weeks of growth at 0.4, 0.9, and 10 μM Zn in both the leaves and roots of lines 2 and 35 (Fig. 7A, B). However, 24 h of resupply of 10 μM Zn^{2+} to plants grown in a low Zn concentration of 0.4 μM Zn for 1 week (Fig. 8) resulted in the down-regulation of *AhHMA4* transcript levels in roots when compared with plants grown in low Zn (0.4 μM Zn) for 1 week. By contrast, in leaves, the expression level remained unchanged.

Different Cd concentrations in the medium had no effect on transcript levels in either roots or leaves (Fig. 9).

AhHMA4 *expression contributes to alterations of* IRT1 *expression under Zn exposure*

Expression of *AhHMA4* in tobacco led to contrasting changes in Zn accumulation, which were dependent on the Zn concentrations in the medium. However, in tobacco, *AhHMA4* was expressed in both roots and shoots under all conditions tested, and transcript levels were altered only in comparisons between low Zn plants and those resupplied with 10 μM Zn (Figs 7, 8, 9). Therefore, it was tested whether *AhHMA4* transgene expression affects the expression of an endogenous tobacco gene known to contribute to Zn accumulation. *NtIRT1* was chosen as a candidate gene because it acts as a multi-substrate cation transporter that contributes to root uptake of Fe^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, and Co^{2+} ions and is similar with the homologues from *A. thaliana* and *O. sativa* (Korshunova *et al.*, 1999; Connolly *et al.*, 2002; Hodoshima *et al.*, 2008; Lee and An, 2009).

As expected, *NtIRT1* was not expressed in leaves (data not shown). However, the transcript was detected in all root samples (Fig. 7B). In wild-type plants, *NtIRT1* transcript levels remained at similar levels under all Zn treatments. By contrast, in *AhHMA4*-expressing transgenics, compared with control conditions, *NtIRT1* mRNA levels were increased under low Zn and, surprisingly, also upon growth at 10 μM Zn. Under control conditions, *NtIRT1* transcript levels were similar in both wild-type and transgenic plants. Compared with plants exposed to low 0.4 μM Zn, a resupply of 10 μM Zn for 1 d to plants grown in low-Zn conditions

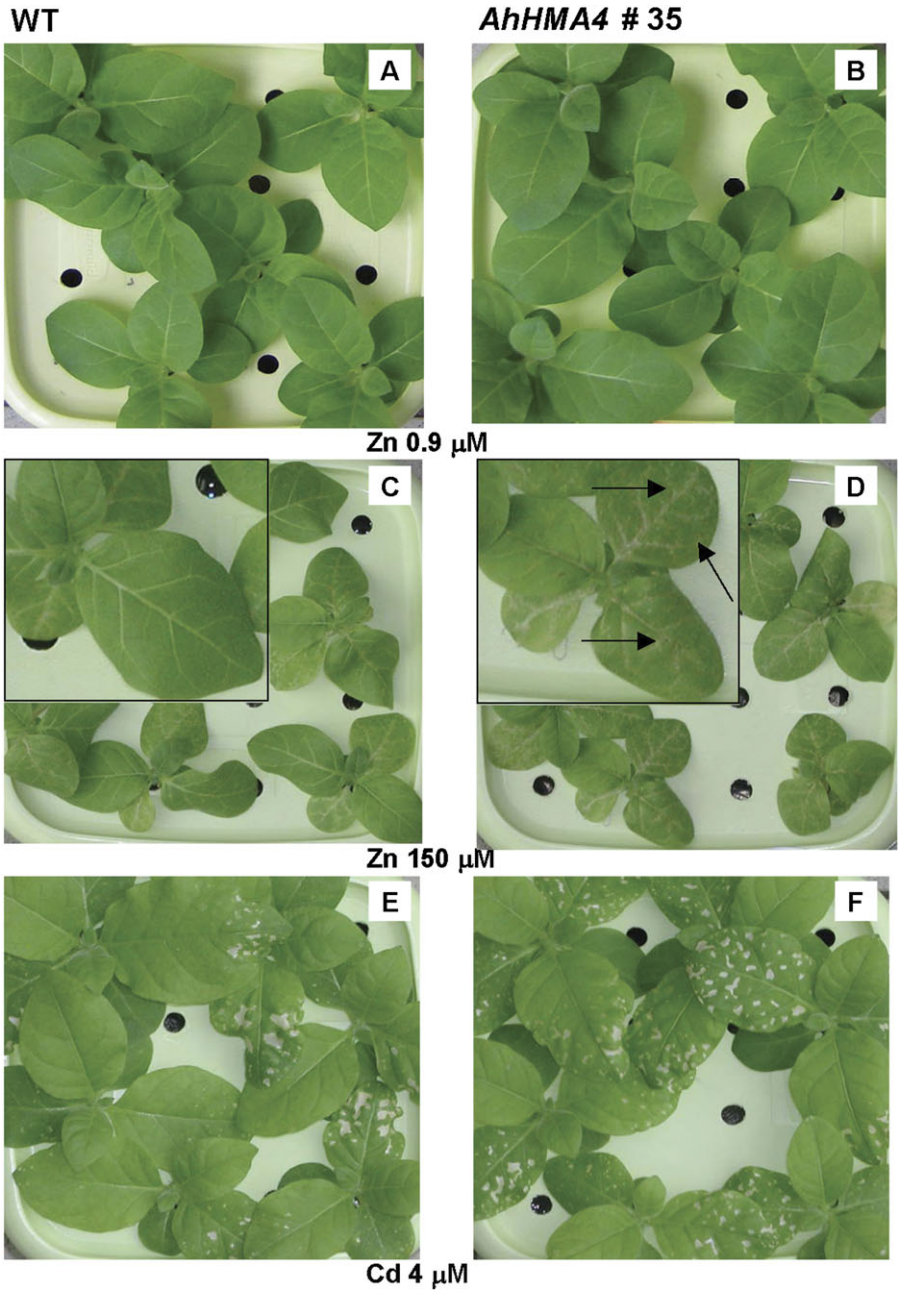

Fig. 4. Photographs of tobacco plants transformed with *AhHMA4* (homozygous T_2 line) and of wild-type (WT) tobacco (var. Xanthi) grown hydroponically under control conditions and in the presence of high Zn (C, D) and high Cd (E, F) concentrations in the medium. Plants were grown in quarter-strength Knop's medium containing 0.9 μM $ZnCl_2$ for 4.5 weeks (A, B); grown in quarter-strength Knop's medium for 3.5 weeks and subsequently exposed to 150 μM $ZnCl_2$ for 8 d (C, D); grown in quarter-strength Knop's medium for 3.5 weeks and subsequently exposed to 4 μM $CdCl_2$ for 6 d (E, F). Arrows indicate necrotic areas on the leaves. Scale: (A–F) 1 cm=1.45 cm; for the enlargements in (C) and (D), 1 cm=2.43 cm.

resulted in a reduction in *NtIRT1* expression in roots of both transgenic and wild-type plants (Fig. 8).

Discussion

In studies aimed at the genetic modification of shoot:root metal partitioning for biotechnological purposes (e.g bio-fortification or phytoremediation), a variety of genes encoding membrane transport proteins or proteins involved in the biosynthesis of heavy metal-cation complexing compounds were introduced into host plants, most often under the control of the strong constitutive CaMV 35S promoter (Pilon-Smits and Pilon, 2002). Different from these approaches, in this study *AhHMA4* was expressed under the control of its native promoter, which governs cell-type-specific expression in *Arabidopsis* species and is at least as strong as the 35S promoter (Hanikenne *et al.*, 2008) in the

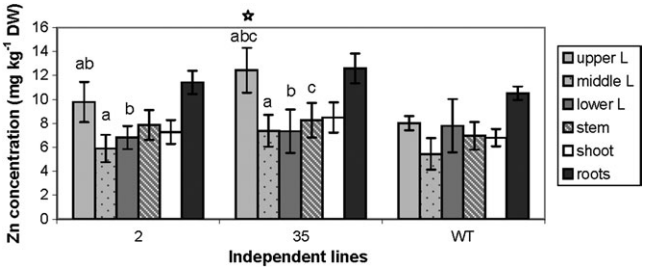

Fig. 5. Zinc accumulation in wild-type and *AhHMA4*-transformed tobacco lines upon long-term growth under low-Zn conditions. Shown are zinc concentrations in the upper leaves (five leaves counting from the top), the middle leaves (four to five leaves below), the lower leaves (five leaves further below), the stems, the shoots (in all leaves and the stem), and the roots of 8-week-old tobacco plants transformed with *AhHMA4* (homozygous lines, T_2 generation) or wild-type (WT) tobacco (var. Xanthi) grown hydroponically in quarter-strength Knop's medium for 1 week and subsequently transferred to low Zn medium (0.4 μM) for 5 weeks. Values correspond to arithmetic means ±SD (n=5); within each plant line statistically significant difference in Zn concentrations between upper, middle, and lower leaves are marked by the same letters; within the same categories values significantly different from the respective WT are highlighted by asterisks ($P \leqslant 0.05$), (evaluated by Student's t test).

heterologous tobacco. Indeed, it was demonstrated here that the *AhHMA4*-promoter is able to direct *AhHMA4* mRNA production in a rather distantly related plant species, tobacco (Fig. 1). In tobacco, however, there was Zn-dependent regulation of *AhHMA4* transcript levels in roots, with a suppression of transcript levels upon Zn resupply compared with plants that remained in Zn-deficient growth conditions (Fig. 8). By contrast, in *A. halleri*, there was little or no metal-supply dependence of *AhHMA4* expression levels (Talke *et al.*, 2006; Hanikenne *et al.*, 2008).

Expression of AhHMA4p1::AhHMA4 *in tobacco generates differing patterns of root/shoot partitioning of micronutrient zinc and non-essential cadmium dependent on metal supply*

Expression of *AhHMA4$_{p1}$::AhHMA4* in tobacco did not contribute to one uniform pattern of Zn and Cd root/shoot partitioning across a range of metal supplies. Zn accumulation in transgenic plants depended on the Zn concentration in the hydroponic medium and, consequently, on plant Zn status (Figs 2, 3). Compared with the wild type, enhanced accumulation of Zn in the youngest leaves was observed upon prolonged cultivation of transgenic plants in 0.4 μM Zn (Fig. 5). In entire shoots, transgenic plants contained lower Zn concentrations than the wild type when grown under control conditions (Fig. 2B) or similar Zn concentrations when grown under low- or high-Zn conditions (Figs 3, 5). In wild-type *A. thaliana* and *A. halleri*, net partitioning of Zn to the shoots is also dependent on the external Zn supply (Talke *et al.*, 2006). However, higher *HMA4* expression in *A. halleri* than in *A thaliana*, was associated with substantially higher shoot:root Zn concentration ratios in *A. halleri* as well as shoot Zn concentrations across the entire range of Zn concentrations in the medium (0.01–100 μM Zn). By contrast, introduction of the *AhHMA4p$_1$::AhHMA4* construct into the tobacco genome overall did not result in significantly higher Zn accumulation in shoot tissues when compared with the wild type. There are several possible scenarios to explain this. It is likely, that the altered metal status in tobacco cells expressing *AhHMA4*, triggers homeostatic mechanisms that counteract the effects of *AhHMA4* expression. Another possibility is that, in tobacco, the *AhHMA4* promoter might respond to environmental stimuli not explicitly tested here in a different manner when compared to *Arabidopsis* species, or might be less active (Talke *et al.*, 2006; Hanikenne *et al.*, 2008). This study provided the first circumstantial evidence for the former possibility (Fig. 7), but did not test the latter one. However, the Zn supply-dependent modifications detected in shoot:root Zn partitioning in transgenic tobacco plants did not correlate with the regulation of *AhHMA4* transcript levels in tobacco (Figs 7, 8), but might be related to the Zn-dependent regulation of other metal homeostasis genes in the transgenic plants, which was not present, or much less pronounced, in the wild type (see Fig. 7).

Moreover, AhHMA4 may be active in tobacco cell types different from those in *Arabidopsis*. *AhHMA4$_{p1-3}$*-GUS (β-glucuronidase) reporter analysis in transformed *A. thaliana* as well as *in situ* hybridization demonstrated a spatial and tissue-specific expression pattern highly similar to that detected in the closely related *A. halleri* (Hanikenne *et al.*, 2008). However, the localization of *AhHMA4* expression in the transgenic tobacco lines is not known. Alternatively or additionally, the AhHMA4 protein might exhibit reduced activity in tobacco because of limited protein production, targeting or activation. This remains to be tested.

Among other factors, the dose-dependent alteration of the zinc accumulation pattern in transgenic plants could also result from modification of the endogenous metal homeostasis network due to *AhHMA4* expression. To date, little is known about the root Zn uptake systems of tobacco. *IRT1* from *Nicotiana tabacum* was cloned and partly characterized (Eide *et al.*, 1996). It is known that IRT1 proteins from various plant species are not specific uptake systems for the primary substrate iron (as a part of the strategy I Fe acquisition), but can transport and act in the accumulation of other divalent cations such as Zn^{2+}, Cd^{2+} or Mn^{2+} (Eide *et al.*, 1996; Korshunova *et al.*, 1999; Vert *et al.*, 2002). Here, *NtIRT1* transcript levels were found to be up-regulated specifically in transgenic tobacco plants upon exposure to Zn deficiency or moderately high Zn concentrations when compared with control conditions or *NtIRT1* transcript levels in the wild type (Fig. 7). Consequently, the regulation of *NtIRT1* transcript levels in the transgenic tobacco can only partially explain the observed distinct effects of *AhHMA4* expression on Zn accumulation under different Zn regimes. Different metal uptake systems

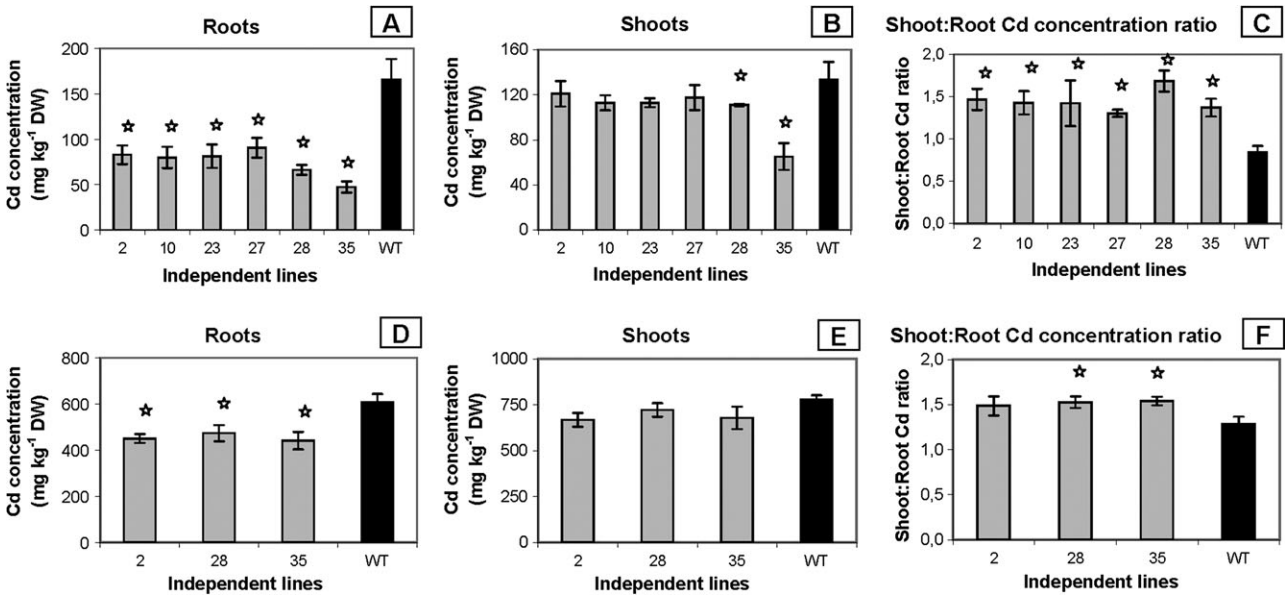

Fig. 6. Cadmium accumulation in wild-type and *AhHMA4*-transformed tobacco lines upon growth in moderate and high Cd concentrations. Shown are cadmium concentrations in the roots, the shoots, and the shoot:root ratios of cadmium concentrations in 6-week-old tobacco plants transformed with *AhHMA4* (homozygous T_2 lines) and wild-type (WT) tobacco (var. Xanthi) grown hydroponically in quarter-strength Knop's medium for 3.5 weeks and subsequently exposed to 0.25 μM $CdCl_2$ for 4 d (A–C) or 4 μM $CdCl_2$ for 6 d (D–F). Lines 10, 23, and 27 are heterozygous (T_1); lines 2, 28, and 35 are homozygous (T_2). Values correspond to arithmetic means ±SD (n=5); those significantly different from WT are indicated by an asterisk ($P \leq 0.05$) (evaluated by Student's t test).

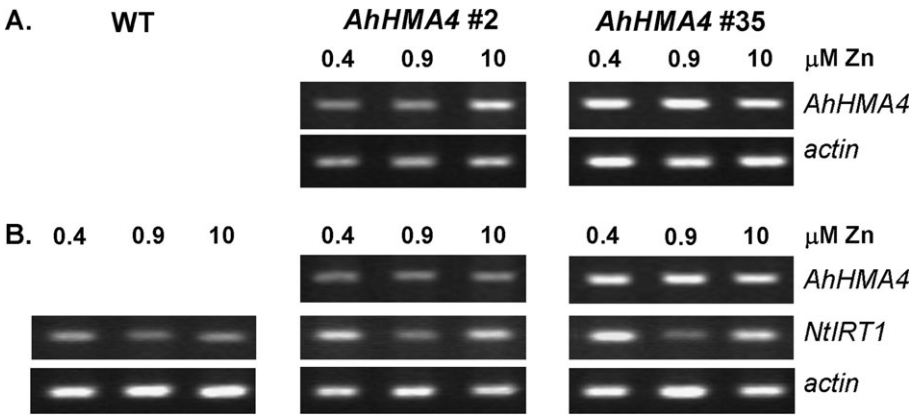

Fig. 7. RT-PCR analysis of *AhHMA4* and *NtIRT1* transcript levels wild-type and *AhHMA4*-transformed tobacco lines grown under different Zn regimes. Shown are gel images of ethidium-bromide stained PCR products of *AhHMA4* and *NtIRT1* for leaves (A) and roots (B) of *AhHMA4*-transformed plants (homozygous lines no. 2 and 35, T_2) and the wild-type (WT) plants grown hydroponically in quarter-strength Knop's medium supplemented with 0.4, 0.9, and 10 μM $ZnCl_2$. Bottom panels show the PCR product for actin as a constitutively expressed control gene.

might contribute or be prevalent for Zn uptake in tobacco plants. In the future, it will also be important to examine the regulation of expression of Zn-deficiency responsive tobacco genes, for example, homologues of *AtIRT3* and *AtZIP4*, so far not cloned in tobacco. As shown by Hanikenne *et al.* (2008), the significant up-regulation of both these genes in the roots of *A. halleri*, which is a consequence of the high level of *AhHMA4* expression in this species, may contribute to Zn hyperaccumulation. An increase in *IRT3* and *ZIP4* transcript levels also accompanied the recapitulation of Zn distribution to the xylem characteristic of *A. halleri* in transgenic *A. thaliana* expressing *AhHMA4*.

There was only a very limited similarity between the modifications of micronutrient Zn and non-essential toxic Cd accumulation in transformed tobacco. Cd accumulation in roots was strongly reduced in transgenic tobacco lines at sub-toxic external Cd concentrations and detectably reduced at toxic external Cd concentrations, whereas there was little change in the shoots compared with the wild type (Fig. 6). Thus, a larger proportion of total cadmium in the plant was allocated to the shoots, leading to an increased

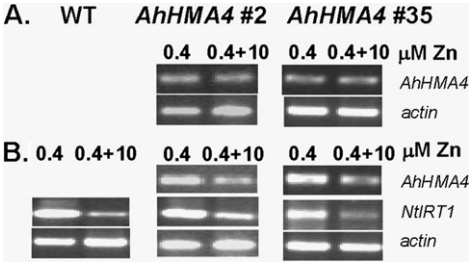

Fig. 8. RT-PCR analysis of *AhHMA4* and *NtIRT1* transcript levels in Zn-deficient wild-type and *AhHMA4*-transformed tobacco lines without and with 1 d of Zn re-supply. Shown are gel images of ethidium-bromide stained PCR products of *AhHMA4* and *NtIRT1* for leaves (A) and roots (B) of *AhHMA4*-transformed plants (homozygous lines no. 2 and 35, T_2 generation) and wild-type (WT) plants grown hydroponically in quarter-strength Knop's medium containing low 0.4 µM Zn for 1 week and subsequently in medium containing 10 µM $ZnCl_2$ for 1 d. Bottom panels show the PCR product for actin as a constitutively expressed control gene.

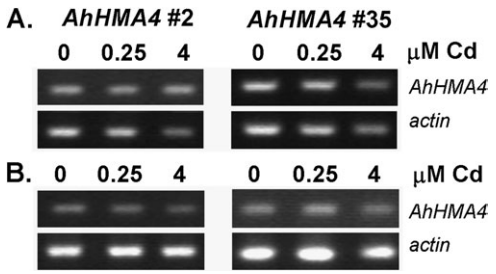

Fig. 9. RT-PCR analysis of *AhHMA4* transcript levels in *AhHMA4*-transformants cultivated in the presence of different Cd concentrations in the medium. Shown are gel images of ethidium-bromide stained PCR products for leaves (A) and roots (B) of *AhHMA4*-transformed plants (homozygous lines no. 2 and 35, T_2 generation) grown hydroponically in quarter-strength Knop's medium supplemented with 0, 0.25 and 4 µM $CdCl_2$ for 3.5 weeks. Bottom panels show the PCR product for actin as a constitutively expressed control gene.

shoot:root ratio of Cd concentrations (Fig. 6C, F), as expected, based on the known functions of *AhHMA4* and *AtHMA4* (Hussain *et al.*, 2004; Hanikenne *et al.*, 2008). This suggests that, among other processes, such as possibly Cd export from the root symplast into the apoplast outside the xylem, *AhHMA4* expression in tobacco did contribute to root-to-shoot Cd transport. At toxic external Cd concentrations, the contribution of non-specific Cd transport though a variety of pathways might increase, thus decreasing the contributions of specific transport systems such as *AhHMA4* (Fig. 6D–F).

In general, the commonality among the alterations in Cd and Zn accumulation by transgenic tobacco plants expressing *AhHMA4* examined in this paper lies in their dose dependence. The importance of the cadmium dose in its root-to-shoot partitioning in transgenic plants was also underlined by Korenkov *et al.* (2007) demonstrating the difference between At*CAX2* and At*CAX4* expressing tobacco cultivated in media containing 0.02 µM Cd (more Cd was retained in the roots) and 3 µM Cd (higher Cd in both roots and shoots). It appears that the introduction of a transgene into a plant does not produce a uniform trait regardless of the growth conditions, but instead, the effect of transgenes is highly dependent on the metal level in the medium. Interestingly, the most pronounced differences in Zn and Cd accumulation between transgenic and wild-type plants (although qualitatively different) due to the *AhHMA4* expression, were detected at low exposure to both metals. In general, at high external concentrations, accumulation patterns in transgenics became similar to those of the wild type. The mechanisms underlying such phenomena may be similar to those behind the suppression of the *A. thaliana* double mutant *hma2/hma4* nutritional deficiency phenotype (Hussain *et al.*, 2004), which was solely compensated by increasing the level of Zn (up to 10 µM in the agar growth medium). The phenotypic complementation of the double mutant by the addition of high external Zn is possibly due to the involvement of a variety of low-affinity transporters, or even a process broadly referred to as 'exclusion breakdown' upon a supply of a metal excess. Finally, in agreement with the results obtained in tobacco expressing *AhHMA4* in this study, Hanikenne *et al.* (2008) suggested, based on their characterization of transgenic *A. thaliana* expressing *AhHMA4*, that this gene is necessary, but not sufficient to generate the metal hyperaccumulation phenotype.

AhHMA4p1::AhHMA4 *expression in tobacco enhances sensitivity to both zinc and cadmium*

Even if *AhHMA4* is expressed in transgenic tobacco plants in the same cell types as in *Arabidopsis* and the encoded protein exhibits full activity, *AhHMA4* expression is likely to be necessary but not sufficient for hyperaccumulation and full Zn and Cd tolerance, similar to the earlier conclusions concerning *AhHMA4* expression in *A. thaliana* (Hanikenne *et al.*, 2008). As shown in this study, the expression of *AhHMA4* in tobacco resulted in enhanced Zn sensitivity (Fig. 4) although at harvest, the final Zn concentration was approximately at the wild-type level (Fig. 3). To compare, heterologous expression of *AhHMA4* in *A. thaliana* under its native promoter also resulted in higher zinc sensitivity, but when expressed under the control of the 35S promoter, an increase in Zn and Cd tolerance was noted in transgenic *A. thaliana* lines (Hanikenne *et al.*, 2008). These two types of transformants also differed in their Zn accumulation patterns. Higher Zn sensitivity of $AhHMA4_{p1}$::*AhHMA4*-transformed *A. thaliana* was accompanied by slightly elevated shoot Zn concentration (1–16-fold), whereas higher Zn tolerance of p35S::*AhHMA4* plants was associated with unaltered or reduced shoot Zn levels (Hanikenne *et al.*, 2008). Only partially in agreement with these data are the results obtained by Verret *et al.* (2004). This study, which was based on the characterization of only a single transgenic line, reported enhanced Zn tolerance in 35S-*AtHMA4* overexpressor lines, but

also enhanced shoot Zn concentrations. The fact that increased Zn sensitivity was observed in both transgenic $AhHMA4_{p1}$::*AhHMA4 A. thaliana* (Hanikenne *et al.*, 2008) and tobacco plants, whereas the use of a 35S promoter enhanced Zn tolerance (Verret *et al.*, 2004; Hanikenne *et al.*, 2008) suggests a crucial role for the cell-type-specific expression and the transcriptional regulation of the introduced transgene.

As shown by Hanikenne *et al.* (2008), not only the full extent of Zn hypertolerance but also, and to a somewhat larger extent, Cd hypertolerance of *A. halleri* depends on *AhHMA4*. The *A. halleri HMA4* RNAi lines exhibited a dramatically enhanced sensitivity to cadmium relative to wild-type *A. halleri* plants. However, the transfer of $AhHMA4_{p1}$::*AhHMA4* to *A. thaliana* slightly lowered the tolerance to this heavy metal. The *AhHMA4*-dependent increase in Cd sensitivity in the leaves of transgenic tobacco lines suggests that *AhHMA4* expression in tobacco results in Cd accumulation at sensitive sites lacking sufficient Cd detoxification capacity, very similar to the earlier observations in *AhHMA4*-expressing *A. thaliana* (Hanikenne *et al.*, 2008). Alternatively, cellular Zn export by *Ah*HMA4, known to be localized at plasma membrane (Courbot *et al.*, 2007), may cause a local signal of nutrient deficiency in the respective cells in transgenic tobacco and *A. thaliana*, which in turn might trigger the release of divalent transition metal nutrient cations from their stores, in particular vacuoles. This could ultimately lead to an increase in cytosolic cadmium concentrations. For example, the increase in cytosolic cadmium was the cause of higher Cd sensitivity of *NRAMP3* overexpressing *A. thaliana* plants due to the non-specific release of Cd from the vacuoles through this transporter (Thomine *et al.*, 2003).

Biotechnology perspective

In summary, transgenic tobacco expressing *AhHMA4* under the control of the native *A. halleri* promoter exhibited a complex pattern of alterations in Zn and Cd accumulation when compared with the wild type. The intended increase in leaf metal concentrations of tobacco plants expressing *AhHMA4*, when compared to the wild type, was only observed when a pool of the youngest (top five) leaves were analysed from transgenic plants grown under low-Zn conditions for 5 weeks (Fig. 5), but not in entire shoots of tobacco plants grown under various Zn conditions (deficiency, control, and excess; Figs 2, 3, 5). For biotechnology purposes, an improved understanding of the molecular mechanisms resulting in such complex dose-dependent effects of transgenes on root/shoot Zn and Cd partitioning in transformed plants is vital, but currently very difficult to achieve in plants of which the metal homeostasis network is less well understood than that of the model plant *Arabidopsis thaliana*. For a future understanding of this phenomenon, it is therefore important to take into account that plant transcript profiles are likely to be specific and different in plants of low, medium, and high Zn status (Puig and Peñarrubia, 2009), as well as in plants exposed to low and high levels of a stress factor, for example, toxic cadmium (Kacperska, 2004). Thus, the phenotype of a transgenic plant exposed to a range of Zn and Cd concentrations probably results from the interplay between the transgene activity and the very different molecular backgrounds of the host plant at varying metal levels in the medium.

Although, in tobacco, the expression of *HMA4* from the Zn/Cd hyperaccumulator *A. halleri* under its native promoter did not cause a dramatic increase in the translocation of the micronutrient zinc from the roots to the shoots, the results obtained are interesting from a biofortification perspective. The transformed plants were more efficient in the translocation of the micronutrient zinc to rapidly growing young leaves of plants grown under zinc deficiency (0.4 μM). In addition, *AhHMA4* expression drastically reduced shoot concentrations of the non-essential, toxic cadmium, and the decrease of contaminants in crop plants is an important secondary goal in biofortification that has been hard to address (Krämer, 2009).

Acknowledgements

This work was supported by the EU through its Sixth Framework Programme for RTD (contract no FOOD-CT-2006-016253). It reflects only the author's views. The Community is not liable for any use that may be made of the information contained therein.

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