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

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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 P₁B-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, P₁BATPase, 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 PI-B-ATPase, both from Arabidopsis 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, AhHMA4 and the homologous AtHMA2 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 hyperaccumulating 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, Warsaw, Poland) was applied as described in Antosiewicz and Henning (2004). Transgenic plants were selected on medium supplemented with 25 μM hygromycin. T0 seeds were collected from the T0 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 T1 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 T1 lines with a 3:1 hyg−:hyg+ 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 T2 plants (nos 2, 28, and 35; details in the Results, section ‘Tobacco expressing 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 T1 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 supply’. 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(NO3)2, 0.375 mM KNO3, 0.312 mM MgSO4, 0.25 mM KH2PO4, microelements: 10 μM NaFeEDTA, 6.25 μM H3BO3, 0.5 μM MnCl2, 0.5 μM ZnSO4, 0.025 μM CuSO4, 0.125 μM Na2MoO4, 1.25 μM KJ, 0.025 μM CoCl2. 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 used (details in the Results). To test the response of tobacco seedlings to various zinc concentrations, seedlings were transferred from the agar plates (as described in the section, ‘Plant growth conditions’).
metal 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 ZnCl₂ 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

Seeds were transferred from the agar plates were pre-cultured on control liquid medium for 3.5 weeks, then exposed to 0.25 and 4 μM CdCl₂ 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 transgenic lines in the T₁ 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 AhHMA4p1 (GenBank accession number EU 382073): forward: 5′-ACGGGGACAGTGAAACAAAG-3′; reverse: 5′-TGCATAACTCCTGCAACAGC-3′ (product 523 bp); (ii) for NtIRT1 (GenBank accession no. AB263746): forward: 5′-TGGTGTGTCGATCGTTGCTGGA-3′; reverse: 5′-AGAAATGCAACCAACAACTGC-3′ (product 588 bp); (iii) for Actin (used as a control; NtAct9, GenBank accession no. X69985): forward: 5′-CTCACATGCTATTTCCCTC-3′; reverse: 5′-AGAGGCTCATTACGACAC-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 indicated 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 CaCl₂ 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% HNO₃, and 39% H₂O₂ (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 Manufacture 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 T₁ generation exhibiting a 3:1 hygR:hygS segregation ratio and cultured 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

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 T₁ 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.
Zn concentrations were equivalent in transgenic and wild-type plants in both shoots and roots (Fig. 2C, D). Expression of \textit{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 T1 generation (nos 10, 23, and 27) was at the same level as in homozygous lines of the T2 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 \( \mu M \) 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 \textit{AhHMA4}-expressing plants.

**Long-term low Zn treatment:** Wild-type plants and transgenic lines grown for 5 weeks in medium containing 0.4 \( \mu M \) 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 \textit{AhHMA4} specifically enhanced Zn accumulation in the youngest, rapidly growing leaves.

\textit{AhHMA4} expression in tobacco decreases Cd accumulation and Cd tolerance

Upon exposure to a low Cd concentration of 0.25 \( \mu M \), root Cd concentrations in \textit{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 \( \mu M \), the differences in Cd partitioning between \textit{AhHMA4}-expressing and
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 AhHMA4p1::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 Zn2+ 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 Fe2+, Zn2+, Cd2+, Mn2+, and Co2+ 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. 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, T2 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 ZnCl2 (A, B) or 500 μM ZnCl2 (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).
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. biofortification 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-Smith 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
heterologous tobacco. Indeed, it was demonstrated here that the \textit{AhHMA4}-promoter is able to direct \textit{AhHMA4} mRNA production in a rather distantly related plant species, tobacco (Fig. 1). In tobacco, however, there was Zn-dependent regulation of \textit{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 \textit{A. halleri}, there was little or no metal-supply dependence of \textit{AhHMA4} expression levels (Talke et al., 2006; Hanikenne et al., 2008).

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

Expression of \textit{AhHMA4p1::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 in similar Zn concentrations when grown under low- or high-Zn concentrations (Figs 3, 5). In wild-type \textit{A. thaliana} and \textit{A. halleri}, net partitioning of Zn to the shoots is also dependent on the external Zn supply (Talke et al., 2006). However, higher \textit{HMA4} expression in \textit{A. halleri} than in \textit{A. thaliana}, was associated with substantially higher shoot:root Zn concentration ratios in \textit{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 \textit{AhHMA4p1::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 \textit{AhHMA4}, triggers homeostatic mechanisms that counteract the effects of \textit{AhHMA4} expression. Another possibility is that, in tobacco, the \textit{AhHMA4} promoter might respond to environmental stimuli not explicitly tested here in a different manner when compared to \textit{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 \textit{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, \textit{AhHMA4} may be active in tobacco cell types different from those in \textit{Arabidopsis}. \textit{AhHMA4p1-3-GUS} (β-glucuronidase) reporter analysis in transformed \textit{A. thaliana} as well as \textit{in situ} hybridization demonstrated a spatial and tissue-specific expression pattern highly similar to that detected in the closely related \textit{A. halleri} (Hanikenne et al., 2008). However, the localization of \textit{AhHMA4} expression in the transgenic tobacco lines is not known. Alternatively or additionally, the \textit{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 \textit{AhHMA4} expression. To date, little is known about the root Zn uptake systems of tobacco. \textit{IRT1} from \textit{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, \textit{NiIRT1} 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 \textit{NiIRT1} transcript levels in the wild type (Fig. 7). Consequently, the regulation of \textit{NiIRT1} transcript levels in the transgenic tobacco can only partially explain the observed distinct effects of \textit{AhHMA4} expression on Zn accumulation under different Zn regimes. Different metal uptake systems
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 \textit{AtIRT3} and \textit{AtZIP4}, so far not cloned in tobacco. As shown by Hanikenne \textit{et al.} (2008), the significant up-regulation of both these genes in the roots of \textit{A. halleri}, which is a consequence of the high level of \textit{AhHMA4} expression in this species, may contribute to Zn hyperaccumulation. An increase in \textit{IRT3} and \textit{ZIP4} transcript levels also accompanied the recapitulation of Zn distribution to the xylem characteristic of \textit{A. halleri} in transgenic \textit{A. thaliana} expressing \textit{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

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**Fig. 6.** Cadmium accumulation in wild-type and \textit{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 \textit{AhHMA4} (homozygous T2 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 (T1); lines 2, 28, and 35 are homozygous (T2). Values correspond to arithmetic means ±SD (n=5); those significantly different from WT are indicated by an asterisk (P <0.05) (evaluated by Student’s \textit{t} test).

**Fig. 7.** RT-PCR analysis of \textit{AhHMA4} and \textit{NtIRT1} transcript levels wild-type and \textit{AhHMA4}-transformed tobacco lines grown under different Zn regimes. Shown are gel images of ethidium-bromide stained PCR products of \textit{AhHMA4} and \textit{NtIRT1} for leaves (A) and roots (B) of \textit{AhHMA4}-transformed plants (homozygous lines no. 2 and 35, T2) 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.
bacco 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 hma2hma4 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 AhHMA4p1::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-AhHMA4 overexressor lines, but...
also enhanced shoot Zn concentrations. The fact that increased Zn sensitivity was observed in both transgenic AhHMA4p1::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, 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 AhHMA4p1::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 AhHMA4, 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).

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