Genes controlling the development and function of plant vacuoles

Li, Y.

Publication date
2017

Document Version
Other version

License
Other

Citation for published version (APA):
Li, Y. (2017). Genes controlling the development and function of plant vacuoles. [Thesis, fully internal, Universiteit van Amsterdam].

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Two *Silene vulgaris* copper transporters residing in different cellular compartments confer copper hypertolerance by distinct mechanisms when expressed in *Arabidopsis thaliana*.

Yanbang Li\(^1\), Mazhar Iqbal\(^4\), Qianqian Zhang\(^3\), Cornelis Spelt\(^1\), Mattijs Bliek\(^1\), Henk WJ Hakvoort\(^2\), Francesca M Quattrocchio\(^1\), Ronald Koes\(^1\) and Henk Schat\(^1\)

\(^1\)Swammerdam Institute for Life Sciences, Dept. of Plant Development and (Epi)Genetics, University of Amsterdam, 1098XH Amsterdam, The Netherlands.

\(^2\)Department of Molecular and Cell Biology, Faculty of Earth and Life Sciences, VU-University, De Boelelaan 1108, 1081 HZ Amsterdam, The Netherlands.

\(^3\)Swammerdam Institute for Life Sciences, Dept. of Plant Cell Biology, University of Amsterdam, 1098XH Amsterdam, The Netherlands.

\(^4\)Current address: Department of Environmental Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

*Published in *New Phytologist* 215, 1102–1114 (2017).*
Summary

- *Silene vulgaris* is a metallophyte of calamine, cupriferous, and serpentine soils all over Europe. Its metallicolous populations are hyper-tolerant to zinc (Zn), cadmium (Cd), copper (Cu), or nickel (Ni), compared to con-specific non-metallicolous populations. These hypertolerances are metal-specific, but the underlying mechanisms are poorly understood.

- We investigated the role of HMA5 copper transporters in Cu hypertolerance of a *S. vulgaris* copper mine population.

- Cu-hypertolerance in *Silene* is correlated and genetically linked with enhanced expression of two HMA5 paralogs, *SvHMA5I* and *SvHMA5II*, each of which increases Cu-tolerance when expressed in Arabidopsis. Most Spermatophytes, except Brassicaceae, possess homologs of *SvHMA5I* and *SvHMA5II*, which originate from an ancient duplication predating the appearance of spermatophytes. *SvHMA5II* and the *Arabidopsis* homolog *AtHMA5* localize in the endoplasmic reticulum and upon Cu exposure move to the plasma membrane, from where they are internalized and degraded in the vacuole. This resembles trafficking of mammalian homologs and is apparently an extremely ancient mechanism. *SvHMA5I*, instead, neo-functionalized and always resides on the tonoplast, likely sequestering Cu in the vacuole.

- Adaption of *Silene* to a Cu polluted soil is at least in part due to up-regulation of two distinct HMA5 transporters, which contribute to Cu-hypertolerance by distinct mechanisms.
Introduction

Naturally or anthropogenically metal-enriched (“metalliferous”) soils occur all over the world. Their vegetations are typically sparse, often without trees, and poor in species. The component species, called “metalophytes”, are either confined to metalliferous soil types (“strict metallophytes”), or occur both on metalliferous and non-metalliferous soils (“facultative metallophytes”).

*Silene vulgaris* (Moench) Garcke (*Caryophyllaceae*) is a prominent facultative metallophyte in western and central Europe. It is particularly common on zinc, lead, or copper mine tailings, or serpentine outcrops (Schat et al., 1996). Its metallicolous populations are extremely metal-tolerant (“hypertolerant”), in comparison with the non-metallicolous populations, though exclusively to the metal(s) that are present at toxic concentrations in their natural environments (Schat et al., 1996; Schat & Vooijs, 1997). Hypertolerances to different metals segregate independently in progenies of crosses between non-metallicolous and multiple hypertolerant plants, and seem to have a simple genetic basis, e.g., one, two, and three or four loci for Cd, Zn and Cu hypertolerance, respectively (Schat et al., 1993, 1996; Schat & Vooijs, 1997). Crosses between metallicolous plants from different populations, or even different subspecies, never yielded progeny with a normal sensitivity (i.e. non-hypertolerance), indicating that metal tolerance in different geographically isolated populations is conferred by genetic change at the same loci (Schat et al., 1993, 1996). Since metal hypertolerance is a product of repeated evolution at a local scale, rather than dispersed from a single hypertolerant ancestral population, it appears that natural selection affected the same loci at different times and places, at least in *Silene vulgaris* (Schat et al., 1993). The responsible genes have not been identified thus far. In general, at the cellular level hypertolerance is based on an enhanced capacity to compartmentalize excess metals into vacuoles or cell walls (Willems et al., 2007; Ueno et al., 2011; Meyer et al., 2015), but the responsible transporters and their precise subcellular localization are poorly characterized.

In this study we address the mechanisms of Cu hypertolerance in *S. vulgaris* from a copper mine tailing near Imsbach, Germany, which shows an extreme level of copper tolerance, even in comparison with other copper mine populations (Schat et al., 1993, 1996). Although restriction of copper uptake seems to play a role, these plants support many-fold higher Cu burdens, both in their roots and shoots, compared to non-metallicolous plants (de Vos et al., 1991; Schat & Kalff, 1992) suggesting that their hypertolerance must be largely based on improved sequestration inside the body, probably driven by altered capacities of one or more ATP-driven Cu pumps (Burkhead et al., 2009).

Four copper-transporting P1B-type ATPases, called AtHMA5 to AtHMA8 (Burkhead et al., 2009), have been described in *Arabidopsis thaliana*. AtHMA5 is upregulated by high Cu exposure and supposed to efflux excess Cu from the cytosol across the plasma membrane (Andrés-Colás et al., 2006), although its precise localization is still elusive. Here we show that Cu hypertolerance in a *Silene* population from a polluted site is associated with increased expression of two HMA5-like genes, *SvHMA5I* and *SvHMA5II*. The encoded protein, *SvHMA5I* and *SvHMA5II*, define two distinct
phylogenetic clades, which predate the appearance of seed plants during evolution and when expressed in *A. thaliana* confer Cu tolerance by distinct mechanisms. SvHMA5I resides in the tonoplast to sequester Cu in the vacuole, while SvHMA5II resides in the endoplasmic reticulum (ER) and relocates to the plasma membrane (PM) upon exposure to Cu, where it probably extrudes Cu from the cell.

**Results**

**Two HMA5 subgroups in the plant kingdom**

The complete genomic DNA sequence of two genes coding for AtHMA5-like proteins in *S. vulgaris*, *SvHMA5I* (JQ904701) and *SvHMA5II* (JQ904703) was obtained from plants from a copper hyper-tolerant *S. vulgaris* population (Imbsbach, Germany) by PCR procedures (see Methods). SvHMA5I and SvHMA5II contain all the main structural features of heavy metal transporting P-type ATPases and share high similarity with the *A. thaliana* AtHMA5 protein (Andrés-Colás *et al.*, 2006) and similar proteins from other angiosperms (Fig. S1).

Phylogenetic analyses showed that angiosperm HMA5 proteins are closely related to mammalian ATP7A and ATP7B, also known after two human Cu metabolism disorders as Menkes disease protein (Petris *et al.*, 1996) and Wilson disease protein (Roelofsen *et al.*, 2000), respectively, indicating a very old evolutionary origin. Within the plant lineage, duplications of this ancient gene gave rise to two clades: HMA7, which includes *A. thaliana* RESPONSIVE-TO-ANTAGONIST1 (RAN1) (Hirayama *et al.*, 1999), and HMA5. The finding that both the lycophyte *Selaginella moellendorffii* and the liverwort *Marchantia polymorpha* possess HMA5 and HMA7 homologs indicates that this duplication happened early in the plant lineage (Fig. S2).

Detailed analysis of SvHMA5I, SvHMA5II and similar proteins from other angiosperms and a gymnosperm (*Pinus taeda*) revealed that the HMA5 family consists of two distinct (sub) clades, which we named HMA5I and HMA5II (Fig. 1a and Fig. S2). All the analyzed angiosperm HMA5I homologs contain 8 exons, except for the HMA5I (XP_011649326.1) from *Cucumis sativus*, which has 10 exons, whereas HMA5II homologs invariably contain only 6 exons, supporting the ancient divergence of HMA5I and HMA5II genes. Furthermore, synteny analysis revealed clear similarity among the genes surrounding either HMA5II (Fig. 1b) or HMA5I (Fig. 1c), supporting that the HMA5I genes and the HMA5II genes of different angiosperms are orthologous. Although the *Marchantia* and *Selaginella* HMA5 proteins always clustered with the angiosperm HMA5 homologs, we could not assign them to either the HMA5I or HMA5II clade (Fig. 1). This suggests that the duplication that gave rise to HMA5I and HMA5II occurred after the separation of the lycophyte and spermatophyte lineages.
Fig. 1 Plant HMA5 proteins constitute two distinct phylogenetic clades. (a) Phylogenetic tree of a selection of HMA5 proteins. HMA5I subgroup, HMA5II subgroup, HMA7 homologues, ATP7A/B and HMA6/HMA8 are grouped with green, gray, yellow, blue and purple backgrounds, respectively. SvHMA5I and SvHMA5II are highlighted in bold. The tree construction was done using Maximum Likelihood after alignment curation with Gblocks. The protein sequences for the different species are those with the highest similarity to SvHMA5I or SvHMA5II. Branch support was calculated on the basis of 1000 bootstraps and bootstrap values above 50% were placed on the tree. Species names: Marchantia polymorpha (M. polymorpha); Selaginella moellendorffii (S. moellendorffii); Pinus taeda (P. taeda); Amborella trichopoda (A. trichopoda); Setaria italica (S. italica); Aquilegia coerulea (A. coerulea); Beta vulgaris (B. vulgaris); Medicago truncatula (M. truncatula); Solanum pennelli (S. pennelli).
pennellii); Theobroma cacao (T. cacao); Daucus carota (D. carota); Eucalyptus grandis (E. grandis); Manihot esculenta (M. esculenta); Populus trichocarpa (P. trichocarpa); Eutrema salsugineum (E. salsugineum); Oryza sativa (O. sativa); Arabidopsis thaliana (A. thaliana); Vitis vinifera (V. vinifera); Homo sapiens (H. sapiens). (b, c) Synteny analysis of the genomic regions containing HMA5II (b) or HMA5I (c) homologs. The arrows indicate genes and their orientation. Similar genes are indicated with the same color and numbers/letters. White fillings and lack of number/letter denote genes occurring only once in this plot. (d) Multiple sequence alignment of the N-terminal of HMA5I and HMA5II proteins from different species. The red asterisks indicate a conserved putative vacuolar localization motif (LxxPLL), and the red rectangle the first metal binding domain.

All analyzed angiosperms possess a single HMA5I gene, but in Brassicaceae HMA5I has been lost (Fig. 1 and Fig. S2) and the genes that flank HMA5I in other angiosperms are in A. thaliana scattered over the entire genome (Table S1). In several angiosperm lineages HMA5II underwent further duplications resulting in small HMA5II gene clusters (Fig. 1a,b, Fig. S2).

HMA5-like proteins display high similarity throughout the protein sequence (Fig. S1), except for their N-terminal regions (Fig. 1d). In this region of the protein, some motifs are clearly conserved among type II proteins and less among type I HMA5s, but there is little similarity across the two groups. Most of the angiosperm HMA5I proteins contain a conserved IxCxxC motif in the first metal-binding domain, whereas HMA5II proteins contain the typical MxCxxC motif. Furthermore, most of the angiosperm HMA5I proteins contain upstream of the first metal-binding domain a conserved LxPLL motif, which was shown to be a vacuolar sorting signal in other types of P-ATPase transporters (Pedrazzini et al., 2013; Li et al., 2016).

Together, these results show that there are two, evolutionary distinct HMA5 subgroups in the plant kingdom and that most species have at least one HMA5I and one HMA5II gene, while in Brassicaceae HMA5I has been lost.

**Expression of SvHMA5I and SvHMA5II in S. vulgaris**

To test the potential role of SvHMA5I and SvHMA5II in Cu tolerance, we examined their mRNA expression patterns in three Silene vulgaris populations originating from (i) a copper mine near Imsbach, Germany, (ii) a Zn smelter waste deposit near Plombières, Belgium, and (iii) a non-metalliferous site at the VU-University campus, Amsterdam, Netherlands. The Imsbach population is hypertolerant to Cu, Cd and Zn, Plombières is hypertolerant to Zn and Cd, but sensitive to Cu, and Amsterdam is sensitive to all these metals (Schat et al., 1996; Schat & Vooijs, 1997). Overall, SvHMA5I was slightly, but significantly, more expressed in roots than in shoots, whereas SvHMA5II was almost exclusively expressed in roots. On average, SvHMA5I expression was about 7-fold higher in Imsbach than in Amsterdam or Plombières (Fig. 2a,b) and SvHMA5II expression in Imsbach was on average 15-fold higher than in Amsterdam and >50-fold higher than in Plombières. Exposure to 2 µM Cu did not significantly affect the expression level or pattern of SvHMA5I and SvHMA5II in the roots, when compared to the normal nutritional 0.1 µM Cu concentration. In the shoot the expression of SvHMA5II was slightly but significantly enhanced in Imsbach and Amsterdam, and that of SvHMA5I slightly but significantly decreased in Imsbach (Fig. 2a,b).
For further analysis we selected 10 hypertolerant and 10 non-hypertolerant plants from segregating F2 families of reciprocal crosses between plants from the Imsbach and Amsterdam S. vulgaris populations. RNA analysis revealed that SvHMA5I and SvHMA5II expression was significantly higher in the Cu hypertolerant progeny than in Cu non-hypertolerant plants (Fig. 2c), indicating that increased SvHMA5 expression co-segregated with Cu-hypertolerance.

**Fig. 2** Expression patterns of SvHMA5I and SvHMA5II mRNA in plants from different S. vulgaris populations. (a, b) Quantitative RT-PCR analysis of SvHMA5I (a) and SvHMA5II (b) mRNAs in shoots and roots of Cu-hypertolerant (Imsbach) and Cu-sensitive (Amsterdam, Plombières) populations of S. vulgaris (means ± SE, n=3). Values were normalized to the expression level in Am shoots at 0.1 µM Cu. 2-week-old seedlings were treated for 48 h with 0.1 µM or 2 µM Cu in hydroponics. Significant differences (P< 0.05) between populations are indicated with different letters, separately for roots and shoots. (c) SvHMA5I and SvHMA5II expression in root of Cu-non-hypertolerant and Cu-hyper-tolerant F2 plants selected from an Amsterdam × Imsbach cross (means ± SE of 10 plants). Significant differences between non-hypertolerant and hypertolerant progeny are indicated by * (P< 0.05), or ** (P < 0.01).
Overexpression of SvHMA5I, SvHMA5II or AthMA5 enhances copper tolerance in A. thaliana

To address whether the increased expression of SvHMA5I and SvHMA5II contributes to Cu-tolerance in the Imsbach population, we expressed both proteins, and AthMA5 as a control, from the constitutive Cauliflower Mosaic Virus 35S promoter in wild type (HMA5) and hma5 mutant A. thaliana (Col-0) plants (SALK_040252) (Andrés-Colás et al., 2006). For each genotype we assayed Cu tolerance in two independent T2 homozygous lines by measuring the root length of 8-day-old seedlings that were grown on MS medium with various Cu concentrations (Fig. 3 and Fig. S3). There was no significant difference between root length of wild type Col-0, hma5 mutant or any of the transgenic lines after growth on MS medium. In the presence of 40 µM Cu, root growth was reduced by 23% in wild type and completely inhibited in the hma5 mutant line, while in the transgenic lines expressing SvHMA5I, SvHMA5II or AthMA5 root growth was not significantly affected. Sixty µM Cu reduced root length in wild type by 47%, but had little or no effect on roots of transgenic plants.

At 100 µM Cu, the root growth of all genotypes was inhibited, though transgenic roots were significantly less affected than wild type roots (Fig. 3). Thus, constitutive expression of SvHMA5I or SvHMA5II increases Cu tolerance to a similar extent as expression of AthMA5. As the latter originates from a Cu-sensitive species, this suggests that it is the increased expression of the SvHMA5 genes, rather than alterations in the encoded proteins, which contributes to the hypertolerance phenotype of S. vulgaris.

Fig. 3 Overexpression of SvHMA5I, SvHMA5II or AthMA5 enhances Cu tolerance of Arabidopsis. (a) Phenotype of 8-day-old seedlings from Col-0, hma5 and overexpression lines grown on vertical MS plates containing 100 µM Cu. Size bar equals 1 cm. (b) Root length of seedlings grown on MS medium supplied with different Cu concentrations for 8 days after germination. For each transgene results from two independent T2 transgenic lines are shown. Values are expressed as mean ± SEM (n=20).
Subcellular localization of SvHMA5II and AtHMA5 is Cu-dependent

To study the subcellular localization of SvHMA5I, SvHMA5II and AtHMA5, we generated transgenic *Arabidopsis hma5* plants expressing GFP fusion proteins. In these lines SvHMA5I-GFP, SvHMA5II-GFP or AtHMA5-GFP accumulated as a single protein of the expected size, which efficiently complemented the *hma5* phenotype (Fig. S4). Hence, these GFP fusions are reliable reporters for the subcellular location of the tagged protein (Quattrocchio *et al*., 2013). To determine the intracellular localization of the HMA5-GFP proteins we grew homozygous T₂ progeny of these transgenic lines on MS medium without additional Cu and used for high resolution imaging protoplasts from 14-day-old seedlings. To mark the ER and the vacuole tonoplast, we transiently transformed those with constructs expressing RFP-KDEL or AtKCO1-RFP (Czempinski *et al*., 2002), respectively. We found that SvHMA5I-GFP co-localized with AtKCO1-RFP in the tonoplast, but not with the ER marker RFP-KDEL (Fig. 4a), whereas SvHMA5II-GFP and AtHMA5-GFP co-localized with RFP-KDEL in the ER, and not with the tonoplast marker AtKCO1-RFP (Fig. 4b,c).

**Fig. 4** Subcellular localization of SvHMA5I, SvHMA5II and AtHMA5 in transgenic *A. thaliana* plants. (a) Confocal images of an *A. thaliana* protoplasts from 14-d-old *hma5* SvHMA5I-GFP seedlings transiently expressing the tonoplast marker AtKCO1-RFP or the endoplasmic reticulum (ER) marker RFP-KDEL (red fluorescence). (b, c) Confocal images of protoplasts from 14-d-old (b) *hma5* 355:SvHMA5II-GFP seedlings, or (c) *hma5* 355:AtHMA5-GFP seedlings transiently expressing the ER marker RFP-KDEL or the tonoplast marker AtKCO1-RFP (red fluorescence). Bars, 10 µm.
Since the subcellular localization of the related P_{1B}-type copper transporters ATP7A (Petris et al., 1996) and ATP7B (Roelofsen et al., 2000) in mammals is Cu-dependent, we examined the subcellular localization of SvHMA5I-GFP, SvHMA5II-GFP and AtHMA5-GFP in roots of 8-day-old seedlings that had been grown on different Cu concentrations. We found that SvHMA5I-GFP localized in the tonoplast (Fig. 5) at Cu concentrations that are inhibitory (140 μM) or non-inhibitory (0-80 μM) (Fig. S4). However, the localization SvHMA5II-GFP (Fig. 5b) and AtHMA5-GFP (Fig. 5c) was Cu-dependent. In roots grown at 0 or 80 μM Cu, SvHMA5II-GFP accumulated in the typical ER localization pattern (Fig. 5b), while in roots grown at 140 μM Cu it resided in the plasma membrane (PM), co-localizing with the fluorescent lipophilic styryl dye FM4-64 (Fig. 5b), which is routinely used as an endocytic tracer in plant cell studies (Geldner et al., 2003; Bolte et al., 2004). We observed a similar change in the localization of AtHMA5-GFP, except that the (re)localization of this protein to the PM occurred already at 80 μM Cu (Fig. 5c).

**Fig. 5** AtHMA5 and SvHMA5II, but not SvHMA5I, relocalize to the plasma membrane in *A. thaliana* upon exposure to Cu. (a-c) Root meristematic cells of 8-day-old seedlings from *hma5 35S:SvHMA5I-GFP* line #1 (a), *hma5 35S:SvHMA5II-GFP* line #1 (b) and *hma5 35S:AtHMA5-GFP* line #1 (b) grown on MS plates containing 0, 80 or 140 μM Cu. Contours of cells were visualized by staining with FM4-64 for 5 min (red fluorescence) before confocal microscopy. Bars, 10 μm.
SvHMA5II and AtHMA5 show polar localization in the plasma membrane

To define the kinetics of the copper-induced redistribution of SvHMA5II-GFP and AtHMA5-GFP, we grew seedlings on MS plates without additional Cu and transferred them after 8 days to liquid MS medium containing 10 µM or 20 µM Cu and examined the subcellular localization of AtHMA5-GFP and SvHMA5II-GFP at different time points. Although in solid MS media 20 µM Cu is needed for toxicity in Arabidopsis (Woeste & Kieber, 2000; Andrés-Colás et al., 2006), 1.3 µM Cu is already sufficient for toxicity in liquid media (Kobayashi et al., 2008). We observed that within 30 min after transfer to liquid MS with 10 µM Cu, both SvHMA5II-GFP and AtHMA5-GFP had lost the ER localization pattern and that after 60 min most SvHMA5II-GFP and AtHMA5-GFP resided in the PM, and remained so for >2 h (Fig. 6a,b). Remarkably, SvHMA5II-GFP and AtHMA5-GFP appeared polarly localized in the PM, accumulating preferentially on the outer lateral side of epidermal root cells in the meristematic zone, whereas the lipophilic fluorescent dye FM4-64 stained the PM evenly (Fig. 6c,d). This indicates that upon exposure to excess Cu SvHMA5II-GFP and AtHMA5-GFP re-localized to the PM to directionally transport Cu out of the root meristematic zone in these transgenic lines.

When we transferred transgenic seedlings to liquid MS medium with 20 µM Cu, SvHMA5II-GFP relocalized from the ER to the PM as observed at 10 µM Cu (Fig. 6a), whereas AtHMA5-GFP relocalized to punctate structures in the cytoplasm within 30 min and remained so during 1 h. Only after 2 h AtHMA5-GFP started to increase in the PM, but the punctate structures remained present (Fig. 6b). Since we did not observe such punctate structures in plants expressing SvHMA5II-GFP, not even when
exposed to 80 µM Cu (data not shown), it seems unlikely that their formation is due to Cu-toxicity. If so, this may suggest that SvHMA5II and AtHMA5 move to the PM along (partially) different pathways.

Addition of 50 µM cycloheximide (CHX), which fully inhibits protein synthesis in Arabidopsis roots (Takano et al., 2010; Liu et al., 2012), did not interfere with the re-localization of AtHMA5-GFP and SvHMA5II-GFP (Fig. S5). This indicates that the Cu-induced relocalization for AtHMA5-GFP and SvHMA5II-GFP was independent of de novo protein synthesis and relied on the redistribution of proteins previously localized on the ER.

SvHMA5II-GFP and AtHMA5-GFP are degraded in the vacuole
Several PM proteins, including for example PIN2 (Kleine-Vehn et al., 2008) and BOR1 (Takano et al., 2010) were shown to be internalized from the PM to an endosomal compartment(s) from which they can cycle back to the PM or move on towards the vacuole for degradation. To investigate whether PM-localized AtHMA5-GFP and SvHMA5II-GFP undergo a similar fate, we examined how their localization is affected by brefeldin A (BFA), a fungal toxin that inhibits vesicle transport driven by ARF-type GDP-GTP exchange factors (ARF GEFs) (Geldner et al., 2003). To this end, we pre-incubated seedlings for 2 hours with 10 µM Cu and 50 µM CHX, to relocate SvHMA5II-GFP and AtHMA5-GFP from the ER to the PM, before adding 50 µM BFA and, to monitor endocytosis, FM4-64. Within 30 min after the addition of BFA and FM4-64, SvHMA5II-GFP and AtHMA5-GFP and FM4-64 were observed in intracellular BFA compartments, suggesting internalization of SvHMA5II-GFP and AtHMA5-GFP from the PM into the trans-Golgi-network (TGN) or early endosomes (EE) (Fig. 7a).

Fig. 7 Internalization and degradation of SvHMA5II-GFP and AtHMA5-GFP in A. thaliana. (a) Internalization of SvHMA5II-GFP and AtHMA5-GFP from the PM to the TGN/EE. 8-day-old transgenic lines were pretreated with CHX (50 µM) and 10 µM Cu for 2 h to chase SvHMA5II-GFP and AtHMA5-GFP out of the ER, followed by treatment for 20 min with CHX (50 µM), BFA (50 µM), FM4-64 and 10 µM Cu. (b) Eight-day-old transgenic lines incubated in liquid MS medium supplied with 10 µM Cu and FM4-64 (5 µM) for 4 h in the absence of light. Arrows indicate GFP signal in the lumen of the vacuole. ep, epidermis; co, cortex. Bars, 10 µm.
To further investigate whether SvHMA5II-GFP and AtHMA5-GFP also reach the vacuole, we transferred 8-day-old seedlings to liquid MS medium containing 10 µM Cu and kept them in the dark to allow the detection of GFP in the vacuole, since GFP in lytic vacuoles is more stable under dark conditions than in the light (Tamura et al., 2003). After 4 hours in the dark we could detect GFP signal within the vacuole, whereas FM4-64 stained the tonoplast, but not the vacuolar lumen (Fig. 7b). We also detected GFP fluorescence in the vacuoles of 8-day-old seedlings on MS plates, which had been constitutively exposed to 100 µM Cu (Fig. S6). The GFP in vacuoles appears to be soluble, suggesting that the fusion protein is cleaved and possibly undergoing degradation.

Together, these results suggest that SvHMA5II-GFP and AtHMA5-GFP internalized from the PM and delivered to the vacuole for degradation via the endocytic pathway.

Co-overexpression SvHMA5I and SvHMA5II result in better root growth then the single expression of each of the two HMA5 genes

Because the Cu non-tolerant *A. thaliana* has only one *HMA5* gene belonging to the type II clade (Fig. 1a), and the encoded protein, AtHMA5, localizes slightly different from SvHMA5II (Fig. 6), we wondered whether the combination of the two proteins from *Silene* could confer copper hyper-tolerance to *A. thaliana*. Therefore we crossed *hma5 35S:SvHMA5I* and *hma5 35S:SvHMA5II* homozygous lines and measured Cu tolerance in the seedlings of F1 progeny. The roots of the double transformant lines are about 20% longer than those of the single transformants when grown on MS medium without Cu addition. In the presence of 100 µM Cu, root growth was inhibited in double transformant lines as well as in wild type and *hma5* mutants, however, roots of *hma5 35S:SvHMA5I 35S: SvHMA5II* plants were about 35% longer than those of single transgenic lines and about 200% longer than wild type Col-0 seedlings (Fig. 8). This indicates that the combined expression of SvHMA5I and SvHMA5II induces root elongation independently from exposure to Cu.

**Fig. 8** SvHMA5I and SvHMA5II co-overexpressing *A. thaliana* show better growth than single transgenic lines. (a) Phenotype of Col-0, *hma5* and overexpression lines growth MS medium or MS medium supplied with 100 µM Cu. Seeds were sown and grown in vertical plates on MS medium. Photographs were taken 8 days after germination. Bars, 1 cm. (b) Root length of plants shown in (a) grown on MS medium or MS medium supplied with 100 µM Cu for 8 days after germination. Values are expressed as mean ± SEM (n=20). Column with different letters indicate significant difference (P<0.01) using Tukey’s test.
**Discussion**

We have analyzed the expression and cellular localization of HMA5 Cu transporters from a non-metallophytic species (*A. thaliana*) and a Cu-hyper-tolerant population of a metallophyte species (*Silene vulgaris*) to understand the mechanism underlying the rare capacity of some plant species to survive in highly polluted sites. We present evidence that the natural variation in Cu sensitivity of *Silene vulgaris* ecotypes results at least in part from the increased expression of *SvHMA5I* and *SvHMA5II*, encoding related P_{1B}-type ATPase copper transporters that reside at different cellular compartments and contribute to Cu tolerance by independent, additive, mechanisms. It is unlikely that the HMA5s confer tolerance to other metals, because loci conferring tolerance to Cu, Zn and Cd segregate independently in *Silene* (Schat & Vooijs, 1997), and because the Plombières accession, which is tolerant for Cd and Zn, but non-tolerant for Cu, expresses HMA5s at a relatively low level.

HMA5I and HMA5II originate from an ancient duplication that predates the appearance of angiosperms, and possibly gymnosperms, and both are today widespread among seed plants. The two characterized HMA5I homologs, *SvHMA5I* and *OsHMA4* confer Cu tolerance when (over)expressed in *Arabidopsis*, and loss of function *OsHMA4* in rice enhances Cu sensitivity (Huang et al., 2016). Since *SvHMA5I* and *OsHMA4* reside in the tonoplast, independent of the environmental Cu concentrations, their main role appears to be to sequester Cu in the vacuole. The transcriptional regulation of *SvHMA5I* shows some striking differences with that of *OsHMA4*. Expression of *OsHMA4* is Cu-inducible and largely restricted to the root, which inhibits Cu transport to the shoot, whereas *SvHMA5I* is expressed at similar levels in both shoot and root and is independent from exogenous Cu. This suggests that *SvHMA5I* protects the aerial tissues from excess copper both indirectly, by sequestering Cu in the root to prevent Cu reaching the shoot, like *OsHMA4*, and directly by vacuolar sequestration of Cu reaching in the shoot. Possibly related to that, natural variation in HMA4-dependent Cu tolerance of rice accessions is due to alterations in the HMA4 protein sequence rather than their expression, whereas the increased tolerance of the *S. vulgaris* Imsbach genotype is at least in part due to increased mRNA levels. It may be argued that the differences in expression levels between tolerant and non-tolerant F2 are much less pronounced than those between the parents (Fig. 2), however, the same applies the difference in tolerance. In fact, only one plant among the F2, out of 600 in total, recovered the fully tolerant Imsbach phenotype (EC_{100} > 250 µM), which is not significantly different from the expectation of two, based on the proposed genetic architecture of the trait, in so far as it segregates in Amsterdam x Imsbach F2 progenies (Schat et al., 1996). The other plants among the 10 most tolerant plants that we selected for expression analysis were all much less tolerant (EC_{100} < 150 µM), the more so because the sensitivity of the sequential test that we used tends to decrease with increasing tolerance (Schat & Bookum, 1992). Thus, our results obtained with the F2 progeny are not in disagreement with the hypothesis that the higher Cu hypertolerance in *S. vulgaris* from Imsbach is at least in part due to increased transcript levels of *SvHMA5I* and *SvHMA5II*. Whether polymorphisms in the *SvHMA5
protein also contribute to different Cu tolerance remains to be established. The loss of the HMA5I homolog in Brassicaceae may account at least in part for the high sensitivity to Cu in Arabidopsis, which shows 50% root growth inhibition at 0.5 µM Cu (Iqbal, 2013), in comparison with non-metallicolous S. vulgaris grown under identical conditions, which shows 50% root growth inhibition at 3.2 µM Cu in the nutrient solution (Schat & Bookum, 1992).

We found that the intracellular localization of the HMA5II proteins AtHMA5 and SvHMA5II is highly dynamic and very different from that of the HMA5I proteins. When expressed in root meristematic cells, SvHMA5II and AtHMA5 both localize in the ER under low Cu conditions, but upon exposure to Cu relocate to vesicular compartment(s) and the PM, most likely to transport Cu out the cell. The mammalian copper P$_{1B}$-ATPase efflux transporters ATP7A and ATP7B share similarity with HMA5I, HMA5II and RAN1 (Hirayama et al., 1999) homologs from plants, suggesting a common evolutionary origin (Fig. 1a and Fig. S2). Mutations in these transporters disrupt the homeostatic copper balance, resulting in copper deficiency (Menkes disease) or copper overload (Wilson disease), respectively (de Bie et al., 2007). The way that the intracellular localization of ATP7A/B is regulated in mammalian cells is strikingly similar to that of AtHMA5 and SvHMA5II. Under high Cu concentration, ATP7A redistributes from the TGN to the basolateral membrane of polarized cells via endocytosis (Ke et al., 2006). Under the same conditions, ATP7B relocates from the TGN to vesicular structures and the apical membrane in polarized hepatoma cells (Roelofsen et al., 2000). The similarity in the redistribution and polar localization upon Cu exposure of plant HMA5II proteins and their mammalian homologs suggests this to be a very ancient mechanism, which already existed in the last common ancestor of plants and animals. This would imply that the vacuolar HMA5I proteins diverged by the acquisition of a vacuolar targeting sequence (Fig. 1d), similar to the evolution of H$^+$-pumping P-ATPases (Li et al., 2016). However, it cannot be fully excluded that the similar HMA5II and mammalian ATP7A/B localization mechanisms arose independently in plants and animals and that the vacuolar localization of HMA5I proteins is the most ancient state.

In order to respond to the supply of specific minerals, the activity of mineral transporters at the PM must be regulated by posttranslational mechanisms, including constitutive endocytic recycling and degradation (Fuji et al., 2009; Zelazny & Vert, 2014). We show that AtHMA5 and SvHMA5II continuously degrade in the vacuole via endocytosis pathway when Cu is present in excess (Fig. 7 and Fig. S6). Other polar ion transporters, such as the A. thaliana iron importer IRT1 (Barberon et al., 2011), is, following ubiquitination, also targeted to the vacuole for degradation depending on the levels of iron. Whether SvHMA5II and AtHMA5 are ubiquitinated prior to vacuolar sorting remains to be established. IRT1 degradation downregulates iron uptake and prevents iron toxicity. However, the vacuolar targeting and degradation of the exporters AtHMA5 and SvHMA5II in response to high Cu levels seems a conundrum, as under these conditions the exporters are most needed for Cu extrusion. One possibility is that AtHMA5 and SvHMA5II continue to contribute to Cu tolerance after internalization from the PM by sequestering Cu into intermediate endosomal compartment(s), which are then delivered
to the vacuole. Furthermore, the continuous turn-over of plasma membrane-based HMA5 proteins may prevent that plants run into an acute copper-deficiency, when exogenous Cu levels suddenly drop to (below) normal levels.

Surprisingly, roots from Arabidopsis plants that constitutively co-express SvHMA5I and SvHMA5II grow faster than those expressing either SvHMA5I or SvHMA5II in media without additional Cu, suggesting interference with some hormone pathways, for example, ethylene perception and signaling, which requires Cu (Hirayama et al., 1999; Woeste & Kieber, 2000), or auxin transport and distribution, which is very sensitive to excess Cu (Yuan et al., 2013). Therefore, resolving the trafficking of copper transporter in response to growth conditions, might open the door to a better understanding of the very different mechanisms by which Cu functions in the regulation of intracellular signaling and seedling growth.
Materials and Methods

Plant material

Seeds of *S. vulgaris* were collected from a copper mine near Imsbach, Germany, a Zn smelter waste deposit near Plombières, Belgium, and a non-metalliferous site at the Vrije Universiteit campus, Amsterdam, Netherlands (Schat *et al.*, 1996). Seeds of a homozygous *A. thaliana hma5* T-DNA insertion mutant line were obtained from the NASC European Arabidopsis Stock Centre (reference number N656401) (Salk_040252C).

An F2 cross was made (n = 1200) through selfing 6 F1 plants of reciprocal crosses between a non-hyper-tolerant (Amsterdam) and a Cu-hyper-tolerant (Imsbach) plant. The F2 plants were screened for Cu tolerance, using “sequential exposure tests”, with 50 µM (~ 600 plants), and 2 µM Cu concentration increments (Schat & Bookum, 1992). Ten F2 plants (out of approximately 600) with the highest tolerance levels (EC100 > 100 µM), and 10 plants (out of ~ 600) with the lowest tolerance levels (EC100 < 8 µM) were selected as described (Schat & Bookum, 1992), and compared for their expression levels of *SvHMA5I* and *SvHMA5II* afterwards. Plant tissues for RNA extraction were taken prior to the test, from plants that were grown for 10 days in hydroponics at 0.1 µM Cu.

Growth conditions

Seeds of *S. vulgaris* were sown on a garden peat soil (Jongkind BV, number 6, Aalsmeer, the Netherlands). After two weeks, seedlings were transferred to a hydroponics system, consisting of 1-L polyethylene pots. The nutrient solution was a modified half-strength Hoagland’s, renewed once per week (Schat *et al.*, 1996). For Cu tolerance testing the same solution was used, but without Fe(Na)EDTA (Schat *et al.*, 1996). *S. vulgaris* was grown in a climate room at 20/15 ºC day/night, photon flux density at plant level 240 µmoles m-2 sec-1, 10 h d-1, air humidity 75% relative humidity (RH).

*A. thaliana* seeds were surface sterilized with 0.8% bleach for 10 min, rinsed five times with sterilized water, sown on Murashige & Skoog (MS) medium or MS medium supplied with the indicated concentrations of CuSO4 and kept in darkness at 4 ºC for 2 days for seed stratification. Then plates were placed vertically in incubators at 22 ºC under a 16/8 light/dark cycle. Plates were photographed 8 days after germination using an EPSON scanner. The root length was measured from the digital images using the ImageJ program (https://imagej.nih.gov/ij/).

DNA/RNA extraction and cDNA synthesis

Fresh root and leaf samples were snap-frozen in liquid nitrogen and stored at -80 ºC until extraction. RNA was extracted using TrizolTM (Invitrogen), following the manufacturer’s instructions, and as previously described (Jack *et al.*, 2007). DNA was isolated as reported (Rivera *et al.*, 1999). Single-stranded cDNA was synthesized in 50 µl containing 2.5 µg RNA, 50 mM Tris-HCl pH8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.8 mM dNTPs, 0.6 µM oligo dT and 100 Units M-MLV reverse transcriptase (Invitrogen) at 42 ºC for 1 h.
Quantitative RT-PCR analyses

The expression of \textit{SvHMA5I} and \textit{SvHMA5II} in root and shoot of \textit{S. vulgaris} after different Cu treatments was determined by quantitative real-time RT-PCR on first strand cDNA using SYBR green 2 × mastermix (Bioline, Taunton, MA, USA) and an Opticon 1 real-time PCR machine (MJ Research, Waltham, MA, USA) following the manufacturer's instructions. The reaction mix was 10 µl SensiMix\textsuperscript{TM} SYBR No-ROX master mix, 250 nM forward primer, 250 nM reverse primer and cDNA (from 85 ng RNA) in a total reaction volume of 20 µl. RT-PCR conditions were as follows: one cycle of 95 ºC for 10 min; 40 cycles of melting at 95 ºC for 10 s, annealing-extension at 60 ºC for 20 s; at the end melting curve from 60 ºC to 90 ºC, read every 0.5 ºC, hold 10 s. Intron spanning primers used to amplify transcripts for \textit{SvHMA5I} and \textit{SvHMA5II} were based on sequence stretches identical for the alleles in the Imsbach and Amsterdam populations. \textit{SvActin2} (JQ435885) served as reference (Supplementary Table 2). Expression values were calculated using the $2^{-\Delta\Delta C_{T}}$ method (Livak & Schmittgen, 2001)

DNA constructs and generation of transgenics

By PCR with two pairs of degenerate primers (Supplementary Table 3) on genomic DNA of Imsbach population we obtained two different products, originating from \textit{SvHMA5I} and \textit{SvHMA5II}, both sharing high similarity with the \textit{A. thaliana} \textit{HMA5} sequences. By 3’RACE and 5’UTR race (Clontech (PT3042-2) Universal Genome walker kit, the 5’ and 3’ UTR of both genes were obtained. Genbank accession numbers for \textit{SvHMA5I} and \textit{SvHMA5II} mRNA and protein sequences are JQ904701 and JQ904703.

The full-size coding sequences of \textit{SvHMA5I} and \textit{SvHMA5II} were amplified from genomic DNA of the \textit{S. vulgaris} population from Imsbach with primers containing attB1 and attB2 site and used to create a Gateway Entry clone by BP reaction with pDONR P1-P2 (Gateway system; Life Technologies, Invitrogen, Carlsbad, CA, USA). Entry clones were recombined with pH7WG2,0 for ectopic expression constructs and with pH7FWG2,0 for GFP fusions.

The full-length coding sequence of \textit{AtHMA5} was amplified from \textit{A. thaliana} genomic DNA using primers containing attB1 and attB2 sites. These fragments were recombined with BP clonase into pDONR P1-P2, behind a rhamnose inducible promoter, and then recombined in pH7FWG2,0 (35S:AtHMA5-GFP) or pH7WG2,0 (35S:AtHMA5). As AtHMA5 was toxic for \textit{Escherichia coli}, the recombination products were transformed to \textit{Agrobacterium tumefaciens} strain GV3101 by electroporation. Transformation of \textit{A. thaliana} plants was carried out by floral dip (Clough & Bent, 1998).

Phylogenetic tree and synteny analysis

HMA5 homologs from different species were identified by the Blastp tool of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Phytozome version 11 (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST). HMA5 homologs from \textit{P.taeda}, were identified by Blastp in a Spruce Genome database (http://congenie.org/blast). Sequence alignments for the phylogenetic tree were generated with MUSCLE in MEGA7, and phylogenetic trees
were constructed using Maximum Likelihood method based on the Le_Gascuel_2008 model (Le & Gascuel, 2008) implemented in MEGA7 with 1000 bootstrap replicates after alignment curation with Gblocks 0.91b (http://www.phylogeny.fr/). A matrix of pairwise distances was estimated using a JTT model, and the tree with the highest log likelihood was chosen with Neighbor-Join and BioNJ algorithms. N-terminal multiple sequence alignments were performed by Clustal Omega method (http://www.ebi.ac.uk/Tools/msa/clustalo/). For alignment shading BOX SHADE version 3.21 was used (http://www.ch.embnet.org/software/BOX_form.html). Synteny was analyzed using the Web-based Phytozoome platform (Goodstein et al., 2012).

Western blotting

Western blotting was done as described (Verweij et al., 2008).

Protoplast isolation and transformation

Protoplasts were isolated from 14-day-old seedlings of the transgenic A. thaliana, lines hma5 35S:SvHMA5I-GFP, hma5 35S:SvHMA5II-GFP and hma5 35S:AtHMA5-GFP, grown on MS medium. Protoplasts were then transiently transformed with RFP-KDEL or AtKCO1-RFP according to the published protocol with some modification (Yoo et al., 2007). Fourteen-day-old A. thaliana seedlings were cut into small strips and digested for 5 h in 10 ml of enzyme solution (1% Cellulase R10, 0.25% macerozyme R100, 0.4 M mannitol, 10 mM CaCl₂, 20 mM KCl, 0.1% Bovine Serum Albumin (BSA) and 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) at pH 5.7). Protoplasts were filtered through 100 µm nylon mesh and centrifuged at 100g for 3 min. The pellets were washed 3 times with chilled W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose and 2 mM MES at pH 5.7). Protoplasts were incubated on ice for 30 min in W5 solution followed by centrifugation for 3 min at 100g. Protoplasts were gently resuspended in MMg solution (0.4 M mannitol, 15 mM MgCl₂, and 4 mM MES at pH 5.7). For each transformation, 250 µl of fresh protoplast (~2 to 10⁵) was added to a mixture of 10 µl of plasmids (~10 µg) and 300 µl fresh prepared PEG solution (40% PEG4000, 0.1 M CaCl₂ and 0.2 M mannitol), gently mixed. After incubation at room temperature for 15 min, the transfection mixture was diluted with 3 ml W5 solution and mix well. After centrifugation for 3 min at 100g, protoplast pellets gently resuspended in 2 ml W5 solution and incubated at room temperature for 20 h.

Microscopy

Confocal images were taken with a Zeiss LSM510 using the following excitation and emission wavelengths: 488 nm and 505-550 nm for GFP; 568 nm and 585-615 nm for FM4-64 and RFP. For all treatments about 10 seedlings were analyzed. In protoplast experiments we imaged at least 10 cells and observed many more for each genotype/treatment.

Seedlings treatments

FM4-64 (Sigma-Aldrich) was prepared as a 50 mM stock solution in DMSO and used at the final concentration of 5 µM. Brefeldin A (BFA; LC Laboratories, Woburn, MA, USA) was prepared as a 50 mM stock solution in DMSO and used at 50 µM. Cycloheximide (CHX; Sigma-Aldrich) was prepared
as a 100 mM stock in DMSO and used at 50 μM. CuSO4·5H2O (Sigma-Aldrich) was prepared as 200 mM stock solution in H2O and used at the concentrations indicated for the different experiments.

**Acknowledgements**

We thank Erik Manders and Ronald Breedijk (Center of Advanced Microscopy, University of Amsterdam) for advice and technical assistance with confocal microscopy; Teun Munnik (University of Amsterdam) for advice on the discussion; Pengwei Wang (Durham University) for advice on the experiment of subcellular localization.

Y.L. and Q.Z. were supported by a fellowship of the Chinese Scholarship Council. M.I. was supported by a fellowship of the Higher Education Commission (HEC), Pakistan.

**Author contributions**

Y.L., M.I., F.M.Q., R.K. and H.S. planned and designed the research. Y.L. performed most of the experiments. M.I. and H.W.J.H. performed the real time RT-PCR. M.I. and K.S. cloned the *SvHMA5I* and *SvHMA5II* cDNAs. Q.Z. performed the *Arabidopsis* crossing. M.B helped with the phylogenetic and synteny analyses. Y.L., F.M.Q., H.S. and R.K. wrote the paper with input from all other authors.
References


Iqbal M. 2013. Molecular mechanisms of heavy metal tolerance and accumulation in hyperaccumulating and nonhyperaccumulating metallophytes.


Supporting Information

Fig. S1 Multiple sequence alignment of SvHMA5I, SvHMA5II and AhHMA5. The red squares indicate the position and name of the conserved domains typical of HMA (Andrés-Colás et al., 2006), including two CxxC N-terminal Cu-binding domains, a CPC(X)_6P transduction domain, an aspartyl kinase domain (DKTGT), and HP motif and an ATP binding domain.
Fig. S2 Phylogenetic tree of a selection of HMA5 proteins. HMA5I subgroup, HMA5II subgroup, HMA7 homologues, ATP7A/B and HMA6/HMA8 are grouped with green, gray, yellow, blue and purple backgrounds, respectively. The tree was built using the Maximum Likelihood after alignment curation with Gblocks. The protein sequences from different species are those with the highest similarity to SvHMA5I or SvHMA5II.

Species names: Arabidopsis thaliana (A. thaliana); Homo sapiens (H. sapiens); Marchantia polymorpha (M. polymorpha); Selaginella moellendorffii (S. moellendorffii); Pinus taeda (P. taeda); Amborella trichopoda (A. trichopoda);Spirodela polyrhiza (S. polyrhiza); Musa acuminate (M. acuminate); Phoenix dactylifera (P. dactylifera); Oryza sativa (O. sativa); Brachypodium distachyon (B. distachyon); Setaria italica (S. italica); Sorghum bicolor (S. bicolor); Aquilegia coerulea (A. coerulea); Daucus carota (D. carota); Mimulus guttatus (M. guttatus); Boechera stricta (B. stricta); Eutrema salsugineum (E. salsugineum); Brassica rapa (B. rapa); Eucalyptus grandis (E. grandis); Manihot esculenta (M. esculenta); Ricinus communis (R. communis); Citrus sinensis (C. sinensis); Carica papaya (C. papaya); Cucumis sativus (C. sativus); Fragaria vesca (F. vesca); Petunia axillaris (P. axillaris); Solanum pennelli (S. pennelli); Medicago truncatula (M. truncatula); Populus trichocarpa (P. trichocarpa); Theobroma cacao (T. cacao); Beta vulgaris (B. vulgaris); Vitis vinifera (V. vinifera);Capsella grandiflora (C. grandiflora).
**Fig. S3** Overexpression of *SvHMA5I, SvHMA5II* or *AtHMA5* enhances Cu tolerance of *Arabidopsis*. (a, b) Root length of transgenic seedlings in Col-0 background (a) or *hma5* background (b) grown on MS medium supplied with different Cu concentrations for 8 days after germination. Two independent T2 transgenic lines are shown for each gene construct. Values are means ± SEM (n=20). Column with different letters indicate significant difference ($P < 0.05$) using Tukey’s test.
Fig. S4 Characterization of GFP fusion proteins. (a) Phenotype of Col-0, hma5 mutant and ectopic expression lines grown on vertical MS plates containing 100 µM Cu. Photographs were made 8 days after germination. Bar = 1 cm. (b) Root length of plants grown on MS medium supplied with 80 or 100 µM Cu, 8 days after germination. Two independent T2 transgenic lines are shown for each gene construct. Values are means ± SEM (n=20). Column with different letters indicate significant difference (P< 0.05) using Tukey’s test. (c) Immunoblot of total proteins from 14-day-old transgenic seedlings stained with anti-GFP antibodies.
**Fig. S5** Redistribution of AtHMA5-GFP and SvHMA5II-GFP was not affected by CHX. Eight-day-old transgenic seedlings, similar to those in Fig. 6a were pretreated with 50 µM CHX for 1 h, followed by a treatment with 50 µM CHX plus 10 µM or 20 µM Cu for the indicated time periods. ep, epidermis; co, cortex. Scale bars in all images equal 10 µm.

**Fig. S6** Localization of SvHMAII-GFP and AtHMA5-GFP in vacuoles. (a, b) SvHMA5II-GFP (a) and AtHMA5-GFP (b) localization in the vacuole of root meristematic cells. Seedlings from transgenic lines were grown on MS plates supplied with 100 µM Cu and transferred to darkness for 4 h before imaging. The red arrows indicate the vacuole. ep, epidermis; co, cortex. Scale bars equal 10 µm.
### Table S1. Position and orientation of homologs of the HMA5I flanking genes identified in other species in the *Arabidopsis* chromosome.

<table>
<thead>
<tr>
<th>indicated letter</th>
<th>Gene ID</th>
<th>position in genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>AT5G19620.1</td>
<td>Chr5:6623204..6627846 forward</td>
</tr>
<tr>
<td>b</td>
<td>AT3G57670.1</td>
<td>Chr3:21370903..21373446 forward</td>
</tr>
<tr>
<td>c</td>
<td>AT3G20870.1</td>
<td>Chr3:7309279..7312752 reverse</td>
</tr>
<tr>
<td>y</td>
<td>AT5G13490.1</td>
<td>Chr5:4335467..4337628 forward</td>
</tr>
<tr>
<td>s</td>
<td>AT4G34890.1</td>
<td>Chr4:16618573..16625057 reverse</td>
</tr>
<tr>
<td>d</td>
<td>AT1G21200.1</td>
<td>Chr1:7421217..7423143 forward</td>
</tr>
<tr>
<td>t</td>
<td>AT2G22410.1</td>
<td>Chr2:9509017..9511231 forward</td>
</tr>
<tr>
<td>r</td>
<td>AT3G63530.1</td>
<td>Chr3:23456157..23458459 reverse</td>
</tr>
<tr>
<td>x</td>
<td>AT5G19350.1</td>
<td>Chr5:6518838..6521474 forward</td>
</tr>
<tr>
<td>n</td>
<td>AT3G20860.1</td>
<td>Chr3:7306147..7308434 forward</td>
</tr>
<tr>
<td>e</td>
<td>AT3G07840.1</td>
<td>Chr3:2501893..2503565 reverse</td>
</tr>
<tr>
<td>f</td>
<td>AT1G12050.1</td>
<td>Chr1:4072752..4076207 forward</td>
</tr>
<tr>
<td>g</td>
<td>AT2G04540.1</td>
<td>Chr2:1581416..1584688 reverse</td>
</tr>
<tr>
<td>h</td>
<td>AT4G02600.1</td>
<td>Chr4:1143962..1147422 forward</td>
</tr>
<tr>
<td>i</td>
<td>AT1G55580.1</td>
<td>Chr1:20764011..20765752 forward</td>
</tr>
</tbody>
</table>

Letters correspond to the numbering of genes flanking *HMA5I* in other Angiosperms, as used in Fig. 1.

### Table S2. Primer sequences used for real time RT-PCR

<table>
<thead>
<tr>
<th>genes</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SvActin2</td>
<td>TCGCTCTATGCCAGTGGTGC</td>
<td>forward</td>
</tr>
<tr>
<td>SvActin2</td>
<td>TCAGATCCCGACCAGCAAGAT</td>
<td>reverse</td>
</tr>
<tr>
<td>SvHMA5I</td>
<td>GAGGCTGCACAATTGGCAAGAG</td>
<td>forward</td>
</tr>
<tr>
<td>SvHMA5I</td>
<td>CCAGTTGCGAACCATGCTAACC</td>
<td>reverse</td>
</tr>
<tr>
<td>SvHMA5II</td>
<td>GAGACGAGTGCTATGCTCATTTCC</td>
<td>forward</td>
</tr>
<tr>
<td>SvHMA5II</td>
<td>CATTTTTTGATCAGTCTGCTGCG</td>
<td>reverse</td>
</tr>
</tbody>
</table>

### Table S3. Degenerate primer sequence used to amplify *SvHMA5I* and *SvHMA5II*

<table>
<thead>
<tr>
<th>genes</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SvHMA5I</td>
<td>RTNRTNGGNTGYCCNTGYGC</td>
<td>forward</td>
</tr>
<tr>
<td>SvHMA5I</td>
<td>GCRCRCTTDATNCCRTNC</td>
<td>reverse</td>
</tr>
<tr>
<td>SvHMA5II</td>
<td>GTKGARAAARGYRTCAARMGKCTBC</td>
<td>forward</td>
</tr>
<tr>
<td>SvHMA5II</td>
<td>GRCTYTGBCCCCAWATRACA</td>
<td>reverse</td>
</tr>
</tbody>
</table>