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Mother plant-mediated pumping of zinc into the developing seed

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Insufficient intake of zinc and iron from a cereal-based diet is one of the causes of "hidden hunger" (micronutrient deficiency), which affects some two billion people[1,2]. Identifying a limiting factor in the molecular mechanism of zinc loading into seeds is an important step towards determining the genetic basis for variation of grain micronutrient content and developing breeding strategies to improve this trait[3]. Nutrients are translocated to developing seeds at a rate that is regulated by transport processes in source leaves, in the phloem vascular pathway, and at seed sinks. Nutrients are released from a symplasmic maternal seed domain into the seed apoplasm surrounding the endosperm and embryo by poorly understood membrane transport processes[4–6]. Plants are unique among eukaryotes in having specific P1B-ATPase pumps for the cellular export of zinc[7]. In *Arabidopsis*, we show that two zinc transporting P1B-ATPases actively export zinc from the mother plant to the filial tissues. Mutant plants that lack both zinc pumps accumulate zinc in the seed coat and consequently have vastly reduced amounts of zinc inside the seed. Blockage of zinc transport was observed both at high and low external zinc supplies. The phenotype was determined by the mother plant and is thus due to a lack of zinc pump activity in the seed coat and not in the filial tissues. The finding that P1B-ATPases are one of the limiting factors controlling the amount of zinc inside a seed is an important step towards combating nutritional zinc deficiency worldwide.

In the quest to identify the membrane-bound transporters that deliver zinc into seeds, heavy metal-transporting P1B-ATPases, which belong to the family of P-type ATPases that actively transports ions and lipids across membranes, are promising candidates. In *Arabidopsis thaliana,* the P1B-ATPase subfamily consists of eight members, AtHMA1–8 (ref. 8). Whereas AtHMA2–4 transport divalent zinc and cadmium cations and AtHMA5–8 transport monovalent copper and silver cations, AtHMA1 has been implicated in the transport of a number of different cations including copper, silver, zinc and cadmium[9,10]. AtHMA3 is localized to the tonoplast and is involved in detoxification by sequestering excess amounts of zinc and cadmium into the vacuole[11]. AtHMA2 and AtHMA4 are structurally very similar to each other and have overlapping functions. Whereas the *hma2* and *hma4* single mutants have wild-type growth rates, the *hma2,hma4* double mutant shows a stunted growth phenotype that can only be rescued by zinc supplementation. Also, the *hma2,hma4* double mutant does not set seed, a phenotype mainly ascribed to a defect in pollen development[12]. Both AtHMA2 and AtHMA4 have been localized to the plasma membrane and function as cellular zinc exporters[12–14]. Their involvement in xylem loading of zinc in roots is well known. They are both expressed in the root pericycle and xylem parenchyma cells and mutants lacking functional AtHMA2 and AtHMA4 accumulate zinc in the pericycle and endodermal cell layers, and exhibit reduced root-to-shoot translocation of zinc[12,13]. The finding that the zinc hyperaccumulator *Arabidopsis halleri* accumulates high levels of zinc in the shoot mainly because of the strongly enhanced expression of *AhHMA4*, caused by activating *cis*-regulatory mutations and gene copy number expansion[15], emphasizes the role of HMA4 in the root-to-shoot translocation of zinc.

To test whether developing seeds depend on *AtHMA2* and *AtHMA4* for proper zinc unloading from the seed coat, we analysed the amount of zinc present in the maternal and filial parts of the seed, respectively, in wild-type and mutant seeds lacking functional *AtHMA2, AtHMA4,* or both. For this purpose, we developed an assay in which mature seeds are separated into seed coat (with the endosperm attached) and embryo fractions, and their respective metal contents are subsequently analysed by inductively coupled plasma mass spectrometry (ICPMS). All seeds used for this analysis were from plants grown side by side in soil watered with 3 mM $ZnSO_4$ twice a week, as these conditions were needed for the *hma2,hma4* double mutant to set seed. At this relatively high zinc supply, trafficking of zinc to the shoot did not differ between genotypes (Supplementary Fig. 1). We determined the total amounts of zinc, magnesium, phosphorus and manganese in batches of ten mature seeds, either intact or separated into seed coat and embryo fractions, using ICPMS. This revealed that the content of the different genotypes or between the content of intact seeds and the combined content of separated seeds for the tested elements showed little variation (Supplementary Fig. 2). As there was no difference between the weight of seeds of different genotypes and the combined weight of their separated parts (Supplementary Fig. 3a), loss of material during the separation procedure was negligible. However, the weight of any ten seeds was variable, which explains the variance seen for the total amounts of element. Instead of weighing samples before ICPMS, which could result in contamination, we used the ratio of seed coat weight to embryo weight as a reference point for normalizing data as it consistently was found to be very similar between and within genotypes (Supplementary Fig. 3b).

We found that in wild-type seeds most of the zinc was present in the embryo fraction (Fig. 1a). Conversely, in the *hma2,hma4* double

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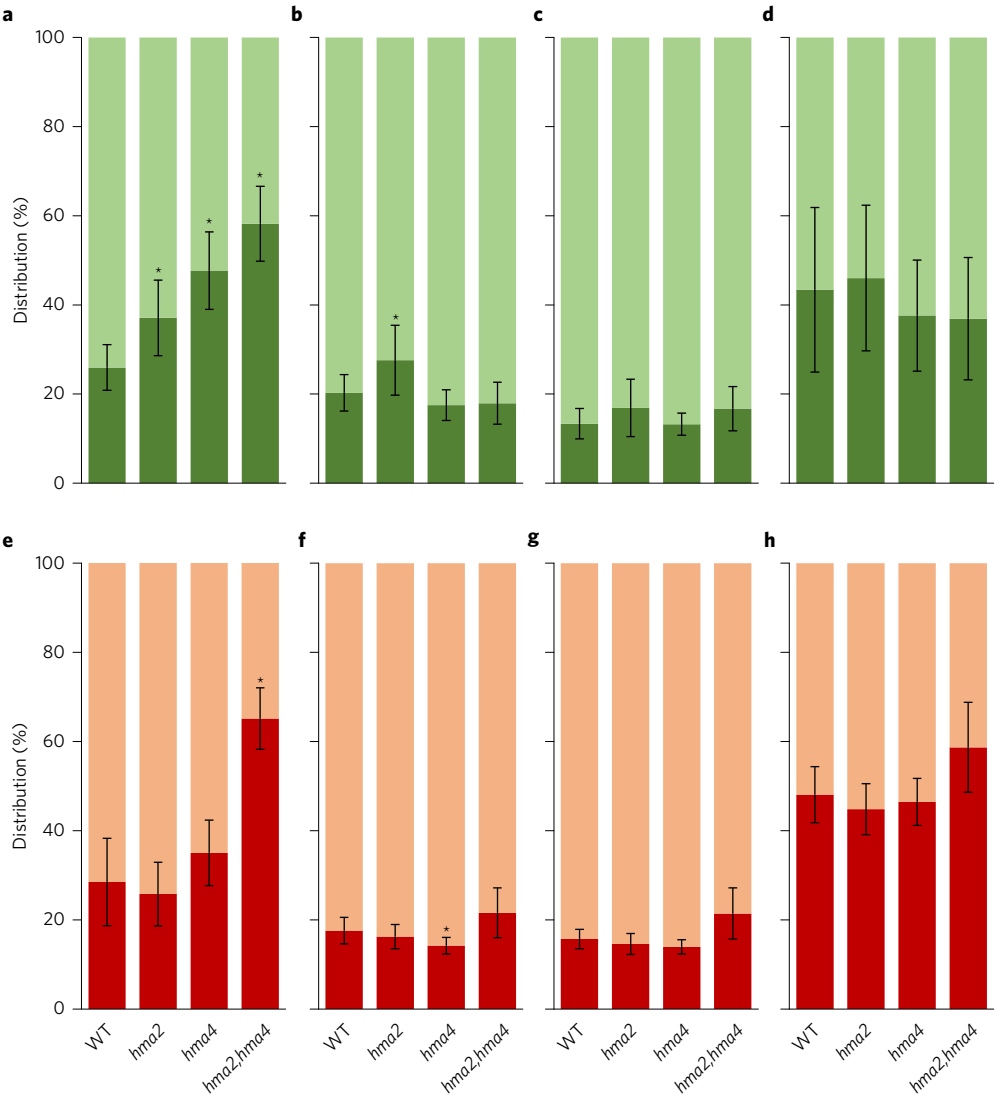

Figure 1 | The distribution of zinc is shifted from the embryo to the seed coat of mature *hma2,hma4* seeds. Microanalytical multi-elemental profiling of batches of ten seeds separated into seed coats and embryos. **a**–**h**, Distribution of elements in seeds from plants grown at high zinc supply (**a**–**d**) and plants grown at low zinc supply (**e**–**h**) (inflorescences of different genotypes had been grafted onto wild-type rootstocks). Bars represent the percentage of the total elemental content that is localized in the seed coat (dark green/red) and embryo (light green/red) fractions. **a**,**e**, Zinc; **b**,**f**, magnesium; **c**,**g**, phosphorus; and **d**,**h** manganese. Data are mean ± s.d. of eight to ten replicates. Statistical analysis indicates significant difference from the wild type (*$p < 0.01$).

mutant, zinc accumulated in the seed coat fraction (Fig. 1a). In *hma2* and *hma4* single mutants, only slightly more zinc accumulated in the seed coat than the wild type, whereas the distribution of magnesium, phosphorus and manganese did not show the same shift (Fig. 1a–d).

hma2,hma4 double mutant plants did not develop seeds unless supplemented with a high amount of zinc (3 mM $ZnSO_4$ twice a week), but under these conditions overall zinc homeostasis of the plant may be perturbed. To make the *hma2,hma4* double mutant set seed under low zinc conditions, we grafted the mutant inflorescences onto wild-type rootstock. When the resulting plants were grown under low external zinc conditions, *hma2,hma4* mutant inflorescences produced seeds, and, compared with the seeds of plants grown under high zinc conditions, contained a much lower zinc content (reduced from around 50–10 ng for 10 seeds, Supplementary Fig. 4). Under these conditions, seeds of *hma2, hma4* double mutant inflorescences accumulated zinc in the seed coat (Fig. 1e). This demonstrates that the *hma2,hma4* double mutant fails to direct zinc towards the embryo both under high and low external zinc supplies. By contrast, no shift in zinc distribution was seen for the *hma2* and *hma4* single mutants at a low zinc supply (Fig. 1e).

To confirm that HMAs are required for export of zinc from the maternal to the filial parts of seeds, we employed micro-X-ray fluorescence (μXRF) and micro-particle-induced X-ray emission (μPIXE) coupled to micro-Rutherford backscattering spectroscopy (μRBS) to obtain spatially resolved images of the distribution of zinc in wild-type and mutant seeds. μPIXE/RBS also allows for quantification of elemental concentrations in the seed sections. For these experiments we obtained seeds from intact plants grown with zinc supplementation. Our analysis of μXRF and μPIXE/RBS maps of all together seven replicate sections imaged per genotype indicated that the spatial distribution of zinc was consistently altered in the *hma4* and *hma2,hma4* mutants compared with the wild type (Fig. 2, Supplementary Fig. 5 and Supplementary Table 1). In wild-type seeds, the distribution of elements was essentially as reported earlier[16,17]. Iron was found to accumulate in the provascular strands, manganese surrounded these strands on the lower side of the cotyledons and calcium was enriched in the seed

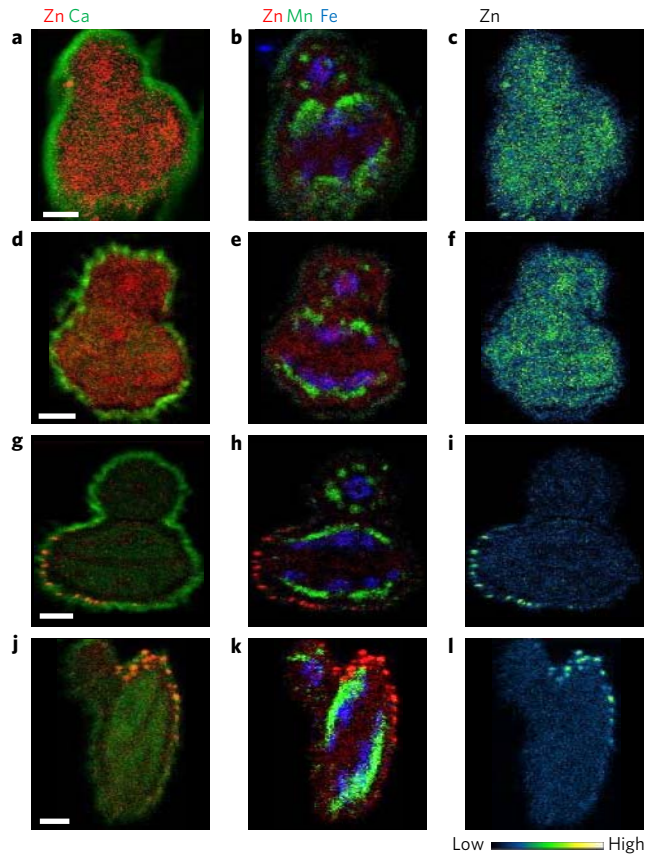

Figure 2 | Zinc accumulates in the seed coat of mature *hma4* and *hma2, hma4* mutant seeds. a–l, μPIXE/RBS elemental profiles of transverse sections of seeds from wild-type (**a–c**), *hma2* mutant (**d–f**), *hma4* mutant (**g–i**) and *hma2,hma4* (**j–l**) double mutant plants grown at high zinc supply. **a**,**d**,**g**,**j**, Two-colour maps showing zinc (Zn) distribution in red and calcium (Ca) distribution in green. **b**,**e**,**h**,**k**, Three-colour maps showing zinc (Zn) distribution in red, manganese (Mn) distribution in green and iron (Fe) distribution in blue. **c**,**f**,**i**,**l**, Temperature colour maps displaying zinc distribution. Scale bars, 50 μm.

coat and showed an even distribution in the embryo, as did zinc (Fig. 2a–c). The pattern of zinc distribution in seeds of the *hma2* single mutant was similar to that of the wild type (Fig. 2d–f). By contrast, zinc distribution was far more uneven across sections of seeds from both the *hma4* single mutant (Fig. 2g–i) and the *hma2,hma4* double mutant (Fig. 2j–l). In these mutants, the zinc signal was reduced in the embryo. By contrast, very high levels of zinc accumulated in the seed coat, with the frequency of high-intensity zinc hot spots gradually declining with increasing distance from the hilum inside the seed coat. There were no consistent changes in the distribution of other elements, such as calcium, iron and manganese, among the replicate sections analysed (Fig. 2 and Supplementary Fig. 5).

To determine whether the ability to provide the embryo with zinc was a maternal property, we performed crosses between wild-type or *hma2,hma4* mother plants and wild-type or *hma2,hma4* pollen. Wild-type plants always produced seeds with an even distribution of zinc in the embryo, regardless of whether they had been fertilized with *hma2,hma4* or wild-type pollen. By contrast, mother plants with a *hma2,hma4* genotype always produced seeds that accumulated zinc in the seed coat, regardless of the genotype of the pollen (Figs. 3, 4 and Supplementary Table 2). This demonstrates that the observed accumulation of zinc in the seed coat is due to the lack of AtHMA2 and AtHMA4 in the seed coat itself. Taken together, our ICPMS data in combination with μXRF and μPIXE/RBS mapping of elemental distribution suggest that cellular export of zinc towards the endosperm and embryo is blocked without the combined function of AtHMA2 and AtHMA4 in the seed coat.

Genome-wide gene activity profiling suggests that *AtHMA2* and *AtHMA4* are both expressed in developing seeds[18], (Supplementary Fig. 6). To confirm the expression of *AtHMA2* and *AtHMA4* at this location, we generated transgenic plants expressing β-glucuronidase (GUS) and investigated the cell-specific expression of the GUS reporter using whole-mount confocal imaging. The *AtHMA2* promoter was active throughout the seed, including both the seed coat and embryo, whereas *AtHMA4* was expressed in the innermost cell layer of the seed coat surrounding the endosperm (the endothelium), in the chalazal region, and also in the embryo (Fig. 5d,e). This expression pattern was detected at all developmental stages (data not shown). When green fluorescent protein (GFP) was fused to the first half of AtHMA4 (HMA4-4TM–GFP) and the resulting construct was expressed under the control of the *HMA4* promoter in transgenic plants, we found GFP expression to be strong in the endothelium (Fig. 5f,g). This is consistent with the finding that, in the maternal tissues of developing seeds, *AtHMA4* is specifically expressed in the seed coat endothelium.

On the basis of our results, we suggest a model in which AtHMA2 and AtHMA4 are involved in the export of zinc from the mother plant seed coat to the filial tissues. The phloem is a long-distance vascular transport system in plants that transports nutrients to reproductive tissues. In *Arabidopsis* seeds, the phloem is symplastically connected to the seed coat. However, for nutrients to proceed further into filial tissues, apoplastic barriers have to be crossed, which requires transport across biological membranes[4], and for zinc export against the inside-negative membrane potential. In the seed coat of the developing seed, an apoplastic barrier is present between the outer and inner integument, as well as between the inner integument and the endosperm, and between the endosperm and the embryo[4]. Whereas *AtHMA2* was expressed in all integuments, *AtHMA4* was mainly expressed in the innermost layer of the seed coat, the endothelium. Thus, AtHMA4 is a prime candidate for mediating the post-phloem translocation of zinc from the seed coat and into the endosperm cavity. The importance of AtHMA4 in this process is evident at a high zinc supply as, in the *hma4* mutant background, zinc accumulates in the seed coat, as shown by both ICPMS and μXRF/μPIXE. This phenotype is even stronger in the *hma2,hma4* double mutant at both a high and low zinc supply, which suggests that AtHMA2 also contributes to this process. The fact that zinc is present in the embryo even in the *hma2,hma4* double mutant indicates that other transporters exist in the seed coat that are able to export zinc into the endosperm cavity. A similar phenomenon is seen in the root, where zinc is loaded into the xylem even in the *hma2,hma4* double mutant[12,13], a transport that at least partially seems to be mediated by the putative transporter AtPCR2 (ref. 19). However, this transport is only efficient at high zinc concentrations, which suggests that these secondary active transporters have a lower zinc affinity than do AtHMA2/4.

AtHMA2 and AtHMA4 belong to the P1B-2 subgroup of heavy metal ATPases, which are common in prokaryotes and plants but absent in animals[7]. Plant cells have substantially higher inside-negative membrane potentials than do animal cells[20], which may be a reason why export of this micronutrient from plant cells cannot be sustained by proton antiporters alone when zinc is limiting, but requires the contribution of primary active transporters. The AtHMA2/4 homologue in *Hordeum vulgare* (barley) is HvHMA2, which functions as a plasma membrane-localized zinc exporter[21]. Expression analysis of laser capture microdissected developing barley grain showed that *HvHMA2* is predominantly expressed in the nucellar projection transfer cells[22], which

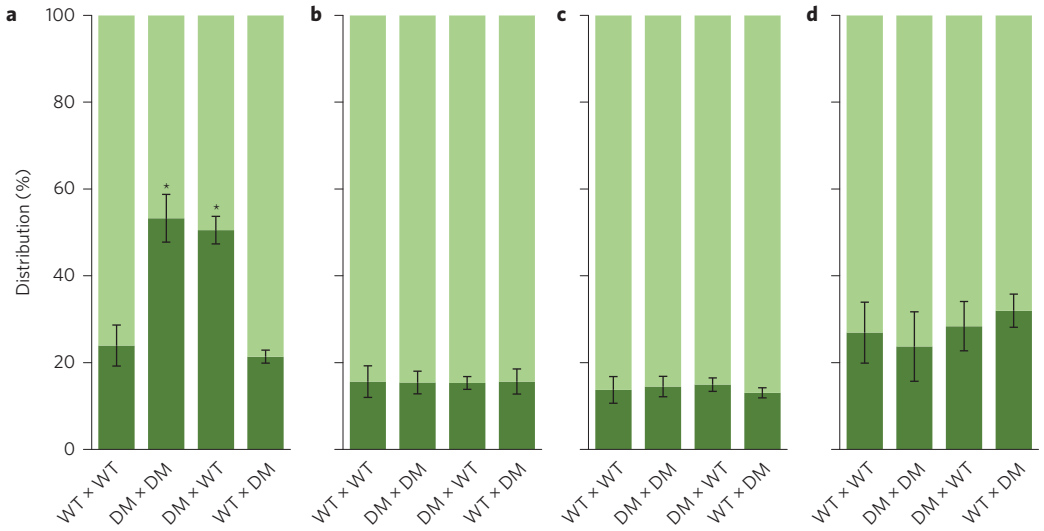

Figure 3 | Lack of zinc pumps in the seed coat but not in filial tissues blocks zinc loading into seeds. Microanalytical multi-elemental profiling of batches of ten seeds separated into seed coats and embryos. Bars represent the percentage of the total elemental content that is localized in the seed coat (dark green) and embryo (light green) fractions of seeds from crosses of plants grown at high zinc supply. Notations represent mother plant × pollen donor. WT, wild type; DM, *hma2,hma4* double mutant. **a**, Zinc; **b**, magnesium; **c**, phosphorus; and **d**, manganese. Data are means ± s.d. of eight to ten replicates, WT × DM of three replicates. Statistical analysis indicates significant difference from the wild type (*$p < 0.01$).

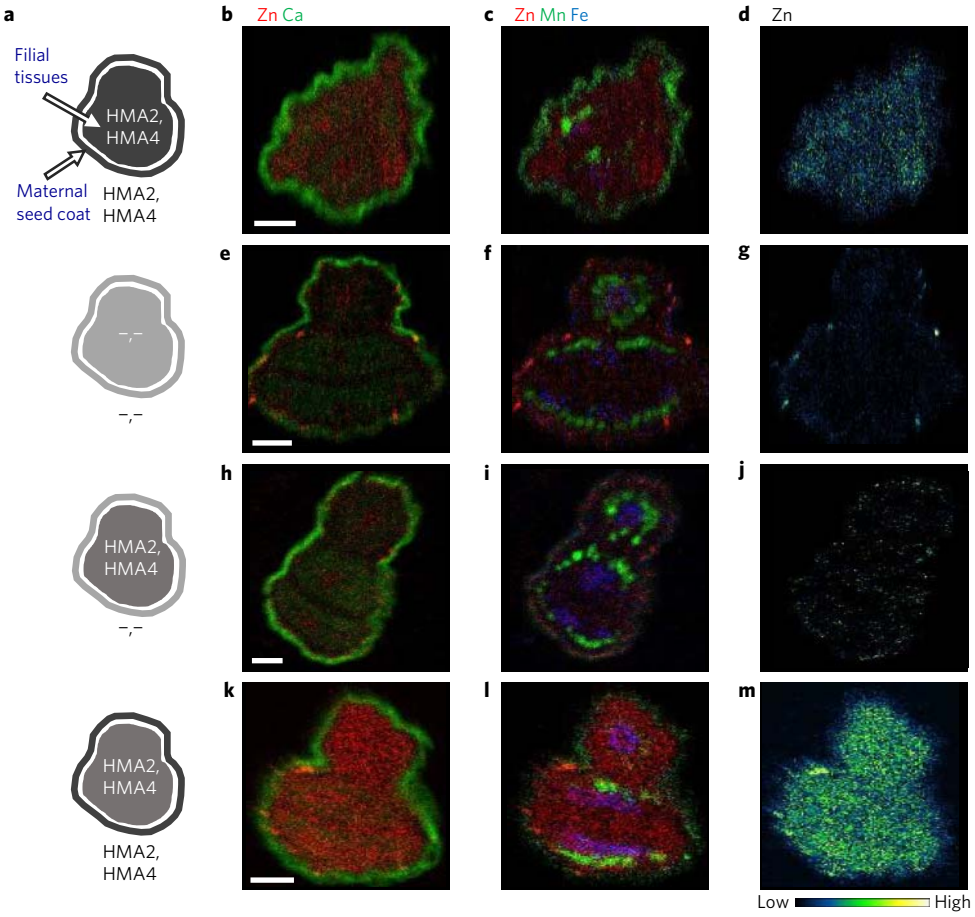

Figure 4 | Zinc export from the seed coat to filial tissues depends on zinc pumps in maternal tissues. μPIXE/RBS elemental profiles of transverse sections of seeds from crosses of plants grown at high zinc supply. **a**, Pictograms indicate presence of AtHMA2 and AtHMA4. Dark grey, AtHMA2 and AtHMA4 present (homozygous *WT*); intermediate grey, AtHMA2 and AtHMA4 present (hemizygous); light grey, AtHMA2 and AtHMA4 absent (homozygous *hma2, hma4*). **b**–**m**, Colour maps showing distribution of elements. Two-colour maps showing zinc (Zn) distribution in red and calcium (Ca) distribution in green (**b**,**e**,**h**,**k**). Three-colour maps showing zinc (Zn) distribution in red, manganese (Mn) distribution in green and iron (Fe) distribution in blue (**c**,**f**,**i**,**l**). Temperature colour maps displaying zinc distribution (**d**,**g**,**j**,**m**). Scale bars, 50 μm.

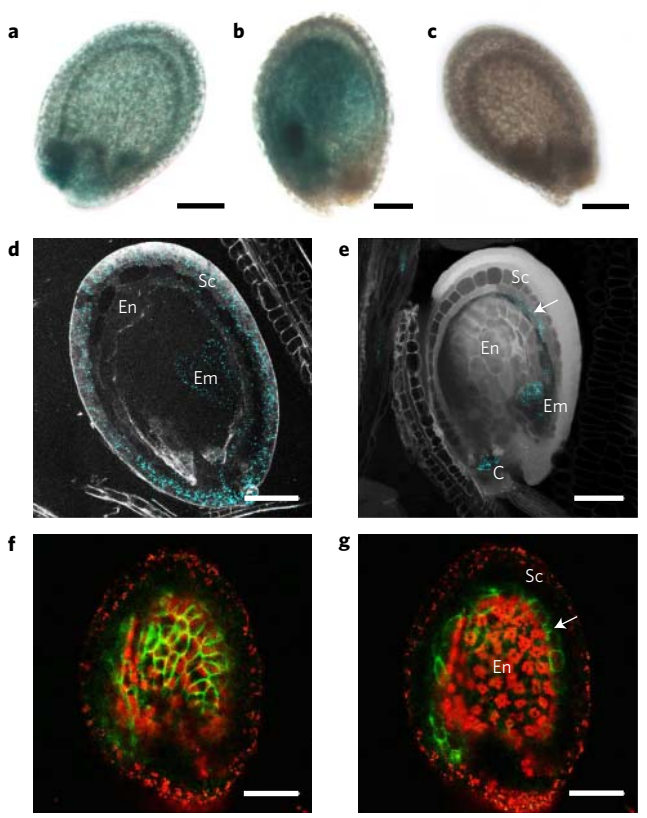

Figure 5 | *AtHMA2* and *AtHMA4* are expressed in developing seeds. a–e, Histochemical staining for GUS activity in developing seeds harbouring reporter gene fusions of the *AtHMA2* promoter (**a**,**d**) and *AtHMA4* promoter (**b**,**e**). (**c**) Wild-type seed. The images show whole seeds (**a**–**c**) and confocal laser scanning optical sections of modified pseudo-Schiff propidium iodide (mPS-PI)-stained seeds (**d**,**e**). **f**–**g**, Two different confocal laser scanning optical sections of the same developing seed expressing a fusion between a part of AtHMA4 and GFP under the control of the *AtHMA4* promoter. Green, GFP fluorescence; red, autofluorescence; C, chalazal region; En, endosperm; Em, embryo; Sc, seed coat. The arrow indicates the endothelium. Scale bars, 100 μm.

correspond to the endothelium of the seed coat in *Arabidopsis*. The homologue in rice is OsHMA2, which functions as a zinc transporter involved in the root-to-shoot translocation of zinc[23–25]. Whether HvHMA2 and OsHMA2 also play crucial roles in the developing grain remains to be investigated; however, it is conceivable that they would have similar functions as AtHMA2–4. In efforts to increase the zinc translocation capacity of plants, *HMA4* from either *A. thaliana* or *A. halleri* has been transgenically expressed in *Arabidopsis*, *Nicotiana tabacum* (tobacco) and *Solanum lycopersicum* (tomato), but with inconsistent results[14,15,26–29]. Notably, none of these studies made use of endogenous *HMA* promoters. Cellular mislocalization of HMA4 is likely to cause undesired export of zinc and interrupt the natural flow of zinc within the plant body. To attain this goal, the natural expression patterns of *HMA2* or *HMA4* should be retained. The finding that localization of AtHMA4 to the seed coat endothelium is crucial for export of zinc into the seed provides us with an essential handle in breeding efforts aimed at fighting hidden hunger.

Methods

Plant material. *A. thaliana* Columbia-0 was used as the wild type. The single mutants *hma2-4* and *hma4-2* and the double mutant *hma2-4,hma4-2* were as described[15]. Plant growth, cloning and plant transformation procedures for creating transgenic GUS and GFP plants are described in the Supplementary Methods.

Multi-elemental analysis. Samples were prepared for multi-elemental analysis as described in the Supplementary Materials and Methods. Multi-elemental analysis of seeds was performed using flow injection analysis (FIA) with the following parameters: ICPMS was set up with an injection volume of 50 μl, a flow of 0.2 ml min^{-1} and a mobile phase of 3.5% HNO_3. For the FIA, a Thermo ICS 5,000 DP pump and an Ultimate 3,000 UHPLC auto-sampler (Thermo Scientific) were used. A triple quad ICPMS (Model 8,800, Agilent Technologies) equipped with an Ari Mist HP nebulizer (Burgener Research International) was used. The ICPMS instrument was run in collision mode using helium as the collision gas; zinc, manganese, magnesium and phosphorus were analysed at m/z ratios of 66, 55, 24 and 31, respectively. Data were acquired and processed using the MassHunter 4.1 Chromatographic software package. For external calibration, a custom-made multi-element standard was used (P/N 4400-ICP-MSCS, CPI International). The standard is non-equimolar and corresponds to the ratio between elements typically found in plants. The digestion procedure and elemental analysis were validated using the certified reference material (CRM) NIST 1,515 Apple Leaf (National Institute of Standard and Technology). For magnesium, phosphorus, manganese and zinc, an accuracy of within ±10% was obtained for samples ranging in size from 200 to 2,000 μg ($n = 8$). *Arabidopsis* seeds were analysed in sample batches of ten intact seeds or ten fractionated seeds divided into seed coats and embryos, typically weighing from 50 to 200 μg. The elemental concentrations in these small sample batches were reported as nanograms of element per ten seeds. The combined masses of the elements measured in the seed coat plus embryo were within ±10% of the masses determined in the whole seed, indicating a negligible level of sample loss or contamination during the analytical procedure.

μPIXE/RBS. Seeds were immersed in a droplet of resin (OCT, Tissue Teck Sakura) and immediately cryo-fixed by plunging into isopentane cooled with liquid nitrogen. Seed cross-sections (30 μm) were done using a cryo-microtome (Leica) and freeze-dried (48 h, −52 °C, 0.01 mbar). Samples were analysed under vacuum at the Atomic Energy Commission nuclear microprobe (Saclay, France). The beamline was operated with a proton source at 3.03 MeV, with a beam focused to 3×3 μm^2 and a current intensity of 500 pA.

GUS analysis. Developing siliques were slit open longitudinally and stained for 3 h at 37 °C in GUS staining solution following 5 min of vacuum treatment. The GUS staining solution contained 50 mM $NaPO_4$ at pH 7.2, 3 mM ferricyanide, 3 mM ferrocyanide, 0.4% Tween20 and 2 mM X-GlcA. The samples were then cleared in 98% ethanol or fixed in fixing solution (50% methanol and 10% acetic acid) for modified pseudo-Schiff propidium iodide (mPS-PI) staining. The mPS-PI staining was as described[30]. Samples were examined using a Leica TCS SP5X confocal microscope with a 20× water immersion objective in sequential mode and were excited with a 488 nm argon laser. The propidium iodide emission signal was collected at 520–720 nm and the GUS reflection signal was collected at 485–491 nm (using the AOBS reflection mode). Cleared developing seeds were imaged by brightfield microscopy (Leica DM 5000B).

GFP analysis. To image developing seeds, siliques were slit open longitudinally and seeds were gently removed using a scalpel, mounted on a microscope slide and imaged immediately. Fluorescence microscopy was performed using an inverted point-scanning confocal microscope (Leica TCS SP5 II) with a 20× water immersion objective. Samples were excited with a 488 nm argon laser; emission was collected at 500–530 nm for GFP and at 660–700 nm for chlorophyll autofluorescence.

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Author contributions

L.I.O., T.H.H., C.L., J.T.H., J.L., S.S., S.C. and V.S. performed the experimental work. L.I.O., T.H.H., C.L., R.D.H., J.L., S.S., U.K., S.H. and M.P. performed data analysis. D.G., U.K., S.H. and M.P. oversaw project planning. L.I.O. and M.P. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional information

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Competing interests

The authors declare no competing financial interests.

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