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RESEARCH PAPER

Overexpression of *METAL TOLERANCE PROTEIN8* reveals new aspects of metal transport in *Arabidopsis thaliana* seeds

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ABSTRACT

- METAL TOLERANCE PROTEIN8 (MTP8) of *Arabidopsis thaliana* is a member of the CATION DIFFUSION FACILITATOR (CDF) family of proteins that transports primarily manganese (Mn), but also iron (Fe). MTP8 mediates Mn allocation to specific cell types in the developing embryo, and Fe re-allocation as well as Mn tolerance during imbibition. We analysed if an overexpression of *MTP8* driven by the CaMV 35S promoter has an effect on Mn tolerance during imbibition and on Mn and Fe storage in seeds, which would render it a biofortification target.
- Fe, Mn and Zn concentrations in *MTP8*-overexpressing lines in wild type and *vit1-1* backgrounds were analysed by ICP-MS. Distribution of metals in intact seeds was determined by synchrotron μXRF tomography.
- *MTP8* overexpression led to a strongly increased Mn tolerance of seeds during imbibition, supporting its effectiveness in loading excess Mn into the vacuole. In mature seeds, *MTP8* overexpression did not cause a consistent increase in Mn and Fe accumulation, and it did not change the allocation pattern of these metals. Zn concentrations were consistently increased in bulk samples.
- The results demonstrate that Mn and Fe allocation is not determined primarily by the *MTP8* expression pattern, suggesting either a cell type-specific provision of metals for vacuolar sequestration by upstream transport processes, or the determination of MTP8 activity by post-translational regulation.

INTRODUCTION

Iron (Fe) and zinc (Zn) deficiencies are widespread nutritional disorders in humans, affecting more than half of the world's population, especially in areas with plant-based diets (Stein, 2010). Besides supplementation and dietary diversification, genetic and agronomic biofortification are promising approaches to overcome this 'hidden hunger' (White & Broadley, 2009). Genetically biofortified crops are enriched in micronutrient density by traditional breeding or transgenic techniques (Bouis *et al.* 2011; Wiegmann *et al.* 2019). Overexpression of metal transporters is a promising way to increase Fe and Zn concentrations of edible parts of crops (Kailasam & Peiter, 2021). For instance, in cassava, Fe concentrations in roots and stems were increased upon expression of the *VACUOLAR IRON TRANSPORTER1* (*VIT1*) of *Arabidopsis thaliana* (Narayanan *et al.* 2015); in wheat, overexpression of *TaVIT2* under control of an endosperm-specific promoter resulted in a more than doubled Fe concentration in white flour (Connorton *et al.* 2017). Moreover, improved micronutrient accumulation in seeds can contribute to seedling vigour, abiotic and biotic stress resistance, and enhanced crop yields (Khoshgoftarmanesh *et al.* 2010; Mari *et al.* 2020). Thus, understanding the mechanisms of micronutrient allocation in the developing seed is of great importance.

In seeds of *A. thaliana*, Fe is concentrated around the embryo's provascular tissue. This storage pattern is dependent on VIT1, which mediates vacuolar Fe sequestration during seed development (Kim *et al.* 2006). An absence of VIT1 results in Fe co-localizing with manganese (Mn), which accumulates in cortical cells of the hypocotyl and subepidermal cells at the abaxial sides of the cotyledons (Chu *et al.* 2017; Eroglu *et al.* 2017). Although VIT1 is able to transport Mn in addition to Fe, the transporter responsible for the specific Mn distribution pattern is METAL TOLERANCE PROTEIN8 (MTP8) (Chu *et al.* 2017; Eroglu *et al.* 2017). In the absence of VIT1, MTP8, which also transports Fe, determines the altered Fe distribution; *vice versa*, when MTP8 is absent, VIT1 mediates a Mn localization around the provascular tissue. Double mutants lacking both VIT1 and MTP8 have dispersed Mn and Fe localization in seeds, confirming that VIT1 and MTP8 can substitute for each other. However, whereas *VIT1* is active from early

stages of seed development, *MTP8* expression, responsible for the vacuolar Mn storage, is confined to later developmental stages, starting from the green cotyledon stage (Eroglu *et al.* 2017).

Apart from their role in human nutrition, micronutrient seed stores are particularly important during seed germination and for seedling vigour, since they supply the developing seedling with nutrients (Andresen *et al.* 2018). While VIT1 is responsible for the import of Fe into vacuoles, the metal is remobilized by NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN3 (NRAMP3) and NRAMP4 at the very beginning of germination, which is necessary for optimal seedling growth (Lanquar *et al.* 2005; Bastow *et al.* 2018). Therefore, VIT1 and NRAMP3 and 4 constitute a functional import/export module (Mary *et al.* 2015). Mis-localization of Fe by a mutation in *VIT1* rescued the Fe deficiency-sensitive phenotype of *nramp3nramp4* mutants.

Like Fe, Mn stored in vacuoles during seed development provides an important resource for the germinating seed (Otegui *et al.* 2002; Eroglu *et al.* 2017). Notably, besides its role in Mn storage during seed development, MTP8 is involved in Fe reallocation to the subepidermal layer from the vasculature during imbibition and early germination. Furthermore, because of its expression in the mature seed and during early germination, MTP8 is also relevant for Mn tolerance during imbibition (Eroglu *et al.* 2017).

The MTPs belong to the Cation Diffusion Facilitator (CDF) family, which is divided in three subgroups, with transporters being either specific to Mn and Fe, Fe and Zn or Zn alone, with most members of the family transporting more than one transition metal (Andresen *et al.* 2018; Alejandro *et al.* 2020). MTPs have been proposed as a potential tool for biofortification (Ricachenevsky *et al.* 2013). A successful enhancement of the nutritional value of grains by overexpression of an *MTP* was achieved in barley (Menguer *et al.* 2017). Thereby, expression of the vacuolar Zn transporter *HvMTP1* in developing barley grains by using an endosperm-specific promoter resulted in increased Zn concentrations in grains, where the metal accumulated in the endosperm.

Based on its involvement in Mn and Fe homeostasis in developing and germinating seeds, we hypothesized that an overexpression of *MTP8* can confer tolerance to high Mn concentrations during imbibition and bring about an increase in Mn and Fe concentration in the seed. The latter would render this transporter a promising target for genetic biofortification. Since high-resolution synchrotron micro X-ray fluorescence (μXRF) tomography provides direct information about the impact of transporters on metal localization (Punshon *et al.* 2013), we employed this technique to investigate the metal distribution in dry seeds of *MTP8* overexpressors, as well as determining bulk metal concentrations. To investigate a potential interference of *VIT1*, overexpressor lines of *MTP8* in a *vit1* knockout background were analysed in parallel.

MATERIAL AND METHODS

Plant material and growth conditions

The transgenic lines 35S:MTP8#OX2 and 35S:MTP8#OX4 of *A. thaliana* have been described previously (Eroglu *et al.* 2016), as well as the *vit1-1* mutant and the *mtp8-1vit1-1* double mutant (Eroglu *et al.* 2017). To obtain *vit1-1*x35S:MTP8#OX2 and 35S:MTP8#OX4x*vit1-1* double mutants, *vit1-1* was crossed with either 35S:MTP8#OX2 or 35S:MTP8#OX4, and homozygous plants were selected by PCR in the F_2 progeny. Plants were cultivated in a standardized soil (ED73; Einheitserde Werkverband, Sinntal-Altengronau, Germany) mixed with 1/3 (v/v) vermiculite (Kammlott, Erfurt, Germany) and placed in the greenhouse under long-day conditions with supplemental lighting (16 h light period). BioMükk (BioFa, Münsingen, Germany) was added to the soil/vermiculite mixture at a concentration of 10 $g \cdot l^{-1}$.

For germination assays, seeds were surface-sterilized with 70% ethanol (1 min) and a solution of 33% NaClO and 0.02% Triton X-100 (5 min), then rinsed four times with sterile water. Sterile seeds were imbibed in distilled water containing 0, 5 or 10 mM $MnCl_2$ and incubated for 3 days at 4 °C in the dark. Thereafter, seeds were rinsed three times with distilled water and sown on ½ strength Murashige and Skoog (MS) medium (M0231; Duchefa Biochemie, Haarlem, the Netherlands) containing 8 $g \cdot l^{-1}$ agar (Phyto Agar P1003; Duchefa Biochemie) with pH adjusted to 5.8. Plants were grown under long-day conditions (16 h light period, 22 °C; 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$; 8 h dark period, 18 °C) and a constant relative humidity of 65%. Percentage germination was recorded after 7 days.

Quantitative RT-PCR

For expression analyses in seeds, around 50 mg of seeds at different development stages were harvested and ground in liquid nitrogen. RNA was extracted with a RNeasy plant mini kit (Qiagen, Hilden, Germany), and 1 μg RNA was transcribed into cDNA using SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA, USA) and random hexamer primers. Realtime PCR was carried out in a realplex^4 MasterCycler system (Eppendorf, Hamburg, Germany) using POWER SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). *MTP8* expression levels were determined against a cDNA standard curve from a dilution series and normalized to *ACTIN2* (At3g18780) as constitutively expressed control.

Determination of metal concentrations

Dried seeds were weighed into PTFE digestion tubes and digested with HNO_3 using a microwave digester (UltraCLAVE IV; MLS-MWS, Leutkirch, Germany). Elemental composition was analysed by sector-field high-resolution ICP-MS (ELEMENT 2; Thermo Fisher Scientific, Bremen, Germany).

Micro X-ray fluorescence (μXRF) tomography

Intact dried seeds were placed on top of a Kapton capillary and samples were kept frozen during the measurements using a cryostream to avoid beam damage. The seeds were analysed at beamline P06 (PETRA III) at DESY (Deutsches Elektronen-Synchrotron, Hamburg, Germany) using the Maia detector, as described previously (Mishra *et al.* 2016). Briefly, the X-ray beam was generated in an undulator, monochromatized using a cryogenically cooled Si(111) double crystal monochromator at 12 keV, and focused with Kirkpatrick-Baez mirrors to approximately 400 × 500 nm^2 spot size. A 384-element Maia detector in backscatter geometry was used to measure X-ray

fluorescence photons emitted from the sample. The intensity of the transmitted radiation was monitored with a passivated implanted planar silicon (PIPS) diode behind the sample. The sample was cooled with a cryostream (Oxford Cryosystems, Oxford, UK) from the top to about 100 K. Single-slice tomograms were measured by scanning lines across the sample at various rotation angles. This was done with a step size of 0.5 μm and a typical dwell time of 1–3 ms per step in each line, and 0.1° between lines, yielding a 360° tomogram. The resulting tomograms were reconstructed using the filtered back projection (FBP) algorithm as implemented in the scikit-image image processing library for Python. Quantification with tomographic standards, including absorption correction and further image processing (smoothing, contrast, colour scales), was performed in ImageJ.

RESULTS

Overexpression of *MTP8* in seeds

It has previously been shown that a knockout of *MTP8* resulted in altered Mn and Fe homeostasis during seed development and germination (Eroglu *et al.* 2017). To investigate whether an overexpression of *MTP8* in *A. thaliana* can confer tolerance to toxic Mn concentrations during imbibition and an increase in seed Mn and Fe concentrations, we first analysed, by qRT-PCR, if *MTP8* is overexpressed in developing and mature seeds of 35S:MTP8 lines (Fig. 1). In the wild type (WT), expression of *MTP8* increased during seed development from 5 days after flowering to mature seed, as has been described before (Eroglu *et al.* 2017). Additionally, in both overexpressors, expression of *MTP8* was strongly increased up to 50-fold as compared to the WT in all tested development stages of the seeds.

Germination after imbibition at toxic Mn levels

Since knockout of *MTP8* causes a hypersensitivity of imbibed seeds to high Mn concentrations (Eroglu *et al.* 2017), we examined if an overexpression can improve Mn tolerance at this stage. Seeds were imbibed with different Mn concentrations for 3 days at 4 °C and plated on ½ MS agar plates after washing. While the germination rate of WT seeds was reduced to around 50% at 5 mM Mn and almost completely abolished at 10 mM Mn, both overexpressor lines were able to maintain germination, even when exposed to 10 mM Mn, albeit germination rates differed between the two overexpressor lines (Fig. 2). 35S:MTP8#OX4 maintained the germination rate completely, while germination of 35S:MTP8#OX2 was affected under toxic levels of Mn. *MTP8* overexpressor lines in the *vit1-1* background showed a similar pattern.

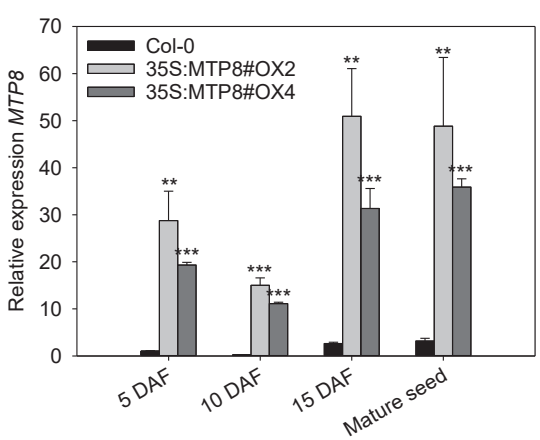

Fig. 1. Expression of *MTP8* in *A. thaliana* seeds is increased in 35S:MTP8 lines. Plants were grown on soil, and seeds were harvested from developing and mature siliques. Expression of *MTP8* was determined by quantitative RT-PCR in seeds at different development stages. Data represent mean ± SE. N = 4 biological replicates. DAF, days after flowering. ** and *** indicate statistically significant differences to Col-0 at $P < 0.01$ and $P < 0.001$, respectively, according to Student's *t*-test.

Metal concentrations in bulk seeds

It has been shown before that *MTP8*-overexpressing lines accumulate more Mn in roots compared to the WT (Eroglu *et al.* 2016). We investigated, using ICP-MS, whether Mn and Fe concentrations in seeds are increased by constitutive ectopic overexpression of *MTP8*. Mn concentration was increased in seeds of line 35S:MTP8#OX4 but decreased in seeds of 35S:MTP8#OX2 as compared to the WT (Fig. 3a). Fe concentration was slightly reduced in the former and unaffected in the latter line. In *MTP8*-overexpressing lines in the *vit1* background, again no consistent results concerning Mn and Fe concentrations were observed (Fig. 3b). *vit1-1*x35S:MTP8#OX2 showed slightly elevated Mn and Fe concentrations, whereas the opposite was the case for 35S:MTP8#OX4x*vit1-1*. The only element whose concentration was consistently increased in seeds of all *MTP8* overexpressor lines was Zn, although MTP8 has not previously been characterized as a Zn transporter (Eroglu *et al.* 2016).

Metal localization in seeds

In seeds, MTP8 governs the distribution of Mn, and also of Fe in the absence of VIT1 (Chu *et al.* 2017; Eroglu *et al.* 2017). We therefore investigated, by synchrotron μXRF tomography, whether overexpression of *MTP8* under the constitutive CaMV 35S promoter affects the allocation of both metals. In the WT, Mn was concentrated mainly in subepidermal cells on the abaxial sides of the cotyledons and the hypocotyl cortex, whereas Fe was localized around the provascular tissues in WT seeds, which confirmed previously published results (Fig. 4a). In 35S:MTP8 seeds, the main accumulation sites of Fe and Mn were not changed (Fig. 4a), although *MTP8* expression was strongly increased (Fig. 1) and activity of the 35S promoter was not confined to those sites. However, in the tomograms, concentration of Fe was increased in the accumulation sites, and an increased Mn accumulation in the seed coat was also observed. These increases were not reflected in the bulk seed analyses (Fig. 3), which may be explained by the fact that the tomography only captures a single slice of an individual seed that may not be representative of the bulk. In all cases, including the WT, Zn was accumulated throughout the whole tissue (Fig. 4). This accumulation increased with *MTP8* overexpression, especially in line 35S:MTP8#OX4, and an additional accumulation in the seed coat was observed. This observation is in accordance with increased Zn concentrations in all lines overexpressing *MTP8* as determined by ICP-MS measurements (Fig. 3).

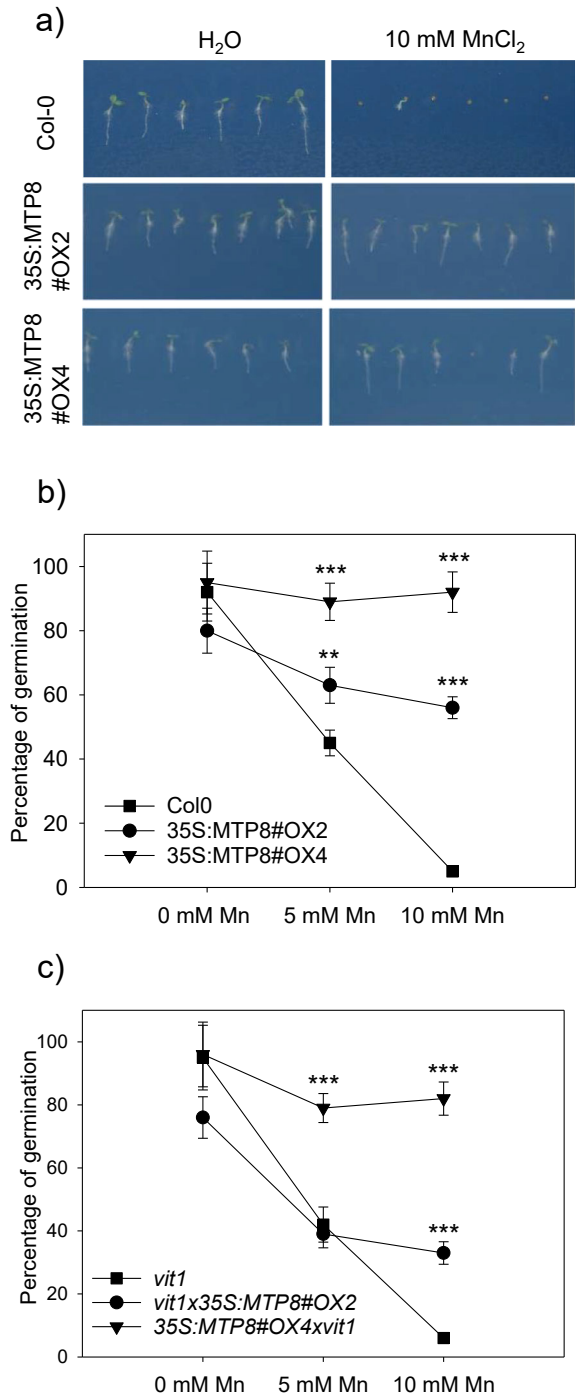

Fig. 2. Overexpression of *MTP8* in *A. thaliana* improves tolerance to imbibition at high Mn concentrations. Seeds were imbibed in either water or Mn concentrations as indicated for 3 days at 4 °C. After imbibition, seeds were washed with distilled water, sown on ½ MS agar plates containing 38 μM $MnSO_4$, and grown for 4 days before images were taken. Germination tests were performed on four ½ MS agar plates per treatment and genotype, containing 40 seeds per plate. (a) Growth of Col-0 and *MTP8* overexpressor lines after Mn imbibition. (b) Percentage of germination after imbibition. Germination rate was recorded 9 days after sowing. Error bars show ±SD of mean (N = 4 replicate plates with 40 seeds per plate). ** and *** indicate statistically significant difference to Col-0 at $P < 0.01$ and $P < 0.001$, respectively, according to Student's *t*-test.

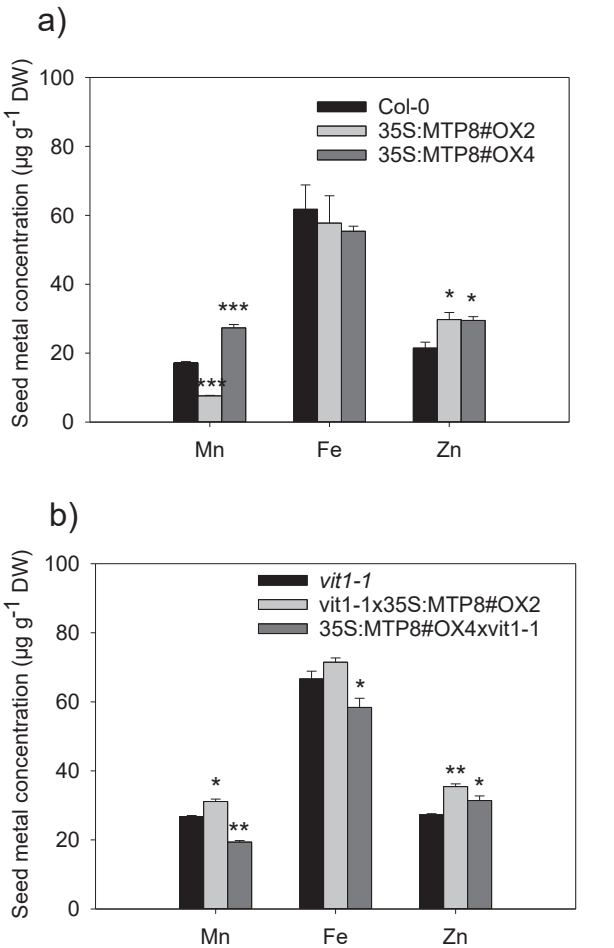

Fig. 3. Metal concentrations in seeds of *MTP8 A. thaliana* overexpressor lines. Mn, Fe and Zn concentrations seeds obtained from 35S:MTP8 plants (a) and 35S:MTP8x*vit1* plants (b). Metal concentrations were analysed by ICP-MS. Data represent mean ± SE. N = 4 biological replicates. *, **, and *** indicate statistically significant difference to Col-0 at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to Student's *t*-test.

To exclude that VIT1 interfered with MTP8-mediated metal allocation in the overexpressors, we analysed metal localization in the *vit1-1* background (Fig. 4b). In all lines lacking VIT1, the Mn localization pattern was unchanged to that in the WT background. However, in those cases Fe was co-localized with Mn. Again, ectopic overexpression of *MTP8* did not affect the distribution of these metals.

DISCUSSION

Overexpression of *MTP8* improves seed germination after imbibition at toxic Mn concentrations

Plants employ two strategies to keep the cytosolic concentration of weakly bound Mn ions low: (i) exclusion of Mn from the cytosol *via* plasma membrane transporters or exocytosis and (ii) sequestration in the vacuole (Peiter *et al.* 2007; He *et al.* 2021). The *A. thaliana MTP8* overexpression lines 35S:MTP8#OX2 and 35S:MTP8#OX4, which have an approximately 7000- and 3000-fold increased expression of *MTP8* in

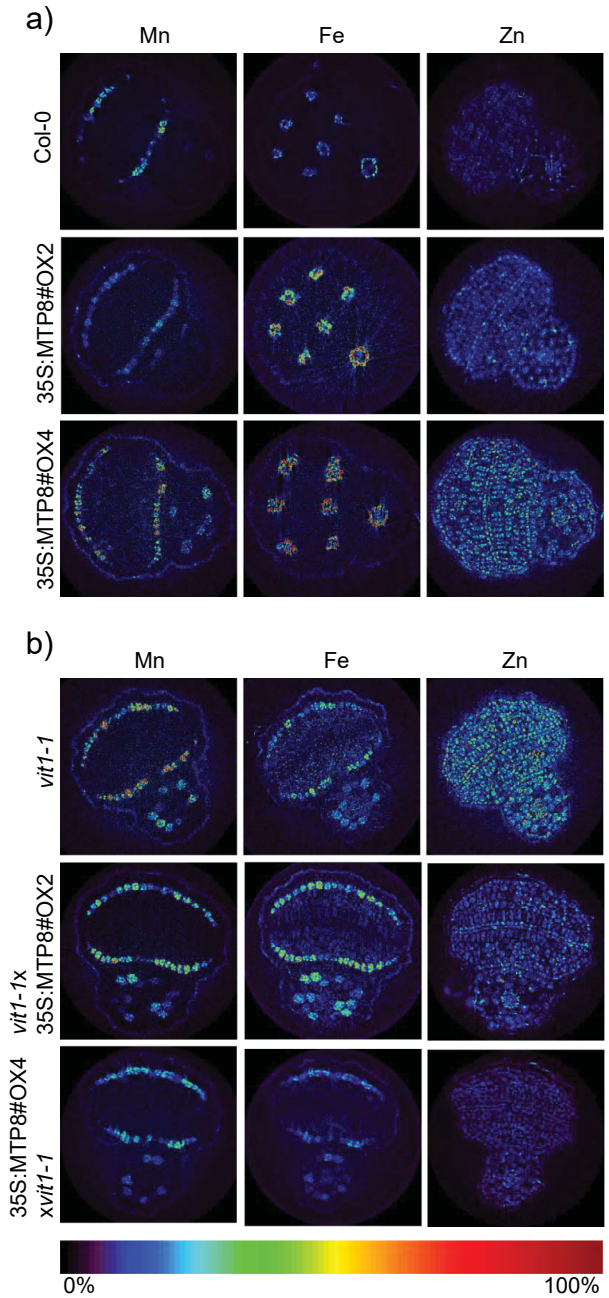

Fig. 4. Metal distribution in *A. thaliana* seeds; (a) Col-0 and 35S:MTP8 lines; (b) *vit1-1* and 35S:MTP8x*vit1-1* lines. µXRF tomographs of intact seeds showing the distribution of Fe, Mn and Zn. The colour scale ranges from 0 to 500 $\mu g \cdot g^{-1}$ for all metals.

roots compared to the WT, accumulated more Mn in roots by sequestration into the vacuole, while overexpression had no impact on Fe accumulation (Eroglu *et al.* 2016). In the current study, an overexpression of *MTP8* by around 40–50-fold was found in developing and mature seeds of those lines (Fig. 1). This overexpression led to improved germination and seedling vigour after imbibition at high Mn levels (Fig. 2). Especially under reducing conditions, *e.g.* waterlogging, Mn levels can strongly increase in the soil (Alejandro *et al.* 2020). This represents a challenge to rehydrating seeds prior to germination, and higher expression of *MTP8* enhances tolerance by sequestering Mn out of the cytosol into the vacuole. Endogenous expression of *MTP8* is high during imbibition and declines during germination (Eroglu *et al.* 2017), supporting the specific role of MTP8 at this early stage of a plant's life, which can be further enhanced by its ectopic overexpression.

Effects of *MTP8* overexpression on metal accumulation and distribution in seeds

The accumulation of micronutrients in seeds is of great importance for seedling vigour and nutritional value (Eggert & von Wirén, 2013). The vacuole represents an important store for Fe and Mn in the seed, whereby VIT1 and NRAMP3/4 constitute a functional module for Fe storage and remobilization (Mary *et al.* 2015), while MTP8 is responsible for vacuolar Mn storage (Chu *et al.* 2017; Eroglu *et al.* 2017). However, VIT1 and MTP8 can compensate for each other because of their ability to transport both Mn and Fe (Eroglu *et al.* 2017). Since *MTP8* is expressed under control of the strong 35S promotor in the overexpressor lines, we expected a homogeneous overaccumulation of Mn and Fe in all tissues of the seed. However, we neither observed a consistently increased accumulation nor a different distribution pattern of Fe and Mn in *MTP8* overexpressors compared to the WT or to the *vit1-1* mutant (Figs 3 and 4). The supply of the embryo relies on the import through its epidermis, following the release into the apoplastic space by the outer integument and the endosperm (Tegeder, 2014). The absence of a robust effect of *MTP8* overexpression on Fe and Mn allocation, even in seeds devoid of VIT1, may be explained by different scenarios. A lack of the vacuolar transporter's substrate, *i.e.* Mn or Fe, would render the transporter irrelevant. Such a substrate limitation is the likely reason why, in root cells overexpressing *MTP8*, only Mn, but not Fe, is overaccumulated (Eroglu *et al.* 2016). Hence, not only sink strength generated by the vacuolar transporters, but also upstream steps of transport may determine metal distribution in seeds. Such hierarchies have been described for other transport processes acting in series. For example, the Mn transporter NRAMP2, operating in the *Trans*-Golgi Network, acts epistatically to the vacuolar NRAMP3/4 in providing Mn to the chloroplast (Alejandro *et al.* 2017). Within chloroplasts, Ca and Mn movement by the thylakoid transporter BICAT1 requires the upstream activity of BICAT2 in the envelope (Frank *et al.* 2019), representing another such genetic interaction.

A sequence of transport steps prior to the metal's arrival at the tonoplast may therefore entail that Mn and Fe are not equally available for all cell types in the developing seed. Such a mechanism may be causal to the finding that in *mtp8vit1* double knockout lines, Mn is still not completely evenly distributed (Eroglu *et al.* 2017). However, the entire embryo is symplastically continuous, with only the provascular system becoming isolated at later stages (Stadler *et al.* 2005). This arrangement renders it unlikely that upstream transport processes within the embryo determine the location of metal storage.

In an alternative scenario, metals may be freely motile and *MTP8* expression evenly distributed within the embryo in overexpressor lines, yet MTP8 may only be active in certain cell types and underlie post-translational regulation mechanisms determining its targeting and/or function. Ineffectiveness of overexpression has been observed before, such as in the small

GTPase RabA2 in *Phaseolus vulgaris* (Blanco *et al.* 2009) or the ferric chelate reductase FRO2 in *A. thaliana* (Connolly *et al.* 2003). In *MTP8* overexpressors, MTP8 might be targeted to the vacuole only in cortical cells of the hypocotyl and abaxial subepidermal cells of the cotyledons. A regulation of transport activity *via* the transporter's insertion in the target membrane has been established as a common mechanism in metal transporters (Agorio *et al.* 2017; Dubeaux *et al.* 2018) and should be examined in this case. Cell type-specific targeting and function may be brought about by essential interacting proteins only present in the respective cell types. One class of interacting proteins are protein kinases, and the regulation of MTP8 by phosphorylation has recently been shown (Zhang *et al.* 2021). It still needs to be established if such potential mechanisms regulate MTP8 in *Arabidopsis* seeds.

We observed a rather unexpected phenotype of the *MTP8* overexpressor lines. Both lines accumulated 10–20% more total Zn in seeds than the WT (Fig. 3). Since the introduction of MTP8 in the Δ*zrc1* yeast mutant did not lead to a complementation of its Zn-hypersensitive phenotype, it is believed that MTP8 is not able to transport Zn (Eroglu *et al.* 2016). On this background, the current results may be explained in two ways. (i) The yeast complementation did not sufficiently resemble the situation in plants, *i.e.* MTP8 might actually transport Zn in plants. There could be many reasons for such a discrepancy, including different competing ligands inside the cells. (ii) Alternatively, the higher Zn accumulation might be an indirect effect of *MTP8* overexpression. This is supported by the fact that a knockout of *MTP8* did not cause a change in seed Zn concentration, while a knockout of the Fe transporter VIT1 did lead to increased seed Zn levels (Eroglu *et al.* 2017). Except for the unloading of Zn from the mother-plant tissue by heavy metal ATPases (Olsen *et al.* 2016), the Zn transporters responsible for accumulating Zn in seeds of *A. thaliana* are largely obscure, and the role of MTP8 also requires further elucidation in this respect.

Taken together, the current study demonstrated that the expression level of *MTP8* in *A. thaliana* determines the resistance of imbibing seeds to Mn. However, the concentration and distribution of Fe and Mn in seeds may not be primarily regulated by *MTP8* expression strength, whereby potential mechanisms of metal conduction in the embryo and post-translational regulation of MTP8 remain to be established.

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