CHAPTER FOUR

Metal Transport in the Developing Plant Seed

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Contents

Abstract

Healthy plant growth depends on a balanced metal homeostasis at the organ, tissue and sub-cellular levels, which is mediated principally by plasma and vacuolar membrane metal transporters. The genetic bases of metal acquisition in developing seeds has long remained poorly understood. Recent technical advances have helped circumvent the difficulties of conducting metal nutrient research on the extremely small seeds of *Arabidopsis thaliana*. The review presents recent advances in our understanding of seed metal homeostasis focussing on this model plant. Metals are loaded from phloem to the seed coat and must pass through the endosperm to reach the embryo. The embryo comprises several apoplastic and symplastic pathways that strictly depend on the changing physiology of the developing seed organs. Metals that reach the developing embryo fuel immediate cellular processes or accumulate in vacuoles to support forthcoming germination. In the mature embryo, metal distribution is homogeneous, with the exception of iron and manganese which localize to distinct cell layers. These metal localizations are strictly dependent on expression of specific tonoplast transporters, with putative functions that go beyond the storage of metals. Accumulating evidence indicates that they can control the timing of metal entry into the embryo.

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1. GENERAL PRINCIPLES IN PLANT METAL HOMEOSTASIS

A healthy plant growth requires a continuous supply of metal nutrients. In plant cells, metals activate enzymes, contribute to protein synthesis and function as signalling molecules. Particularly, transition metals mediate electron transport reactions, such as photosynthesis, respiration, and detoxification of reactive oxygen species, due to their ability to take several different charges (Andresen, Peiter, & Küpper, 2018). In hyperaccumulator species, metals can accumulate in leaf vacuoles at concentrations that are poisonous for insects, thereby contributing to the protection of the plant from herbivory (Boyd, 2007). However, meeting the demand for metals first requires the scavenging of positively charged metal cations from the negatively charged soil particles and subsequently their mobilization from roots to sink tissues. Whenever plants fail to provide sufficient amounts of metals to sink tissues, growth is restricted. In agriculture, plants often cannot meet the need for metals (e.g. iron and zinc), making metal deficiencies one of the most widespread problems of agricultural production.

Membrane metal transporters determine the majority of metal content in plant organs, tissues, and organelles. For example, whether ferroportin2 (FPN2) transporter is truncated or full length can account for differential cobalt levels in shoots of distinct *Arabidopsis thaliana* natural accessions (Morrissey et al., 2009). How plants gain metal hypersensitivity (Castaings, Caquot, Loubet, & Curie, 2016; Gao et al., 2017) or hypertolerance (Morrissey et al., 2009) can often be explained by assessing a change in activity of their membrane transporters. Comparison of hypertolerants with their non-hypertolerant relatives shows a striking difference in the expression of metal transporters upon metal stress. For example, in roots under cadmium stress, *cation exchanger1* (*CAX1*) of hypertolerant *Arabidopsis halleri* was upregulated 15 times more than in *A. thaliana* (Baliardini, Meyer, Salis, Saumitou-Laprade, & Verbruggen, 2015; Becher, Talke, Krall, & Krämer, 2003). Sufficient amounts of metals must be taken up from the soil and transported to the sink tissues in order to complete the plant's life cycle. Metal transport from the soil to the seeds involves (i) root acquisition, comprising uptake from the rhizosphere to the cytosol of epidermal and cortex cells of the root, (ii) long-distance transport, including loading from root pericycle into the xylem, root-to-shoot transport via the xylem and its subsequent unloading, (iii) remobilization from source (e.g. leaves) to sink organs (e.g. developing seeds) via the phloem and (iv) transport within the seed,

including translocation from maternal to filial tissues and storage. At each of these steps, metals must pass through several membranes with the help of diverse families of metal transporters.

Metal transporters belong to several protein families such as the natural resistance-associated macrophage proteins (NRAMPs), the metal-tolerance proteins (MTPs), the vacuolar iron transporter-like family (VTLs), the ZRT/IRT-like proteins (ZIPs) and yellow stripe1-like family (YSLs). NRAMPs transport divalent cations, particularly Mn^{2+} and Fe^{2+} into the cytosol (Thomine, Wang, Ward, Crawford, & Schroeder, 2000). Their primary physiological function appears to be maintaining iron (Fe) and manganese (Mn) homeostasis. Particularly, NRAMPs are associated with the acquisition and radial transport of Fe and Mn from the outer to inner parts of the root (Castaings et al., 2016) and their remobilization from the vacuole (Mary et al., 2015). MTPs localize to the tonoplast to detoxify excess of metals from the cytosol by sequestering them into the vacuole (Delhaize, Kataoka, Hebb, White, & Ryan, 2003). According to their type, MTPs principally catalyze Mn, Mn/Fe or zinc (Zn) transport (Montanini, Blaudez, Jeandroz, Sanders, & Chalot, 2007). MTP expression profile often overlaps with the presence of large concentrations of metals in vacuoles of different tissues such as shoots of hyperaccumulators (Gustin et al., 2009; Persans, Nieman, & Salt, 2001), the metal-rich aleurone layer of wheat kernels (Vatansever, Filiz, & Eroglu, 2017) or the Mn-rich subepidermis of *A. thaliana* embryos (Eroglu, Meier, von Wirén, & Peiter, 2016). Thus, MTPs are good targets for developing biofortification strategies due to their metal sequestration capacity (Ricachenevsky, Menguer, Sperotto, Williams, & Fett, 2013). vacuolar iron transporter1 (VIT1) is an ortholog of yeast tonoplast Fe/Mn transporter CCC1 (Kim et al., 2006; Li, Chen, Ward, & Kaplan, 2001). Both VIT1 and a group of proteins that share homology with VIT1, the family of VTLs, transport Fe and Mn into the vacuole and participate in Fe homeostasis (Gollhofer, Timofeev, Lan, Schmidt, & Buckhout, 2014). The ZIP family comprises 15 members in Arabidopsis, which transport Zn, Fe, Mn and Cu (Milner, Seamon, Craft, & Kochian, 2013). Several ZIP homologs are induced by Zn or Fe deficiency, suggesting their involvement in homeostasis of these metals (Waters & Sankaran, 2011). Although a number of ZIP proteins have not been yet characterized, a protein belonging to this group, iron regulated transporter1 (IRT1), represents one of the most extensively characterized metal transporters and is reviewed elsewhere (Brumbarova, Bauer, & Ivanov, 2015; Jeong, Merkovich, Clyne, & Connolly, 2017). YSLs transport metals that are chelated with nicotianamine (NA), a nonpeptidyl chelation agent. NA is found in phloem sap and can form

stable complexes with metals under neutral or slightly basic pH, suggesting that it is the principal metal chelator in the phloem (Stephan & Scholz, 1993; von Wirén et al., 1999). Thus, the crucial role of YSLs in long-distance transport and seed metal loading is emerging (Curie et al., 2009; Waters et al., 2006). Members of the oligopeptide transporter (OPT) family are characterized as peptide carriers and share a considerable homology with YSLs (Koh et al., 2002). In contrast to other members of the family which generally carry glutathione or glutathione complexes (Wongkaew et al., 2018), OPT3 transports Fe ions (Zhai et al., 2014).

Acquisition of Fe by the root is well understood and provides a good example of how transporters operate cooperatively to retrieve metals from the rhizosphere. First, Fe has to be mobilized, as most of the Fe in soil is immobile due to its precipitation in sparingly soluble oxides. Fe is mobilized through acidification by the activity of proton pumps, particularly AHA2 (Santi & Schmidt, 2009), and chelation by low molecular weight compounds, such as coumarins, which are secreted into the rhizosphere (Schmid et al., 2014; Tsai & Schmidt, 2017). The mobilized ferric Fe [Fe(III)] diffuses in the apoplast, followed by the reduction of Fe(III) to ferrous Fe [Fe(II)] via plasma membrane-bound ferric chelate reductase enzyme (Robinson, Procter, Connolly, & Guerinot, 1999) and coumarins (Rajniak et al., 2018; Sisó-Terraza et al., 2016). From the apoplast, Fe enters the cytosol of epidermal and cortex cells through divalent cation transporter IRT1. IRT1 is the only high-affinity Fe transporter involved in root Fe acquisition (Korshunova, Eide, Clark, Guerinot, & Pakrasi, 1999). In contrast to IRT1, NRAMP1 and perhaps other low-affinity plasma membrane transporters contribute to Fe transport provided that extracellular Fe concentration is sufficiently high (Castaings et al., 2016).

IRT1 shows a broad range of affinity for divalent cations, which has direct consequences on Fe acquisition and general plant health. While taking up Fe, IRT1 also non-specifically takes up other metals as indicated by the strong correlation of IRT1 expression with accumulation of Mn, Co, Zn, Cd, and Ni in the root (Korshunova et al., 1999; Nishida et al., 2011). This poses a heavy metal stress unless these metals are detoxified by sequestration into the vacuole (Thomine & Vert, 2013). Vacuolar sequestration is mediated by tonoplast-localized metal transporters that belong to diverse families (Arrivault, Senger, & Krämer, 2006; Eroglu et al., 2016; Morrissey et al., 2009; Schaaf et al., 2006). Interestingly, expression of these transporters is tightly connected to that of IRT1 by a common transcription factor, FER-like iron deficiency induced transcription factor (FIT), suggesting that

heavy metal intake concomitant with Fe intake is coupled with subsequent sequestration of the former into the vacuole (Colangelo & Guerinot, 2004). In addition, similar to a safety fuse, Fe accompanying metals may shut down Fe acquisition machinery to curb further intake. This can be achieved either by inhibiting IRT1 (Dubeaux, Neveu, Zelazny, & Vert, 2018) or ferric chelate reductase activity (Eroglu et al., 2016). Therefore, strategies in the roots provide a perfect example of how several transporters and other helper proteins have to work in concert, to ensure that an adequate amount of Fe is taken up while the inhibitory or toxic effects of other metals are suppressed.

In the cytoplasm, free Fe triggers oxidative stress through Fenton reactions; therefore, most of the Fe should be kept chelated. Fe species differ at the tissue and subcellular levels, depending on the availability and concentration of their chelators and the stability of complexes in that particular pH. In plastids, Fe is stored in protein nanocages called ferritins which are suggested to release Fe for photosynthetic reactions (Briat, Duc, Ravet, & Gaymard, 2010; Harrison & Arosio, 1996).

Plant embryos are young plants that do need metals for development. Unlike mature plants, they do not possess functional roots; thus, the developing embryo completely depends on the metal flow that is derived from the maternal tissues. Knowledge of seed metal homeostasis at the molecular level has been very poor until recently, due to technical difficulties in quantifying metals in small seeds of Arabidopsis. However, recent technical advances now permit measurements in these organs with higher accuracy and increased resolution. This review will discuss recent advances in seed metal nutrient homeostasis focussing on Arabidopsis.

2. ARABIDOPSIS SEED METAL HOMEOSTASIS

Metal translocation from the mother plant to the seed and its subsequent distribution within the seed is critical for successful germination and for food safety. Traditionally, seed metal nutrient research has ignored Arabidopsis, since the tools and techniques that were available were simply too limiting to conduct analyses on such small seeds. However, recent advances seem to put Arabidopsis at the centre of seed nutrient research. These advances include imaging techniques with increased resolution (Punshon, Ricachenevsky, Hindt, Socha, & Zuber, 2013; Zhao, Moore, Lombi, & Zhu, 2014), the availability of transcriptomes of individual seed tissues (Belmonte et al., 2013; Dekkers et al., 2013; Le et al., 2010), and

protocols that allow collecting higher biomass in a shorter time (Bates, Jewell, & Browse, 2013; Perry & Wang, 2003; Raissig, Gagliardini, Jaenisch, Grossniklaus, & Baroux, 2013).

Metal transporters must ensure that filial tissues (embryo and endosperm) receive sufficient amounts of metals so that the metal demand of the developing seed is fulfilled, but not exceeded. The developing seed requires metals for both immediate use and use after detachment from the mother plant until the root of the germinating seed sets in. Regarding its immediate use, the developing seed is not totally different from the mature plant, since it carries out photosynthesis (although the rate is much lower than in the mature leaves and its function may be rather to provide oxygen to heterotrophic embryos; Rolletschek, Weber, & Borisjuk, 2003), it respires and detoxifies radicals all of which require metal-bearing enzymes. Characterization of metal transport mutants which can be rescued by additional metal supply has indicated that embryos might be aborted when metal supply to the seed is not sufficient (Stacey, Koh, Becker, & Stacey, 2002; Waters et al., 2006; Wong, Jarvis, Sherson, & Cobbett, 2008). In addition, harvested seeds contain fairly constant levels of metals, independent of how the mother plant was fed (Rengel, Batten, & Crowley, 1999). As an example, in nature and in agronomic practice, limited, i.e., basic soils, or excess Fe, i.e., acidic or fertilized soils, can impact seed yield dramatically but not the final seed Fe concentration (Tyler, 1998; Wiersma, 2005, 2012). Alternatively, recent findings show that seed Fe concentrations are regulated genetically, by expression of certain transcription factors such as basic helix-loop-helix 104 (bHLH104), Brutus (BTS), and Brutus-like (BTSL). Increase in expression of bHLH104 (Li, Zhang, Ai, Liang, & Yu, 2016) and decrease in expression of BTS (Li et al., 2016; Long et al., 2010) as well as its two paralogs, BTSL1 and BTSL2, resulted in a striking increase in Fe concentration of the seed (Hindt et al., 2017; Li et al., 2016; Long et al., 2010) Future research will have to pinpoint the metal transporter genes that are regulated by these transcription factors.

3. POST-PHLOEM METAL TRANSPORT

Upon fertilization, the ovule transforms into a seed and becomes a sink for metal nutrients. The micropyler region, which represents the open space where the pollen tube enters into the ovule to fertilize the egg, subsequently fuses to close the seed. The seed coat contains two integuments of the ovule. Its chalazal region is connected to the silique via the funiculus. The funiculus itself contains vascular bundles, comprising xylem, and phloem. Due to the low level of transpiration in the seed, the xylem does not contribute

significantly to the feeding of the seed. Thus, the phloem has been suggested as the main source of metals arriving at the seed coat, and ultimately in the embryo (Fig. 1).

In phloem, metal nutrients can be carried in chelated forms. Because the phloem sap is neutral to basic, metals such as Fe tend to precipitate unless they are chelated. A major chelator of metals in phloem is nicotianamine (NA) (Ishimaru et al., 2010; Schmidke & Stephan, 2006; von Wirén et al., 1999). Consistent with the chelation function of NA, plant lines that carry a mutation in NA-metal carrier proteins, YSLs, may show differential

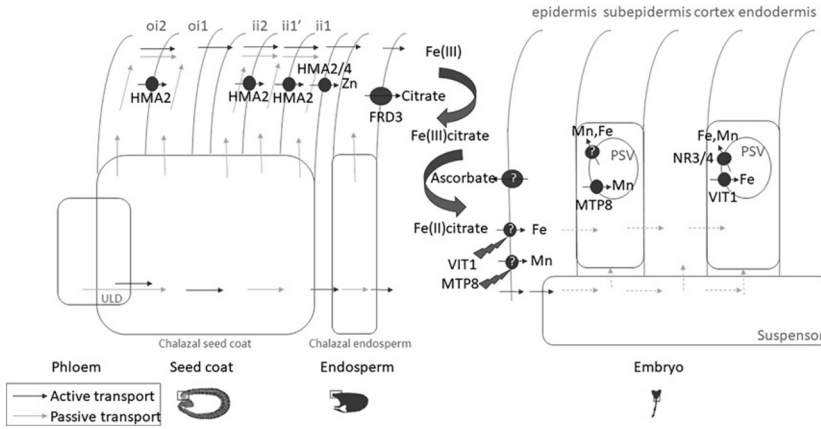

Fig. 1 Metal translocation from phloem to the embryo inside the Arabidopsis seed. Phloem ends in unloading domain of the chalazal seed coat. Metals that are unloaded here diffuse through the outer and inner integuments, and chalazal seed coat. HMA2 is expressed in all integuments, whereas HMA4 is mainly expressed in the innermost layer of the seed coat, the endothelium. Both transport Zn. FRD3 transports citrate to the space between the endosperm and embryo and chelates Fe. FRD3 expression localizes to the protodermis of the embryo (not shown in the figure) and in the endosperm. Chelated Fe(III) is reduced by ascorbate and taken up into the embryo cells. VIT1 and MTP8, which are tonoplast metal transporters, control Fe and Mn intake to the embryo. Metals can be either directly taken up by the embryo or via the suspensor. The embryo and suspensor form one single symplast at the globular stage, but as the embryo develops, several distinct symplastic domains appear. In mature seeds, the suspensor gets degraded. Fe is taken into vacuoles of endodermal cells of the embryo by VIT1 and in subepidermal cells of the abaxial side of the cotyledons by MTP8. Fe in endodermal cells is effluxed by NRAMP3/4. Red sketches at the bottom are seed organs and zoom outs of the regions above them. Green arrows represent symplastic (passive) pathways while red arrows represent apoplastic barriers, where active transport is necessary. Dashed green arrows represent symplastic pathways that only exist during the early stages of developing embryos. *Oi's*, outer integument cell layers; *ii's*, inner integument cell layers. Lightning-like symbol indicates signalling. *ULD*, unloading domain. *NR3/4*, NRAMP3 and 4. *PSV*, protein storage vacuole.

metal concentrations in the seed. For instance, *ysl1* single and *ysl1 ysl3* double knock out lines in Arabidopsis and a *ysl2* mutant in rice exhibit lower Fe accumulation in seeds (Jean, Schikora, Mari, Briat, & Curie, 2005; Koike et al., 2004; Waters et al., 2006). In order to address why *ysl* mutants show such seed metal phenotype, Fe distribution in the shoot of NA-less Arabidopsis mutants was investigated (Schuler, Rellán-Álvarez, Fink-Straube, Abadía, & Bauer, 2012). NA-less mutant leaves accumulated an excess of Fe in the vascular tissues although the leaf mesophyll was severely Fe-deficient, suggesting a role for NA in the unloading of Fe from the phloem to the nearby tissues (Schuler et al., 2012). It is possible that YSLs that are localized to the phloem are also needed to unload NA-metal complexes in the chalaza of the seed coat.

4. FROM SEED COAT TO THE ENDOSPERM, AND FURTHER TO THE EMBRYO

Metals need to move from the seed coat to the endosperm in order to reach the developing embryo. Since metal trafficking inside seed layers cannot be observed in real time, the specific regions that are responsible for efflux and subsequent uptake must be inferred from indirect evidence. These include (i) the presence and density of plasmodesmatal connections between cells, which indicate whether tissues are symplastically connected, (ii) the cell morphology, i.e., whether cells are specialized as transfer-cell-like and (iii) whether tissues are covered with a waxy substance, such as suberin, which blocks solute transport. Wherever tissues are not symplastically connected, metal transport will depend on plasma membrane transport proteins. Therefore, identifying new metal transporters and localizing the site of their expression will aid our understanding of metal trafficking between seed tissues.

Metals that are unloaded in chalazal seed coat are destined for translocation to the endosperm (Fig. 1). Metals can then diffuse from the chalaza throughout the three cell layers of the outer integument which are symplastically connected (Stadler, Lauterbach, & Sauer, 2005). From the innermost cell layer of the outer integument, metals are transferred to the outer cell layer of the inner integument by means of an active transport. They subsequently diffuse throughout the two cell layers of this integument (Stadler et al., 2005). It should be noted that, even in tissues that are symplastically connected, the additional presence of an apoplastic pathway cannot be ruled out (Fig. 1). Such a pathway has been proposed for sucrose and aminoacid

transport, based on expression of membrane transport proteins between inner cell layers of each of the integuments (Chen et al., 2015; Karmann, Müller, & Hammes, 2018; Müller et al., 2015).

From the innermost cell layer to the endosperm, metals must be transported via active processes (Stadler et al., 2005). Two proteins involved in this process, heavy metal ATPase2 and 4 (HMA2/4), were first identified in the vasculature of mature plants and associated with xylem loading of Zn (Hussain et al., 2004; Verret et al., 2004). In seeds, expression of the two proteins localized to the seed coat: HMA2 is expressed in all cell layers, whereas HMA4 is mainly present in the innermost layer. In addition, in *hma2/4* double knock-out seeds, Zn translocation from the seed coat to the developing embryo was reduced (Olsen et al., 2016). However, HMA2/4 are not the only Zn exporters in the seed coat, as the embryo of double knock-out lines can still receive Zn. Thus, lower affinity transporters may come into play upon accumulation of Zn in the seed coat (Olsen et al., 2016).

The endosperm continuously transforms throughout seed development. From the coenocyte stage where it is formed of a single multinuclei cell harbouring a large central vacuole, the endosperm goes through uneven cellularization events which lead to gradual shrinking of the central vacuole. Depending on the location of cellularizations, the endosperm can be subdivided as micropyler (where the pollen tube enters the ovule), chalazal (above the end of vascular bundles) and peripheral (starting from the peripheries then extending to the centre) endosperms (Olsen, 2004). In contrast to the others, the chalazal endosperm is cellularized much later and contains structures resembling those of transfer cells (Nguyen, Brown, & Lemmon, 2000). Furthermore, the chalazal endosperm contains a high number of mitochondria and an abundant endoplasmic reticulum (Nguyen et al., 2000).

The above properties point to the chalazal endosperm as a promising candidate for nutrient transport between the seed coat and the developing embryo or the micropyler endosperm. A direct transport pathway from the chalazal endosperm to the embryo proper and/or the suspensor (a single cell layer attaching the embryo proper to the micropyler endosperm) has been suggested to operate during the very early phase of embryogenesis. This pathway would involve the central vacuole that is protruded into the chalazal endosperm at one end, and at the other end placed in close proximity of the embryo proper (Otegui, Capp, & Staehelin, 2002). However, such a direct transport may only be operational during the early stages of seed development; since, later on, the central vacuole shrinks, and the embryo becomes completely surrounded by the micropyler endosperm.

Seed tissues are covered with waxy substances known to restrict metal transport in certain organs of mature plants. In particular, the cuticle, which is a hydrophobic film over epidermis, is a major factor decreasing the effect of foliar fertilizers (Fernández & Eichert, 2009). Hydrophilic nutrients can still pass through the cuticle, albeit at much lower rates. Recent research showed that in the developing Arabidopsis seed, both the endosperm (Beeckman, De Rycke, Viane, & Inzé, 2000; De Giorgi et al., 2015) and the embryo proper (Delude, Moussu, Joubès, Ingram, & Domergue, 2016) are covered with a cuticle. Very recently, the cuticle surrounding the endosperm has been reported to have gaps in the micropyler and the chalazal regions (Loubéry, Giorgi, Utz-Pugin, Demonsais, & Lopez-Molina, 2018). Besides cuticle, suberin is another waxy substance found in seeds. In contrast to the cuticle which localizes to the outer surface of cell walls, suberin localizes to their inner side. In roots, suberization of the endodermis was recently suggested as nutrient-stress responsive, thereby regulating nutrient acquisition (Barberon et al., 2016). In seeds, suberization occurs in the outer integument (Molina, Ohlrogge, & Pollard, 2007) and chalazal plug (Franke et al., 2009; Lashbrooke et al., 2016), and is mostly developmentally regulated. The exact distribution of waxy substances and their implication in nutrient acquisition of seed organs should be addressed in future research.

Nutrient transfer from endosperm to embryo may involve the suspensor. This assumption is based on indirect evidence including the differentiation of suspensor cells with transfer cell-like features in some species, absence of the cuticle layer in suspensor in contrast to the cuticle-covered embryo proper and expression of nutrient transporter gene families in the suspensor (Ingram & Nawrath, 2017; Stacey et al., 2008; Yeung & Meinke, 1993). Furthermore, histochemical analyses by Roschzttardtz, Conéjéro, Curie, and Mari (2009) have revealed a Fe accumulation in the suspensor of developing Arabidopsis embryos. However, this Fe accumulation extends to the symplastically connected embryo proper (Stadler et al., 2005), making it impossible to interpret whether and to which extent the embryo proper and the suspensor are responsible for Fe intake. The suspensor can only feed the embryo proper for a limited time since its symplastic connections are weakened and then crushed by the developing embryo following heart stage (Stadler et al., 2005). Thus, it is believed that, later on, the embryo proper acquires nutrients from the surrounding endosperm tissues autonomously.

Metal acquisition by the embryo appears to be controlled in a nutrient-specific manner and by complex mechanisms, having similarities and distinctions to root metal acquisition. In the xylem sap of roots, the main chelator

of Fe is citrate (Durrett, Gassmann, & Rogers, 2007; Rellán-Álvarez et al., 2009). It is secreted to the xylem sap by an efflux transporter called ferric reductase defective3 (FRD3) (Durrett et al., 2007). In addition to the root vasculature, *FRD3* is expressed in the chalazal endosperm throughout development (Belmonte et al., 2013) and in the aleurone (Roschzttardtz, Séguéla-Arnaud, Briat, Vert, & Curie, 2011), a single cell layer that is the remainder of consumed endosperm in mature seeds. A direct evidence that citrate chelates Fe in seeds came from the analysis of Fe ligands in the liquid endosperm of pea (Grillet et al., 2014). Therefore, it has been suggested that FRD3-mediated citrate efflux mobilizes Fe in the apoplast between the endosperm and embryo (Grillet et al., 2014; Roschzttardtz et al., 2011). In roots, Fe acquisition is dependent on a Fe(III) reduction step carried out by a membrane-bound ferric chelate reductase (Robinson et al., 1999) and reductants, i.e., coumarins, that are exuded to the rhizosphere (Mladěnka et al., 2010; Rajniak et al., 2018). Interestingly, the maturing embryo is also able to perform a Fe(III) reduction in vitro, albeit by a different mechanism involving an efflux of ascorbate. Fe(III) reduction capacity is impaired in the presence of ascorbate scavengers in in vitro essays and mutants that are defective in ascorbate synthesis could accumulate lower Fe levels (Grillet et al., 2014). Well-known members of the Fe acquisition machinery of roots (IRT1, FRO2, etc.) that are all regulated by FIT are not expressed in the embryo (Belmonte et al., 2013). Therefore, the proteins determining Fe uptake in the embryo seem to be distinct from those in roots and are yet to be elucidated.

Comparison of elemental concentrations of developing embryos revealed that embryo acquisition of Fe takes place prior to that of Mn (Otegui et al., 2002; Roschzttardtz et al., 2009; Socha, 2016). While Fe enters early into the developing embryo, i.e., at torpedo stage (Roschzttardtz et al., 2009; Socha, 2016), Mn is sequestered in the endoplasmic reticulum of chalazal and micropyler endosperms and not released until the early phase of the photosynthetic bent-cotyledon stage (Otegui et al., 2002). In mature plants, Mn is transported into the endoplasmic reticulum by a P-type Ca^{2+} pump named ECA1 (Liang, Cunningham, Harper, & Sze, 1997). Since the corresponding gene is also transcribed in the chalazal and micropyler endosperms (Belmonte et al., 2013); ECA1 might mediate the transitory accumulation of Mn in these tissues. Because Fe and Mn have similar atomic radii and charges, membrane transporters often cannot differentiate between these two ions. Thus, transient storage of Mn in the endosperm could be a strategy to prioritize Fe transport to the embryo since essentiality of Fe for an organism is more widespread.

5. METAL TRANSPORT WITHIN THE EMBRYO

Metals are essential for metabolic processes occurring in rapidly developing embryos especially during early embryogenesis. Later on, metals are directed to storage compartments, as a preparation for desiccation stage. This is well illustrated in the case of Fe and photosynthesis. In green leaves of mature plants, 80% of Fe is located in the chloroplast (Terry & Abadía, 1986). In seeds, chloroplasts develop and the embryo becomes photosynthetically active at late globule stage (Tejos, Mercado, & Meisel, 2010). Therefore, similar to what occurs in leaves, a large part of embryonic Fe is stored in chloroplasts to support photosynthesis. In agreement with this view, Perls/DAB staining revealed an enrichment of Fe under the shoot apex of torpedo stage embryo (Fig. 2) showing an overlap with the region

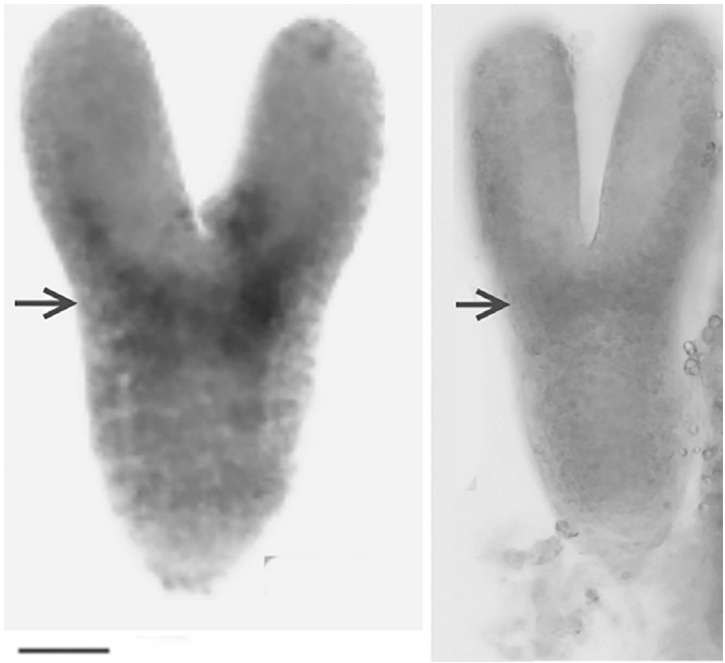

Fig. 2 Overlap of Fe and chlorophyll distribution in torpedo stage embryos. On the left, an isolated embryo was stained by Perls'/DAB and observed under a microscope. Fe is revealed as a black band under the shoot apex. Note that chlorophyll was removed during the staining protocol. On the right, distribution of chlorophyll in a similar embryo was observed under the microscope without any treatment. Bar is 50 μm. Red arrows indicate preferential Fe (left) and chlorophyll (right) accumulation under the shoot apex.

where strong chlorophyll accumulation is observed (Fig. 2 and see the chlorophyll fluorescence in fig. 2R in Tejos et al., 2010). However, Fe pool in the chloroplasts is obscured as the embryo develops, because the massive amount of Fe localizes around the provascular strands and also possibly chloroplast distribution no longer shows an obvious pattern (Fras, Smolen, & Maluszynska, 2008; Roschzttardtz et al., 2009; Tejos et al., 2010). During the subsequent maturation stages, Fe is effluxed from chloroplasts (Divol et al., 2013). Therefore, prior to desiccation stage, the largest Fe pool is localized around provascular strands which accounts for half of total seed Fe (Ramos, Khodja, Mary, & Thomine, 2013). This means that the use of metals that enter the embryo progressively shifts from maintaining metabolic needs to building up reserves.

Several genetic factors that mediate metal homeostasis within the embryo have recently been identified. Fe pools in dedifferentiating chloroplasts in maturing embryos are mobilized by two YSL proteins, YSL4/6 (Divol et al., 2013). In support of this, mutant plants that showed inactivation of these two genes were susceptible to Fe toxicity and contained an excess of Fe in the chloroplasts. Furthermore, at least one of these YSLs, YSL6 accumulated in seeds during late maturation and localized to the chloroplast membrane in embryos (Divol et al., 2013). However, it should be noted that this chloroplast localization could not be confirmed by a different study in which YSL6-GFP localized instead to the tonoplast or endoplasmic reticulum (Conte et al., 2013). The release of Fe from the chloroplast may contribute to Fe accumulation around the provascular strands. Transport of Fe into this hotspot has been shown to be mediated by VIT1, an ortholog of the vacuolar metal transporter CCC1 in yeast, based on complete loss of Fe enrichment around provascular strands in *vit1* knock out mutants (Kim et al., 2006). Vacuolar Fe stores are mobilized during germination in order to support metabolism, such as photosynthesis, before the root metal uptake machinery steps in (Bastow et al., 2018; Lanquar et al., 2005). Mobilization of Fe stores from the endodermal vacuoles depends on two tonoplast-localized proteins from the NRAMP family, NRAMP3/4 (Lanquar et al., 2005). In germinating seeds of wild-type plants, the staining of Fe around provascular strands disappears during the first 3 days (Roschzttardtz et al., 2009) and thereafter, root iron acquisition takes over, according to promoter activity of IRT1 (Lanquar et al., 2005). By contrast, in a *nramp3/4* double knock-out, Fe release from the endodermis was delayed (Roschzttardtz et al., 2009) and germinating seeds could not survive under Fe deficiency. Interestingly, in a *vit1* background, where Fe stores are mislocalized, the susceptibility of *nramp3/4* to Fe deficiency during early germination disappears. Thus, it is

proposed that NRAMP3/4 specifically mediate an efflux of Fe from endodermal cells, and together with VIT1 constitute an influx-efflux unit in these cells (Mary et al., 2015).

Among the micronutrients investigated so far, in addition to Fe, only Mn appears as clearly enriched in certain cell types of the embryo. This enrichment is observed in the subepidermal cell layer of the abaxial sides of the cotyledons and in hypocotyl cortex. It is mediated by the tonoplast metal transporter MTP8 (Eroglu, 2015; Eroglu et al., 2017, 2016). MTP8-mediated enrichment accounts for one-third of total Mn in the seed (Ramos et al., 2013). Characterization of seeds that show genetic inactivation of *VIT1*, *MTP8* and other metal transporter genes resulted in seeds that mislocalized Fe and Mn in several different ways (Table 1). In contrast to the single large lytic vacuole in cells of mature plants, embryo cells contain several smaller vacuoles with one or few globules in each, containing phytic acid salts that chelate metals (Bolte et al., 2011; Feeney, Kittelmann, Menassa, Hawes, & Frigerio, 2018). Although Fe and Mn were differentially localized at cell-layer level; at the subcellular levels, both were observed in these globules (Eroglu et al., 2017; Lanquar et al., 2005; Roschzttardtz et al., 2009), probably associated with phytate (Bruch, Thomine, Tabares, & Un, 2015). Cell layer-specific distribution of Fe and Mn immediately raised the question of whether these highly specialized localizations have any kind of adaptive significance.

Table 1 Iron and Manganese Enriched Tissues in Mature Embryos of Mutant Arabidopsis Lines

Genotype	Fe Hotspots	Mn Hotspots	References
Col-0	e	s	Kim et al. (2006)
vit1	s	s	Kim et al. (2006)
mtp8	e	e	Eroglu (2015)
vit1 mtp8	h	h	Eroglu et al. (2017)
cax1/3	e	s (l.p)	Punshon et al. (2012)
nramp3/4	e	s + e	Socha (2016)
mtp8 nramp3/4	e	h + e	Socha (2016)
Imbibed Col-0	e + s	s	Eroglu et al. (2017)
Imbibed *mtp8*	e	s	Eroglu et al. (2017)

The table mostly refers to dry seeds. Imbibed seeds were incubated in water for 2 days. The metal distribution is described as follows: h: homogeneously distributed, e: enriched in endodermal cell layer, s: enriched in sub-epidermal cell layer, (l.p): enriched but less pronounced than in wild type (Col-0).

6. DO TONOPLAST TRANSPORTERS CONTROL METAL ACQUISITION IN THE EMBRYO?

The functional significance of cell-layer specific accumulation of Fe and Mn in the embryo is yet unclear. None of the cell layers, including the Mn-enriched subepidermal or the Fe-enriched endodermal ones, differ from each other under the microscope, regarding the number, distribution, and size of storage organelles (Busse & Evert, 1999), thereby failing to indicate any kind of specialization for these cell layers. Although a few studies have suggested that Fe enrichment in the endodermis is critical for germination under Fe deficient conditions (Gollhofer et al., 2014; Kim et al., 2006; Mary et al., 2015), mechanistic explanations for the phenotypes are lacking. In addition, the specific enrichment of Mn in subepidermal cells could not be associated with any relevant physiological function.

Alternatively, the two transporters responsible for the cell layer specific accumulations, VIT1 and MTP8, may be involved in embryo metal loading. Three lines of evidence suggest that VIT1 and MTP8 affect metal translocation from the endosperm to the embryo. Firstly, the activities of the *VIT1* and *MTP8* promoters coincide with the entry into the embryo of Fe and Mn, respectively (Belmonte et al., 2013; Eroglu et al., 2017; Otegui et al., 2002). *VIT1* is expressed as early as the torpedo stage, in contrast to *MTP8*, which is not expressed until the bent-cotyledon stage. Secondly, qualitative X-ray fluorescence imaging has shown that the absence of VIT1 delays Fe loading to the embryo (Punshon et al., 2013; Socha, 2016). Whereas Fe can be observed in endodermal cells of wild-type embryos, at the same developmental stage of *vit1* seeds, it is localized in the coat. Thirdly, the absence of MTP8 somehow triggered embryo to switch on Mn uptake in the early phase of germination (Eroglu et al., 2017). In wild-type plants, seed embryos take up external Mn at millimolar concentrations only, suggesting that during germination Mn transport into the embryo is mediated by a low-affinity transport system. In contrast, *mtp8* embryos efficiently accumulate Mn at micromolar concentrations, suggesting that an alternative, high-affinity acquisition system is activated. Taken together, these data suggest that tonoplast transporters can control metal acquisition in the embryo possibly by signalling the embryo metal status to plasma membrane transporters expressed at its surface (Fig. 1). Further exploration of this model offers a promising research direction.

Seed is the final target for nutrients in the life cycle of annual plants, including staple crops and model plants such as *A. thaliana*. Up to date, many metal transporter genes have been characterized in mature plants, but it has

been neglected most often, to take one step further to investigate whether these metal transporter genes also contribute to seed homeostasis. Therefore, despite mature plants have been extensively studied, metal transportation to the seed and within the seed is still poorly understood. Yet, metals in seeds must be optimized for their concentration and localization to improve stress tolerance in agriculture and to combat micronutrient deficiencies in humans. The present review compiled the relevant literature and drew a picture of our current understanding of seed metal nutrition. By interpreting existing data and discussing knowledge gaps, we tried to generate new hypotheses and pointed out some new research directions, which will hopefully be fruitful.

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