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Facing the challenges of Cu, Fe and Zn homeostasis in plants

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Plants have recently moved into the spotlight owing to the growing realization that the world needs solutions to energy and food production that are sustainable and environmentally sound. Iron, copper and zinc are essential for plant growth and development, yet the same properties that make these transition metals indispensable can also make them deadly in excess. Iron and copper are most often used for their redox properties, whereas zinc is primarily used for its ability to act as a Lewis acid. Here we review recent advances in the field of metal homeostasis and integrate the findings on uptake and transport of these three metals.

Understanding the fundamentals of plant growth in order to meet the demands for food, fuel and fiber is of the utmost importance. Metals have vital roles in a plant's life cycle, yet there are many impediments to proper metal homeostasis. Here we focus on some of the challenges of maintaining Fe, Zn and Cu homeostasis, and on the strategies that plants use to meet these challenges. These three transition metals, along with Mn, Mo and Ni, are the metal micronutrients considered essential for plants (**Table 1**). Although reviews often cover each of these transition metals separately, by comparing and contrasting what we have learned about each of these important metals, we can achieve a more integrated picture of how plants manage their ionome.

Fe and Cu both exist in multiple redox states, readily accepting and donating electrons from their d orbitals. As such, Fe and Cu serve as critical cofactors for components of the electron transport chain in the mitochondrion and in the chloroplast[1]. Fe is also found in the center of Fe-S clusters, which act as electron acceptors and donors in a number of key cellular processes including photosynthesis, respiration, sulfate assimilation and ethylene biosynthesis[2]. The most abundant Cu protein in plants is plastocyanin, a protein that transfers electrons from the cytochrome b_6f complex to photosystem I (PSI). Cu is used as a cofactor by proteins involved in protection from reactive oxygen species, lignification of the cell wall, pollen formation, proper carbohydrate metabolism, and formation of phenolics in response to pathogen attack[1]. Cu is also required by the ethylene receptor for proper signaling.

Zn, unlike Fe and Cu, is not redox active. This property, combined with the pronounced Lewis acid characteristics of the Zn^{2+} ion and the flexibility of the coordination sphere with respect to geometry and the number of ligands, helps explain why Zn has so many different roles inside cells[3]. It is required as a cofactor in over 300 enzymes, including RNA polymerase, superoxide dismutase, alcohol dehydrogenase and carbonic anhydrase[4]. It also has key structural roles, such as its use in the Zn finger family of transcription factors for formation of the DNA binding domain that interacts with the major groove of DNA[4,5].

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Mechanisms of metal mobilization and uptake

The primary source of metals for the plant is the soil (**Table 1**); thus, efficient uptake is essential for life. Even when abundant, metals can be inaccessible in the soil owing to their tendency to be present predominantly in an insoluble form. Zn and Cu are primarily insoluble in soils because of adsorption to clay, $CaCO_3$ or organic matter, whereas Fe is predominantly found as Fe hydroxides[1]. Insolubility is particularly pronounced at the higher pH of alkaline soils, which represent approximately 30% of the world's soils. To deal with the inaccessibility of some metals, nongraminaceous plants rely primarily on a reduction-based strategy of uptake (**Fig. 1**), whereas graminaceous plants (grasses) more commonly use a chelation-based method (**Fig. 2**).

Acidification of the soil. To overcome the challenge of insolubility in alkaline soils, plants can use ATPase activity to extrude protons into the rhizosphere to decrease the pH of the soil under metal-limiting conditions[6]. As the pH of the soil decreases, the increased concentration of protons helps to generate free metal. For example, Fe^{3+} is released from insoluble oxides with the concomitant formation of water:

$$Fe(OH)_3 + 3H^+ \leftrightarrows Fe^{3+} + 3H_2O$$

Acidification can have a major impact, as a unit drop in pH increases the solubility of Fe by 1,000-fold[7]. The ATPases responsible for proton extrusion and soil acidification under Fe deficiency have not yet been confirmed, but they are likely to be members of the AHA (*Arabidopsis* H^+ ATPase) family[8]. Of the 12 family members in *Arabidopsis*, AHA1, AHA2 and AHA7 are the most likely candidates, as they are all expressed in the roots and are upregulated under Fe deficiency[9]. Acidification of the soil would also result in an increase in the solubility of Zn and Cu, by encouraging cation exchange and

Table 1 Essential metal micronutrients for plants

Element	Biologically relevant oxidation states	Lithosphere[a] (mg kg^{-1})	Typical plant[b] (mg kg^{-1})	Transporter family	Examples
Cu	Cu^{+}, Cu^{2+}	50	6	COPT, HMA	Plastocyanin, cytochrome oxidase, SOD
Fe	Fe^{2+}, Fe^{3+}	45,000	100	FRD3, NRAMP, OPT, VIT, YSL, ZIP	Cytochromes, Fe-S proteins, SOD
Mn	Mn^{2+}, Mn^{3+}, Mn^{4+}	950	50	CAX, NRAMP, ZIP	Water-splitting enzyme in PSII, SOD
Mo	Mo^{4+}, Mo^{6+}	1.5	0.1	MOT	Nitrate reductase, sulfite oxidase, xanthine dehydrogenase, aldehyde oxidase
Ni	Ni^{2+}	80	0.1		Urease
Zn	Zn^{2+}	75	20	ZIP, HMA, MTP	RNA polymerase, alcohol dehydrogenase, carbonic anhydrase, SOD

[a]Figures taken from Table 1.3 in *The Handbook of Trace Elements*[100]. [b]Figures taken from Table 1.3 in *Mineral Nutrition of Higher Plants*[1].

releasing the divalent metals from insoluble chelates with soil particles. ATPase activity also allows for the establishment of a negative membrane potential, along the order of –100 to –250 mV, which serves to drive cation uptake[8].

Reduction-based strategy. Once freed from insoluble chelates, the transition metals are more accessible for uptake. However, the transporters in many plants have a specific affinity for a particular oxidation state of each metal. Many plants address this problem by using a reduction-based strategy for metal uptake. Whereas Zn is always found in the +2 oxidation state under physiologically relevant conditions, both Fe and Cu need to be reduced for uptake by their respective transporters, IRT1 and COPT1 (ref. 10). For example, Fe is transported into the cell in the divalent form, despite being present in the soil primarily in the trivalent form. To accommodate this, Fe^{3+} is reduced to Fe^{2+} by the ferric chelate reductase FRO2, which transports electrons from NADH bound to cytoplasmic binding sites across the membrane via heme to reduce Fe^{3+} (ref. 11). Consistent with this role, FRO2 is expressed in the plasma membrane and shows increased accumulation and activity under Fe deficiency[11,12]. When induced by Fe deficiency, FRO2 is also able to reduce Cu, but FRO2 expression is not upregulated under Cu deficiency.

Chelation-based strategy. In contrast to the reduction-based strategy, grasses primarily use a chelation-based strategy for Fe uptake. This strategy employs the release of chelators into the rhizosphere, known as phytosiderophores, to bind Fe^{3+} for transport into the plant[13]. Phytosiderophores are synthesized from methionine and are usually referred to collectively as belonging to the mugineic acid family (the MAs). Expression of the genes involved in MA biosynthesis is upregulated under Fe deficiency[14], resulting in increased release of MAs. In barley, MAs are also thought to play a role in mobilizing Zn in addition to Fe (ref. 15). Roots of Zn-deficient barley plants have increased expression of genes involved in the biosynthesis of MAs, and there is increased secretion of MAs from the roots of these plants[15]. In addition, Zn-deficient barley is not deficient in Fe, as had previously been suggested to explain phytosiderophore release under Zn deficiency. Furthermore, using a positron-emitting tracer imaging system (PETIS) to follow the movement of radiolabeled Zn, more Zn was shown to be taken up when plants were supplied with Zn-MAs than when supplied with free Zn^{2+}, which suggests that MAs secreted as a result of Zn deficiency are effective in absorbing Zn from the soil. In rice, however, similar experiments showed that phytosiderophores play a role in the distribution of Zn within the plant rather than in the absorption of Zn from the soil[16]. So far, there is no suggested role for phytosiderophores in Cu uptake.

Transporters involved in metal uptake from the soil. Though ions are able to diffuse into the space between the cell wall and the plasma membrane (the apoplast) of some cells in the root, apoplastic transport is blocked by the impermeable Casparian strip in the endodermal layer. At this point, metals must be actively transported across the plasma membrane into the symplastic space where they can then move via plasmadesmata to the inner root cell layers for subsequent loading into the vasculature[17] (**Figs. 1** and **2**).

Transport of metals into the symplast of the epidermis is carried out via members of several transporter families. Fe is taken up primarily through the high-affinity transporter IRT1 (refs. 18–21). This essential member of the ZIP (ZRT, IRT-like proteins) metal transporter family localizes to the plasma membrane of the root epidermis and is required for seedling survival[19–21]. The lethal phenotype of *irt1* mutants can be rescued by addition of exogenous Fe, which indicates that its primary role is in uptake of Fe (refs. 19,20). Like *FRO2*, *IRT1* has been found to be upregulated in response to Fe deficiency, and substantial protein accumulation occurs only under Fe deficiency[22], which suggests both transcriptional and post-translational control[23]. The mechanism for the post-translational regulation has recently been elucidated; IRT1 has been shown to be ubiquitinated at specific lysine residues, which leads to proteasome-mediated degradation[23].

Though it has long been thought that grasses use only the chelation-based strategy for Fe uptake, work in recent years has identified orthologs of IRT1 that are upregulated under Fe deficiency in rice and can rescue Fe transport in yeast mutants[24]. Further overturning the dogma that grasses only transport chelated Fe, a recent paper has demonstrated that rice is able to take up Fe^{2+} when nicotianamine (NA) synthesis is compromised[25]. NA is the precursor for the synthesis of MAs. This ability of grasses to take up Fe^{2+} would be particularly advantageous in plants such as rice that are often grown in flooded soils where Fe would be less oxidized and more likely to exist as Fe^{2+}. This finding changes the way we categorize metal uptake strategies, emphasizing a greater plasticity than was originally thought.

Although the essential role of IRT1 is in Fe uptake, it can also transport other divalent metals[26], and *irt1* plants have reduced levels of Zn as well as other cations[19,21]. Because *irt1* plants are able to survive without the addition of excess Zn, it is likely that Zn is primarily taken up into the plant via other transporters. It is not yet known, however, which proteins are responsible for Zn uptake from the soil.

Unlike Fe and Zn, Cu is not taken up primarily as Cu^{2+}, but instead is transported as Cu^{+} by COPT1 (ref. 27). COPT proteins are the *Arabidopsis* orthologs of the yeast transporter CTR1. COPT1 can complement yeast *ctr1* mutants and is upregulated under Cu deficiency in plants, and mutant plants show decreased Cu accumulation as well as upregulation of genes that respond to Cu limitation[27]. Though Cu^{2+}

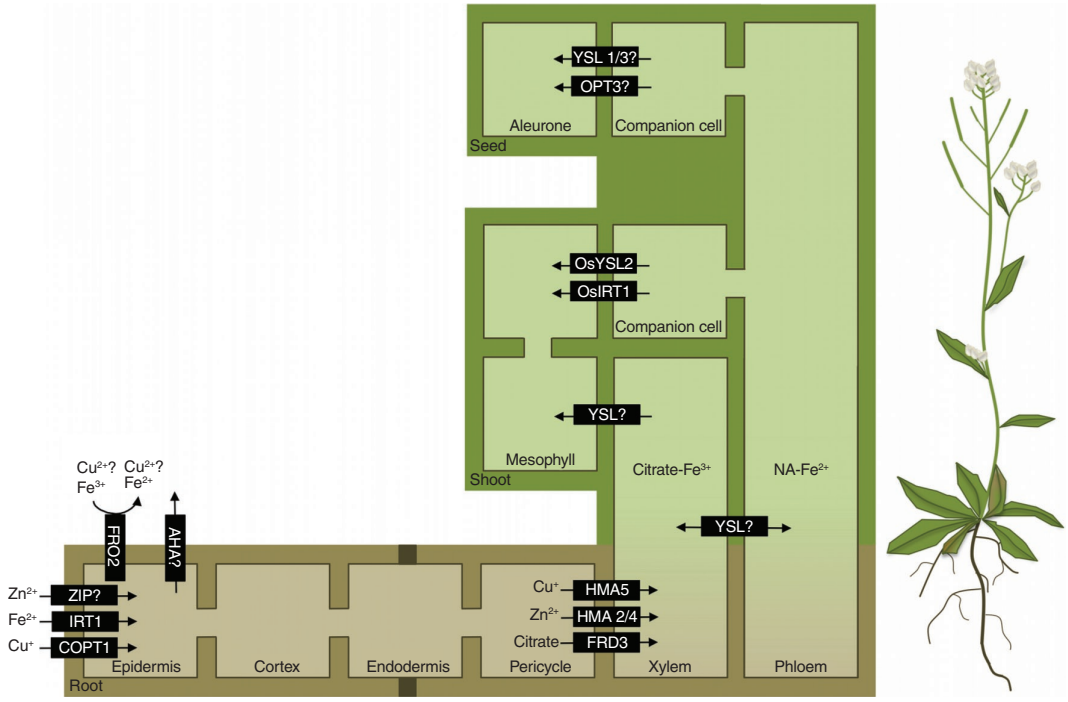

Figure 1 Intercellular metal transport in dicots. Fe, Zn and Cu are taken up into the symplast by transporters in the epidermis. Reduction of Fe and possibly Cu by FRO2 and acidification of the soil by an AHA contribute to increased metal uptake. Metals can then travel through the symplastic space to the vasculature, bypassing the waxy Casparian strip on the endodermis. Transport into the xylem is still not fully characterized but is thought to involve members of the HMA family and the citrate effluxer FRD3. In the xylem, metals are carried to the shoot through the transpiration stream where they are unloaded into the shoot, most likely by a member of the YSL family. YSLs may also translocate metals to the phloem, where they can then be delivered to the seed. The dark brown boxes represent the Casparian strip. NA, nicotianamine.

is more commonly found in the soil than Cu^{+} (ref. 10), it is possible that Cu^{2+} is reduced by FRO2, as reduction of Cu^{2+} is lost in *frd1-1* mutants and can be restored when *FRO2* expression is restored[11]. In addition to uptake of Cu^{+} through COPT1, plants may also take up Cu as the more abundant Cu^{2+} via a member of the ZIP family, a transporter family known to preferentially transport divalent cations. Both ZIP2 and ZIP4 are known to be upregulated by Cu deficiency and can complement *ctr1* yeast mutants for Cu uptake[28], but further research with loss-of-function mutants is needed to test the involvement of these transporters in Cu uptake.

As mentioned above, Fe is primarily taken up as chelated MA complexes in grasses. The transporter responsible for Fe-MA uptake, YS1 (yellow stripe 1), was originally identified in maize[29] using the *ys1* mutant, which is defective for phytosiderophore uptake. YS1 is expressed in the roots in response to Fe deficiency and localizes to the plasma membrane, as would be expected for an uptake transporter[30]. In rice, OsYSL15 (yellow stripe like) is the primary Fe-MA uptake transporter[31]. Members of the YSL family also are involved in metal distribution within the plant, as will be discussed below.

Transport between tissues

Although initial uptake into the plant is clearly critical, many of the essential roles of metals are in photosynthetic shoot tissue. Metals must therefore be transported throughout the plant, from uptake at the roots to the tissues where they are required. Once within the root epidermal cell after uptake from the soil, ions can move through symplastic passages from the epidermis to the pericycle to be loaded into the xylem[17]. Metals must be actively loaded into the xylem and transported by the transpiration stream to shoot tissue[32]. Some tissues such as the seed are not fed by the transpiration stream and must rely on the phloem for nutrients. Other tissues such as developing leaves do not yet have fully differentiated xylem and must receive the necessary metals through the phloem, which differentiates more quickly[32]. Proper loading and unloading of the vasculature is essential for metal transport in the plant (**Figs. 1** and **2**).

Root-to-shoot transport. The transporter that loads Fe into the xylem is not yet known, but Fe is most likely transported chelated to other molecules. Candidates include citrate and NA. NA is found in all higher plants studied thus far, and as mentioned earlier, it is the precursor for formation of phytosiderophores in grasses. The pH of the xylem favors the chelation of Fe to citrate rather than NA (ref. 32), and it is known that Fe exists as Fe^{3+}-citrate chelates in the xylem[33]. FRD3 (ferric reductase defective), a citrate transporter that localizes to the plasma membrane of the pericycle and the vascular cylinder[34], has been recently shown to efflux citrate into the xylem and is required for Fe transport to the shoot[33]. *frd3* plants show reduced citrate levels in the xylem as well as the shoot, and they hyperaccumulate Fe in the root, thus emphasizing the necessity of FRD3 for long-distance transport of Fe. Rice also relies on a *FRD3*-like gene, *OsFRDL1*, for efficient translocation of Fe to the shoot[35]. Fe is thought to be unloaded from the vasculature into developed tissue through yet-unknown mechanisms.

Zn is effluxed into the xylem for long-distance transport by the heavy metal transporters HMA2 (heavy metal ATPase) and HMA4, which localize to the plasma membrane of the root and shoot vasculature[36]. *hma2hma4* mutants show decreased shoot Zn and increased root Zn,

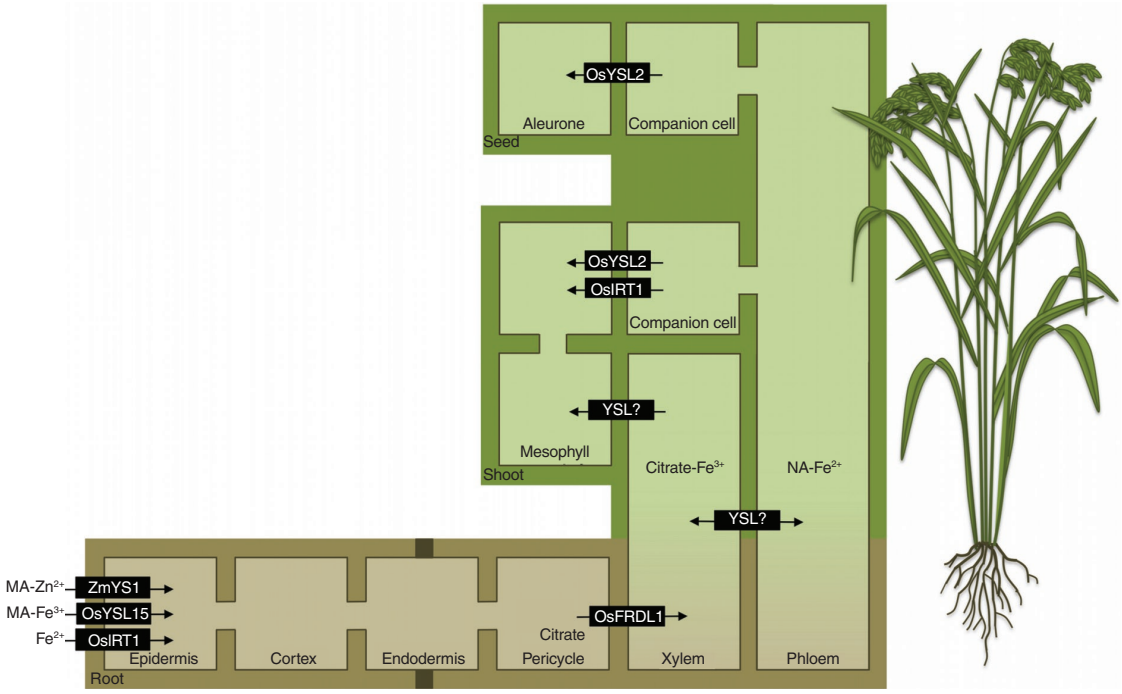

Figure 2 Intercellular metal transport in monocots. Fe and Zn are taken up as phytosiderophore chelates by YSL transporters in the epidermis. Fe can also be taken up by OsIRT1. Metals move through the symplastic space to the vasculature, bypassing the waxy Casparian strip on the endodermis. The citrate effluxer FRDL1 is important for loading of citrate into the xylem and subsequent Fe transport to the shoot through the transpiration stream. YSL transporters also may play a role in unloading the xylem into the shoot and the phloem. Fe is unloaded from the phloem by OsYSL2 and OsIRT1 into shoot and seed tissue. The dark brown boxes represent the Casparian strip. MA, mugineic acid; NA, nicotianamine.

which supports the role of HMA2 and HMA4 in xylem loading[36]. HMA4 was also identified as a gene with increased expression in the Zn hyperaccumulator *Arabidopsis halleri*[37,38]. Hyperaccumulators are plants that can take up and tolerate levels of metals that are toxic to non-accumulators. Recent work has uncovered the mechanism of increased HMA4 expression in *A. halleri*; surprisingly, the increase in expression is not due to *trans* factors but rather to a triplication of the gene and changes to *cis* regulatory elements driving *HMA4* expression[39]. The group also demonstrated that HMA4 is the primary means of Zn shoot hyperaccumulation in *A. halleri* by showing that plants that hyperaccumulate Zn in the shoot show higher levels of *HMA4* expression and that knockdown of *HMA4* by RNA interference abrogates the hyperaccumulation in the shoot. Interestingly, the group also showed a separation between the ability to accumulate elevated levels of Zn in the shoot and the ability to tolerate these levels. By expressing *AhHMA4* in *A. thaliana* under control of the *A. halleri* endogenous promoter, the group was able to show that these transformed plants recapitulated the Zn distribution patterns of *A. halleri* and showed increased shoot Zn levels, but they also showed signs of Zn toxicity. This emphasizes that additional genes are required in hyperaccumulators to confer tolerance to high levels of metals. Hyperaccumulators have been discussed in depth elsewhere[40–42]. The ligand for transport of Zn to the shoot is likely to be NA or organic acids[6].

Efflux of Cu into the vasculature is also thought to occur through an HMA family transporter. Work has implicated HMA5 in Cu efflux by showing that HMA5 is predominantly expressed in the root and is strongly and specifically induced by excess Cu. *hma5* mutants overaccumulate Cu in the root, which suggests a compromised efflux system when HMA5 is absent[43]. Further evidence in support of the role of HMA5 in Cu translocation from the roots to the shoots comes from a study of natural variation in Cu tolerance among *Arabidopsis* accessions, which identified HMA5 as a major QTL associated with Cu translocation capacity and sensitivity[44]. Cu is likely chelated to NA for translocation from the root to the shoot, based on the biochemical properties of NA and the phenotypes of the *chloronerva* mutant of tomato, which cannot synthesize NA (ref. 32).

Shoot-to-seed transport. Fe^{2+} and Zn^{2+} are thought to be transported in the phloem as NA chelates, and although the transporters involved in phloem loading and unloading are not fully known, they are thought to include members of the YSL group, a subfamily of the oligopeptide transporter (OPT) family of transporters[32]. One of the better characterized members of this subfamily is YSL1, which localizes to the shoot vasculature as well as the siliques, pollen grains and the developing seeds[45]. *ysl1* mutants accumulate reduced levels of Fe in the seeds, and these seeds show germination defects on low-Fe soil, which suggests a role for YSL1 in metal loading of the seed[45]. YSL3 is also expressed in the shoot vasculature as well as in the pollen. A recent study on the double mutant *ysl1ysl3* demonstrated that both of these transporters have a role in Fe, Cu and Zn remobilization from leaf tissue[46]. Seeds accumulate these metals at reduced levels in the absence of YSL1 and YSL3. In addition, YSL2 has been shown to complement Fe and Cu uptake yeast mutants when supplemented with NA (ref. 47), although a second group failed to see complementation by YSL2 (ref. 48). In further support of a role in transporting complexes into the vasculature, both groups found that YSL2 localizes to the lateral plasma membrane[47,48]. Furthermore, the rice ortholog, OsYSL2, also localizes to the vasculature and has been shown to transport Fe-NA when expressed in *Xenopus laevis* oocytes[49].

Although OPT proteins are named for their ability to transport oligopeptides, a recent study has demonstrated that OPT3 may serve a role in transporting Fe (ref. 50), and while it has not been established what form of Fe is transported, it is likely to be chelated to NA or an oligopeptide. This group showed that *opt3-2* mutants have reduced seed Fe content and abrogated seedling growth under Fe-deficient conditions, which suggests a role of OPT3 in seed Fe loading. Although yeast studies with OPT3 have suggested that it can transport Cu as well[28], OPT3 does not seem to play a role in Zn or Cu loading, as *opt3-2* seeds actually accumulate increased levels of these two metals[50].

Intracellular transport

Once transported to the proper tissue, metals must be properly distributed at the subcellular level to ensure sufficient levels to the necessary compartments while safely storing metals under times of excess (**Fig. 3**).

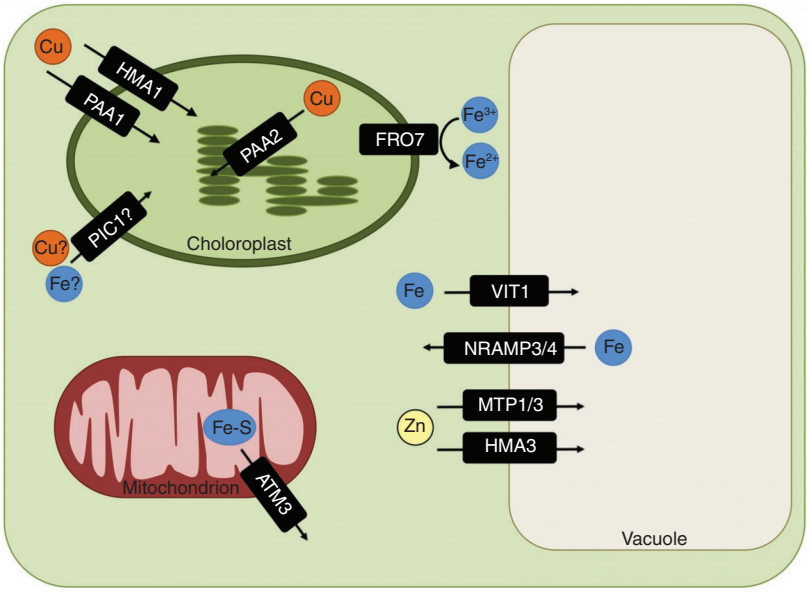

Figure 3 Intracellular metal transport. Fe is transported into the vacuole by VIT1, Zn is transported into the vacuole by MTP1 (or MTP3) and HMA3, and Fe is remobilized from the vacuole by NRAMP3 or NRAMP4. Transport into the chloroplast is best characterized for Cu, which is transported into the chloroplast by HMA1, PAA1 and possibly PIC1. PAA2 is thought to transport Cu across the thylakoid membrane. Transport of Fe into the chloroplast is known to require reduction by FRO7 and may involve transport by PIC1. Very little is known about transport in and out of the mitochondria, though ATM3 is well established as an Fe-S exporter.

Chloroplast. Nearly 90% of the Fe in the plant is localized to the chloroplast, where it is required for use in the electron transport chain and the synthesis of chlorophyll, heme and Fe-S clusters[17]. In addition, Cu and Zn or Fe are required in the chloroplast as cofactors for superoxide dismutases (SODs), which catalyze the conversion of superoxides to hydrogen peroxide, preventing cellular damage by the extremely reactive hydroxyl radical species normally produced from the electron transport chain[51]. Recent work has shown that the two chloroplastic Fe-SODs, FSD2 and FSD3, are required to protect the chloroplast from ROS damage during chloroplast development[52]. CuZn-SODs are the other type of SOD found in the chloroplast, and under limiting conditions of metals, plants can make either CuZn-SOD or Fe-SOD (refs. 10,53). The mechanism of this fluidity of metal use as cofactors involves differential control of Cu enzymes by microRNAs[54,55], which are, in turn, regulated by the transcription factor SPL7 (squamosa promoter binding protein like)[56]. Interestingly, transcripts encoding essential proteins requiring Cu such as plastocyanin are not targeted for degradation by the microRNAs, whereas CuZn-SOD transcripts are targeted for degradation under Cu-limiting conditions. This allows plants to allocate Cu to the most essential functions under Cu deficiency while using other metals for enzymes that carry out equivalent functions. This type of flexibility has also been observed in *Chlamydomonas*, where expression of the heme containing cytochrome c_6 is induced under Cu deficiency while the Cu-dependent plastocyanin is actively degraded[57].

Although the necessity of metals in the chloroplast has been clearly established, the transporters responsible are still not all identified. The permease PIC1 localizes to the inner chloroplast envelope and is critical for chloroplast development[58]. PIC1 was able to complement the yeast Fe uptake mutant *fet3fet4* as well as the yeast Cu uptake mutant *ctr1*, which suggests that it may also transport these metals in plants, although whether PIC1 can transport Fe and/or Cu in plants has yet to be shown. An alternative role for PIC1 has been suggested, as it has also been identified as a component of a protein translocation complex[59]. In addition to the requirement for a transporter, it has been recently shown that reduction of Fe by FRO7 is required for uptake into the chloroplast[60]. *fro7* mutants have reduced Fe in the chloroplast and show photosynthetic defects, including perturbed photosystem components and compromised electron transport. Most importantly, FRO7 is required for seedling survival under Fe-limiting conditions. The requirement for reduction at the chloroplast[60], along with (i) the identification of a reductase in the mitochondrial proteome[61], (ii) the abundance of other FROs[62] and (iii) the transport of Fe as Fe^{3+}-citrate chelates[33], raises the possibility that Fe must be reduced at each of the membranes that it must cross.

Although the chloroplastic transporters for Fe and Zn are not yet determined, more is known about Cu transport into the chloroplast. Previous work identified PAA1 (HMA6) and PAA2 (HMA8), two members of the Cu-transporting P_{IB}-type ATPase family, as critical for Cu delivery to plastocyanin in the chloroplast[63,64]. PAA1 localizes to the inner chloroplast envelope, and PAA2 localizes to the thylakoid membrane. Cu transport into the chloroplast is not completely abolished in *paa1paa2* mutants, which suggests the action of other transporters. In addition to PAA1, another family member that is a possible candidate is HMA1, which localizes to the chloroplast envelope and shows increased ATPase activity in the presence of Cu and Zn (refs. 65,66). Found to be a Ca^{+}/heavy metal pump in yeast, HMA1 may play a specialized role in Cu delivery to superoxide dismutase, as *hma1* mutants show reduced chloroplastic CuZn-SOD activity but normal plastocyanin content[65,66].

Mitochondria. Fe and Cu must also be transported into the mitochondria to function in the respiratory electron transport chain and in synthesis of Fe-S clusters. As with the chloroplast, the mitochondrial transporters for these metals have not yet been identified. While no importer has been found, STA1/AtATM3, an ABC transporter orthologous to the yeast ATM1p, has been implicated in the export of Fe-S clusters and can rescue yeast *atm1* mutants[67]. Though the other two ATM proteins in *Arabidopsis*, ATM1 and ATM2, also localize to the mitochondria, they are not able to rescue the mutant and most likely do not

play a role in Fe-S cluster export in plants[68]. Little more is known for Cu transport. The Cu chaperone Cox17 has been implicated in Cu delivery within the mitochondria[69], but no transporter has been identified yet. Zn is most likely transported by a ZIP that localizes to the mitochondria, but as of yet no ZIP transporters have been assigned this function.

Vacuole. The vacuole is emerging as an essential metal storage compartment in seeds, functioning during early seedling development as an initial store of metals for the plant before uptake from the environment is possible. Fe is known to be transported into the vacuole by the transporter VIT1, which has been recently shown to be critical for proper localization of Fe in the seed[70]. Remobilization of Fe from the vacuole is thought to be mediated by the actions of NRAMP3 and NRAMP4 (ref. 71), which have been shown to be upregulated under Fe deficiency and which localize to the vacuolar membrane[71,72]. Though single mutants of either NRAMP3 or NRAMP4 show no phenotype, *nramp3nramp4* mutants show a 90% lethality rate when germinated on Fe-deficient soils, which suggests that these proteins are functionally redundant and are required for Fe mobilization during early seedling development[71].

Zn has been shown to be transported into the vacuole by members of the MTP (metal tolerance protein) family—also referred to as CDF (cation diffusion facilitator) proteins. Both MTP1 and MTP3 localize to the vacuolar membrane[73–76], and overexpression of MTP1 or MTP3 confers resistance to high levels of Zn (refs. 73,74). Loss of expression of MTP1 or MTP3 confers Zn hypersensitivity, which further supports a role for these transporters in Zn vacuolar loading. The transporters responsible for Zn remobilization from the vacuole are not yet known. A proteomic analysis of a vacuolar membrane-enriched fraction from rice roots identified two transition metal transporters, ZIP2 and COPT5 (ref. 77). These transporters are thus candidates for transporting metals into the vacuole, but there are as of yet no functional data to go along with this localization.

Strategies for dealing with toxicity of metals

The same qualities that make these transition metals so essential as cofactors can also make them highly toxic within the cell. For example, free Fe can generate high levels of oxygen and hydroxyl free radicals through the Fenton reaction[78]. The main strategies that the plant uses to combat this toxicity are sequestration and chelation to carrier molecules.

In addition to sequestration within the vacuole, Fe has been shown to be stored in plastids in ferritin, a protein nanocage that can store up to 4,500 atoms of Fe^{3+} in its interior as an Fe oxide mineral[79]. In animals, ferritin is the primary storage form for Fe, but recent work has suggested that in *Arabidopsis* the role of ferritin is solely to deal with excess Fe and prevent oxidative damage[80], much like the detoxifying role of ferritin in bacteria[81] and *Chlamydomonas*[82,83]. Of the four ferritins found in *Arabidopsis*, only FER2 is found in the seed, whereas FER1, FER3 and FER4 are expressed in shoot tissue and FER1 is found in the root[84]. When FER2 was knocked out, seed Fe levels remained unchanged, but the seeds showed increased susceptibility to oxidative stress during germination. Likewise, when FER1, FER3 and FER4 were all knocked out, there was clear evidence of oxidative stress in the shoot, while shoot Fe levels remained unchanged[80]. This suggests that, unlike in animals, most plants use ferritin primarily to detoxify Fe rather than as a major storage unit. Some plants, however, do use ferritin as a storage unit, and an exciting new study has shown that oceanic diatoms use ferritin to safely store Fe for later use[85]. This finding is remarkable not only because it identified ferritin in a lineage that had not previously been known to have ferritin, but also because it demonstrated that its presence in these diatoms confers a competitive growth advantage over other oceanic diatoms that do not have ferritin[85].

Many transition metals are complexed with carriers as another strategy to prevent toxicity. In animals, Fe, Cu and Zn are all found associated with carrier molecules—notably transferrin for Fe and albumin for Cu and Zn (ref. 1). In plants, there is no known chaperone for Zn or Fe, although a group recently identified an Fe chaperone in humans[86]. In plants, Fe is often found chelated to NA, citrate or phytosiderophores[32]. Cu is found bound to chaperones that deliver it to particular compartments or proteins where it will be used[10]. CCH binds Cu^{+} and is thought to recycle Cu from senescing tissue[87]. Another Cu chaperone, CCS1, is thought to deliver Cu to superoxide dismutase in the chloroplast[88]. Yet another, COX19, increases under Cu treatment or induction of ROS production and may deliver Cu to cytochrome *c* oxidase in the mitochondria[89]. We should also point out that metallothioneins and phytochelatins can both bind metals and probably function in protecting plants from metal toxicity[90,91].

Current technology for visualizing metals

As we think about advances in the field regarding metal transport and localization, it is important to note the challenge for researchers of tracking metal transport, given the limits of metal imaging techniques currently available. Methods such as inductively coupled plasma mass spectrometry (ICP-MS) allow for sensitive metal detection[92], but ICP-MS requires destructive preparation of the samples, thus limiting its use in following metal localization. As mentioned above, PETIS technology, which uses gamma rays emitted from positrons (β+), has been used to visualize and quantify the uptake and translocation of radiolabeled ^{52}Fe and ^{62}Zn in grasses[15,16,24,93], but such imaging requires specialized equipment and cannot give cellular and subcellular resolution. Synchrotron X-ray fluorescence (SXRF) also allows the nondestructive spatial visualization of metal abundance at the tissue level and has the great advantage that it can be used to detect multiple metals simultaneously at high resolution[70,94]. However, to easily examine metal localization *in vivo*, one would really like metal-specific fluorophores. Fluorescent small molecules that respond to metal ions in the cell with appropriate selectivity and sensitivity offer the ability to probe the cell biology of metals with spatial and temporal fidelity[95]. Though a fluorescent sensor has been used for localization of Zn in *Arabidopsis* roots[96], the fluorescent sensors for Cu have not been used in plants, and so far there is no suitable fluorescent sensor for Fe.

Remaining questions

Despite the recent advances in understanding metal homeostasis in plants, there are still many questions that remain to be resolved. Plants have clearly overcome the many challenges of metal homeostasis, from uptake to transport to localization to toxicity. Of these, we understand the most about uptake and overcoming toxicity. Transport between tissues and subcellular localization still pose many questions. For example, it is still unclear how the vasculature is unloaded and reloaded in the shoot—a critical step in getting metals to the places where they are required. At the cellular level, the transporters involved in mitochondrial and chloroplastic transport have yet to be fully understood. Given the essential function of Fe and Cu in both of these compartments, it will be of great interest to know what transporters regulate movement of these metals. In addition, it is known that metals exist in multiple reduction states, and many transporters and chelators are specific to a particular valency. It is very likely that yet-uncharacterized reductases are present at transition areas where metals must be reduced for transport or binding, and future studies will be needed to identify essential players.

Beyond the scope of this review are the many more unanswered questions regarding regulation of metal homeostasis; Fe regulation has been recently reviewed[97]. Another challenge that was not discussed in this

review is how metalloproteins acquire the correct metal. In the case of Cu, it appears that chaperones deliver Cu, but is this also true for other metals? Recent work in cyanobacteria documents that the compartment where a metalloprotein folds can determine which metal it binds[98,99]. In addition, many new advances in other plant lineages have not been discussed here, and comparisons among diverse species will lead to a better understanding of metal transport and distribution.

Given the importance of metals to the survival and proper function of plants, and given the importance of plants to nutrition and energy, it is imperative that research address the many unknowns that remain in the field of metal homeostasis.

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