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Research review

Forging a symbiosis: transition metal delivery in symbiotic nitrogen fixation

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Summary

Symbiotic nitrogen fixation carried out by the interaction between legumes and rhizobia is the main source of nitrogen in natural ecosystems and in sustainable agriculture. For the symbiosis to be viable, nutrient exchange between the partners is essential. Transition metals are among the nutrients delivered to the nitrogen-fixing bacteria within the legume root nodule cells. These elements are used as cofactors for many of the enzymes controlling nodule development and function, including nitrogenase, the only known enzyme able to convert N_2 into NH_3. In this review, we discuss the current knowledge on how iron, zinc, copper, and molybdenum reach the nodules, how they are delivered to nodule cells, and how they are transferred to nitrogen-fixing bacteria within.

Introduction

Nitrogen is one of the most difficult-to-acquire macronutrients in the biosphere. Its most abundant form, dinitrogen (N_2), can only be accessed by a small and phylogenetically diverse group of bacteria and archaea that synthesize the enzyme nitrogenase (Dos Santos *et al.*, 2012). This metalloenzyme complex is unique in its capacity to catalyze the reduction of N_2 to ammonia, one of the most energy- and catalytically demanding reactions in biology. Non-nitrogen fixers must ultimately obtain their nitrogen from either free-living or symbiotic diazotrophic bacteria, from geological/atmospheric events (such as volcanoes or lightning), or, since the early 20th century, from polluting and costly synthetic chemical fertilizers (Gruber & Galloway, 2008).

Amid the different strategies to get ahead in the competition for assimilable nitrogen, the endosymbiosis established 60 million years ago between legumes and rhizobia is one of the most sophisticated and best studied (Oldroyd, 2013; Downie, 2014). Rhizobia are diazotrophic bacteria that, through a complex and partner-specific signaling process, promote the development of root nodules (Oldroyd, 2013; Xiao *et al.*, 2014). These bacteria colonize the nodule cells establishing organelle-like structures, the symbiosomes, in which rhizobia differentiate into bacteroids surrounded by the symbiosome membrane, originating from the host cell (Wang *et al.*, 2010; Kereszt *et al.*, 2011; Ivanov *et al.*, 2012). Only fully mature bacteroids synthesize nitrogenases, which in turn produce the ammonia that will be delivered to the host plant in exchange for other mineral nutrients and photosynthates (Udvardi & Poole, 2013). Efficient nutrient exchange is essential for the maintenance of the symbiotic partnership. When nitrogen delivery to the host is stopped or no longer necessary, the symbiosis is aborted (Hahn *et al.*, 1984; Goh *et al.*, 2019). Similarly, if the host plant does not provide photosynthates or mineral nutrients (such as transition metals, phosphate, or sulfate), the endosymbiont dies and nitrogen fixation is interrupted (Watson *et al.*, 1988; Krusell *et al.*, 2005).

Transition metals, such as iron, molybdenum, copper, or zinc, are among those nutrients that must be transferred from the host to the symbionts (Johnston *et al.*, 2001). These elements are catalytic cofactors and structural components of around a third of the proteins of a typical cell, participating in almost every biological process (Finkelstein, 2009; Foster *et al.*, 2014). Nodule cells have an even greater demand since many of the key enzymes of symbiotic nitrogen fixation are highly expressed metalloenzymes. Iron and molybdenum are components of the ubiquitous bacteroid-expressed Fe, Mo-nitrogenase metallic clusters (Fe_4-S_4, Fe_8-S_7 or

P-cluster, and FeMo-co Fe_7-S_9-C-Mo-homocitrate; Burén *et al.*, 2020). Moreover, cytosolic iron-containing leghemoglobins are used to maintain the microaerobic conditions required to balance the strictly aerobic bacteroid metabolism with the oxygen-sensitive nitrogenase (Appleby, 1984; Ott *et al.*, 2005). To be able to sustain oxidative respiration in this low-oxygen environment, bacteroids synthesize specialized iron- and copper-dependent cytochrome oxidases with high O_2 affinity (Preisig *et al.*, 1996) that are essential to satisfy the high energy demands of symbiotic nitrogen fixation. Other metalloproteins are involved in signal transduction (such as NADPH-oxidases) or free radical control (catalases or superoxide dismutases, for instance), among several other processes in the symbiosis (Dalton *et al.*, 1998; Rubio *et al.*, 2004; Arthikala *et al.*, 2014). The importance of metals for this symbiotic nitrogen fixation cannot be overstated considering that nodules represent *c.* 5% of the total plant biomass, but could contain 25–30% of the total plant transition metal content (Burton *et al.*, 1998; O'Hara, 2001). Therefore, it is not unexpected that metal deficiency in soils negatively impacts nodulation and symbiotic nitrogen fixation (O'Hara *et al.*, 1988; Tang *et al.*, 1991, 1992; Johnston *et al.*, 2001). In this review, we present our current understanding of transition metal delivery to legume nodules, together with the outstanding questions in the area, expanding on previous revisions focused just on one element (Brear *et al.*, 2013; Day & Smith, 2021) or dated before the recent advances on our understanding of metal transport in nodules (Johnston *et al.*, 2001; González-Guerrero *et al.*, 2014).

Metal uptake from soil

Transition elements, particularly iron and zinc, are limiting nutrients for plants in many regions, including some of the main agricultural areas of the world (Chen & Barak, 1982; Alloway, 2008). This is typically the consequence of low metal bioavailability in calcareous soils, rather than their low abundance. Low metal availability is also an added challenge for nitrogen-fixing nodules, which rely on the plant for metal supply.

Plants have developed precise and exquisitely controlled mechanisms for soil metal uptake and delivery/distribution within the plant (Mendel, 2011; Olsen & Palmgren, 2014; Connorton *et al.*, 2017; Andresen *et al.*, 2018). As dicots, legumes possess a metal uptake network similar to that of *Arabidopsis thaliana* (Fig. 1). The rhizosphere is acidified by H^+-ATPases that increase metal solubility (Santi & Schmidt, 2009). Then, ferric reductase oxidases (FRO) reduce Fe^{3+} and Cu^{2+} to more soluble Fe^{2+} and Cu^+ (Robinson *et al.*, 1999; Bernal *et al.*, 2012), which are transported into the epidermal cell through specific transporters, typically of the ZRT1-, IRT1-like protein (ZIP) and natural resistance-associated macrophage protein (NRAMP) families for divalent cations and copper transporter (COPT) family for Cu^+ (Vert *et al.*, 2002; Sancenon *et al.*, 2004; Castaings *et al.*, 2016). Specifically in legumes, FRO1-mediated reduction of Fe^{3+} seems to be the main contributor for iron uptake (Grusak *et al.*, 1990a; Waters *et al.*, 2002; Andaluz *et al.*, 2009). However, the identity of the specific transporters responsible for legume metal uptake from soil still remains unsolved due to a lack of localization data, although a number of publications have studied legume ZIP (López-Millán *et al.*, 2004; Stephens *et al.*, 2011), COPT (Wang *et al.*, 2021), or NRAMP transporters (Qin *et al.*, 2017). Nevertheless, it has been proposed that pea RIT1 protein would be functionally equivalent to *A. thaliana* IRT1 (Cohen *et al.*, 2004). In plants, most metal uptake genes are typically upregulated under metal deficiency (Colangelo & Guerinot, 2004; Bernal *et al.*, 2012; Lilay *et al.*, 2021). Nodulation elicits a similar response in legumes, as the plant prepares for the increased metal demand of symbiotic nitrogen fixation (Terry *et al.*, 1991).

While phenylpropanoid-derived coumarins have been identified as important elements for plant iron uptake (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2016; Tsai *et al.*, 2018), they do not seem to play the same role in the model legume *Medicago truncatula*. In fact, releasing coumarins to the rhizosphere might be disadvantageous to legumes, as it could lead to decreased expression of rhizobial nodulation genes (Peters & Long, 1988). Comparative transcriptomic analysis of iron-deficient roots of Arabidopsis and *M. truncatula* indicates that flavins could functionally substitute coumarins in legume species (Rodríguez-Celma *et al.*, 2013). However, further characterization of the legume iron deficiency response, of their root exudates, and of the combined effect of flavins/flavonoids on nodulation must be carried out.

Metal delivery to nodules

Iron and likely other transition elements travel from the epidermis to the stele following three alternative pathways: the symplastic pathway, the apoplastic pathway, and the less-characterized transcellular pathway (Barberon & Geldner, 2014; Curie & Mari, 2017; Fig. 1). In the first one, metals within the epidermal cell will transit through plasmodesmata to the root pericycle cells. In the apoplastic pathway, metals will move from the epidermis to the stele following a concentration gradient. Along the way, some of these cations can be stored associated with negatively charged cell walls to be used under metal-deficient conditions (Curie & Mari, 2017). Finally, the transcellular pathway would require the polar distribution of efflux and uptake transporters to ensure a directionality of metal transport, as it has been illustrated for boron (Barberon & Geldner, 2014). Metals using either of the latter two pathways would be largely stopped at the Casparian strip in the endodermis. Delivering these metals to sink organs would require an additional step of metal import into the endodermal cells. For long-distance transport, metals are released to the xylem, through which they travel as metal complexes and are delivered to the shoots.

Since nodules are in close contact with the soil, it could be expected that metals are acquired directly through the nodule epidermal cells, using the mechanisms described previously. However, diffusion barriers, similar to a Casparian strip, surround the nodule cortical cells (Hartmann *et al.*, 2002; Minchin *et al.*, 2008), limiting metal transit to the symplastic pathway to reach the nitrogen-fixing cells. This model of metal transport from the nodule-soil interface would be compatible with the developmental pattern of determinate type nodules (such as those of *Phaseolus* and *Glycine*), in which the whole nodule matures quite

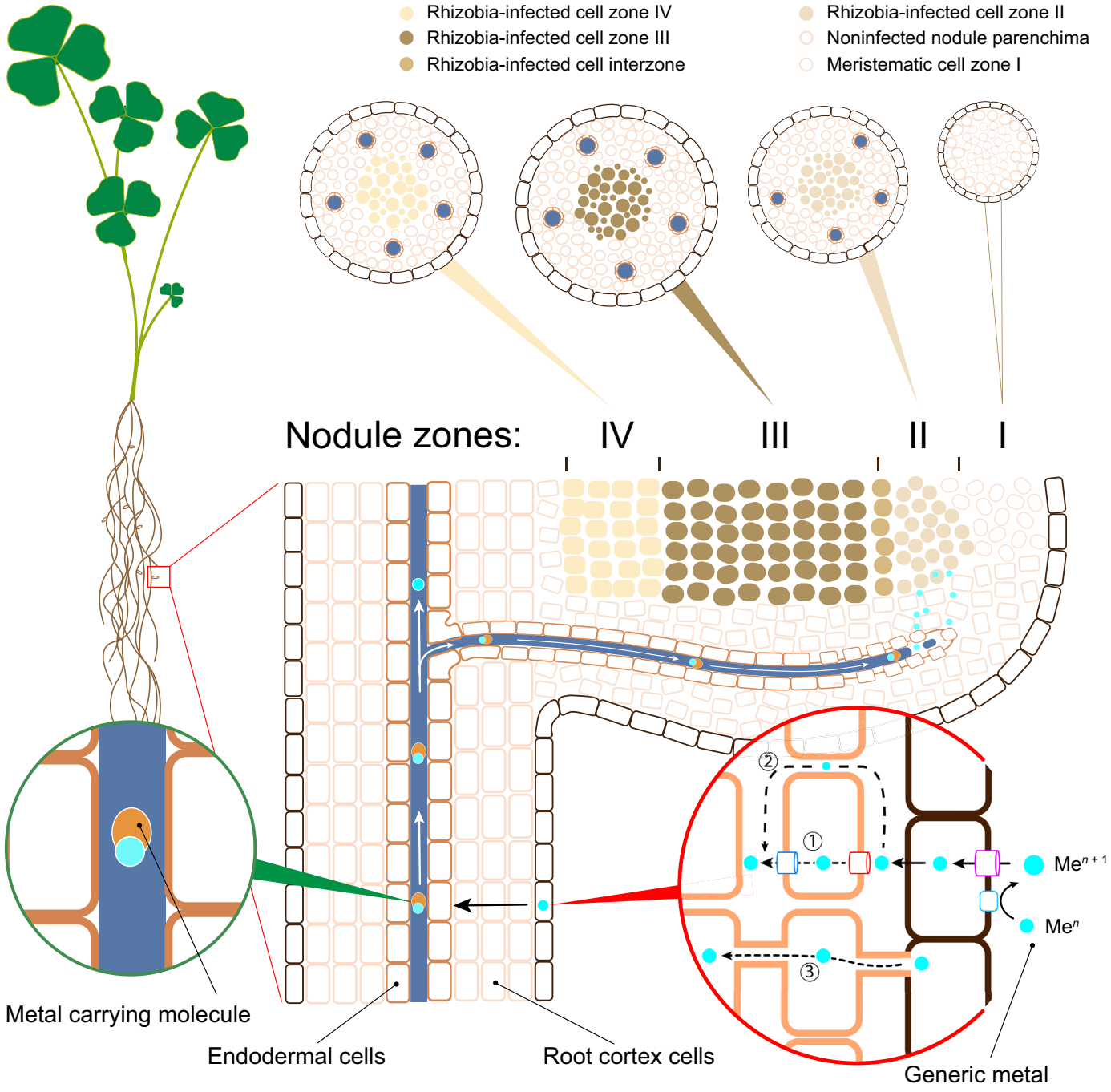

Fig. 1 Metal delivery to indeterminate-type nodules. Diagram illustrating how transition metals are introduced from soil, a process that may require reduction and the use of flavins. Transition metals are delivered to the vasculature following through three possible pathways: transcellular (1), apoplast (2), or symplast (3). Upon reaching the endodermis, transcellular and apoplastic metals have to be transported into the cells. Transition metals are then released to the vasculature, where they bind to metal carriers (e.g. citrate or nicotianamine) to be delivered to the infection/differentiation zone of the nodules.

uniformly, and metal demands would be similar throughout the nodule (Hirsch, 1992). However, not all legumes develop nodules in this way; some (such as *Medicago* or *Pisum*) retain their apical meristem, which leads to a continuous, indeterminate, growth and to the establishment of different developmental zones along the nodule axis (meristem, infection/differentiation zone, interzone, fixation zone, and senescent zone; Vasse *et al.*, 1990; Fig. 1). Each of these areas would have different metal demands depending on the stage of bacteroid differentiation and nitrogenase synthesis. In indeterminate-type nodules, metal delivery should be more focused on the regions where symbiosis is being established, where oxygen concentration is being reduced and nitrogenase synthesized. To this end, a more targeted, vascular, metal delivery strategy would be advantageous. Accordingly, metals would be primarily delivered to

the differentiation/early fixation zone of the nodules, where metalloprotein synthesis, particularly nitrogenase components, is very active (Vasse *et al.*, 1990). Furthermore, vascular metal delivery allows the coordination of metal partitioning between leaves and nodules, arguably the two main metal sinks in the legume vegetative stage. Unfortunately, no isotopic studies have been carried out to date that would allow us to discern between vascular vs epidermal metal delivery pathways, although these methodologies have been used for non-nodulated legumes in the past (Oliveira *et al.*, 2014). However, insights have been gained from metal imaging and metal transporter localization studies.

Synchrotron-based X-ray fluorescence studies on elemental distribution in the indeterminate nodules formed by *M. truncatula* support the targeted vascular metal delivery hypothesis (Rodríguez-Haas *et al.*, 2013), as iron localization changes in the different nodule developmental zones. In the differentiation zone, iron accumulates in the apoplast, whereas in the fixation zone, iron is mostly present within the rhizobia-containing cells, matching the symbiosome distribution. These observations suggest that iron, and likely other metal nutrients, is released from the vasculature to the nodule infection/differentiation zone and later incorporated into the symbiosomes of rhizobia-infected cells as the fixation zone develops (Fig. 1). Further evidence for this model comes from the localized expression in the late differentiation zone-early fixation zone of many of the transporters involved in metal entry into rhizobia-containing cells (Tejada-Jiménez *et al.*, 2015; Abreu *et al.*, 2017; Senovilla *et al.*, 2018), as well as the phenotypes of mutants in nodule vascular metal transport (Castro-Rodríguez *et al.*, 2020; Escudero *et al.*, 2020a), detailed in the following sections. Furthermore, vascular transport is also important in determinate type nodules, as mutants causing iron retention in the xylem result in less iron available to the nodule (Takanashi *et al.*, 2013).

However, other authors have suggested that under some circumstances, such as iron deficiency, direct metal uptake from the soil surrounding the nodules could be possible (Slatni *et al.*, 2012). This is based on the detection of proteins immunoreactive to an antibody raised against *A. thaliana* IRT1 in the nodule cortex of common bean (*Phaseolus vulgaris*). However, these immunoassays do not necessarily detect the orthologue of Arabidopsis IRT1, the protein responsible for iron uptake from soil. The antibodies raised could be detecting any other ZIP protein that could be transporting not only Fe^{2+}, but also Zn^{2+}, or Mn^{2+} into other compartments/cell layers. In addition, no epidermal localization of this putative ZIP protein could be clearly discerned in the images. Overall, we consider that stronger transporter localization data and/or isotopic tracing studies are still required to conclude the existence of direct metal uptake from soil by the nodule epidermis.

Little is known on the specifics (genes, chelates, or the tissues involved) of vascular transport of metals to nodules. Considering that most of the metals are acquired via uptake into root cells, it would be expected that a system similar to the one used to transport metals to the shoot would be in place (Puig & Peñarrubia, 2009; Conte & Walker, 2011). This means that metals would traffic through the pericycle into the xylem where they would form complexes with citrate and other organic acids (Flis *et al.*, 2016). However, studies using fluorescent probes to trace water transport in the root-nodule system indicate that the nodule core is not apoplastically connected to the root, and that water is distributed to the nodules via the phloem (Bederska *et al.*, 2012). Once in the nodule, solutes will move to the nodule endodermis, which is apoplastically disconnected from the inner nodule layers. Additional support for a symplastic delivery of metals to nodules is the prominent role of nicotianamine (NA) and metal-NA transporters of the Yellow Stripe1-Like (YSL) family in symbiotic nitrogen fixation (Fig. 2). NA is the preferred metal chelator in the phloem (Flis *et al.*, 2016), where it can bind to iron, copper, or zinc. These NA–metal complexes would be introduced in the cell by YSL proteins (Curie *et al.*, 2008). Castro-Rodríguez *et al.* (2020) showed that *M. truncatula* YSL3 protein is required for iron and zinc delivery to nodules. Loss of this transporter results in less iron reaching the fixation zone of the nodule, and in zinc being retained in the vasculature. More recently, Wu *et al.* (2023) have proposed that soybean YSL7 could also be involved in iron-NA transport to nodules at the vascular levels. This is largely based on the ability of GmYSL7 to complement a *fet3fet4* iron uptake yeast mutant when iron is theoretically provided as a NA complex, as well as on the increase in iron toxicity of a *ccc1* iron detoxification yeast mutant when using the same substrate. However, iron–NA complexes are difficult to form *in vitro*, and free iron, not bound to NA, could be abundant. This is suggested by having similar results when transforming the yeast mutants with Fe^{2+}-transporting IRT1. Moreover, iron-NA-dependent complementation could not be achieved by other laboratories working on the same GmYSL7 or the *M. truncatula* orthologue (Castro-Rodríguez *et al.*, 2021; Gavrin *et al.*, 2021; see below). Regardless of the specific role of GmYSL7, the overall involvement of YSL transporters in metal allocation to nodules is further supported by the need of NA synthesis in nodules by NA synthases (NAS). Nitrogenase activity is severely reduced in *M. truncatula nas2* mutant (Escudero *et al.*, 2020a). As also found in *ysl3* mutants, iron is mostly retained in the apical zone (infection/differentiation) in *nas2* mutants, with less of it reaching the fixation zone. *MtNAS2* mutation also changes iron speciation in the fixation zone as observed with X-ray absorption spectroscopy, with a reduction in the percentage of iron associated with sulfur (Fe-S clusters, Fe-glutathione, …) in favor of an increase in Fe^{2+} species associated with O/N (typically organic acids or amino acids). Interestingly, no significant differences in iron coordination are observed in the vasculature, suggesting that other NAS may be compensating for the lack of NAS2.

Molybdenum cannot be delivered using a NA/YSL system, in contrast to iron, copper, and zinc. This is the consequence of molybdenum being most commonly available in the biosphere as molybdate oxoanion rather than as a cation, thereby requiring alternative transporters. In plants, high-affinity molybdate transport is carried out by proteins of the molybdate transporter1 (MOT1) family (Tejada-Jimenez *et al.*, 2013). To support the enhanced demands of nitrogen fixation, the MOT1 family has expanded in legumes compared with other dicots (Tejada-Jiménez *et al.*, 2017). While *A. thaliana* encodes two MOT1 genes, *M. truncatula* has five family members. One of them, MOT1.2 is

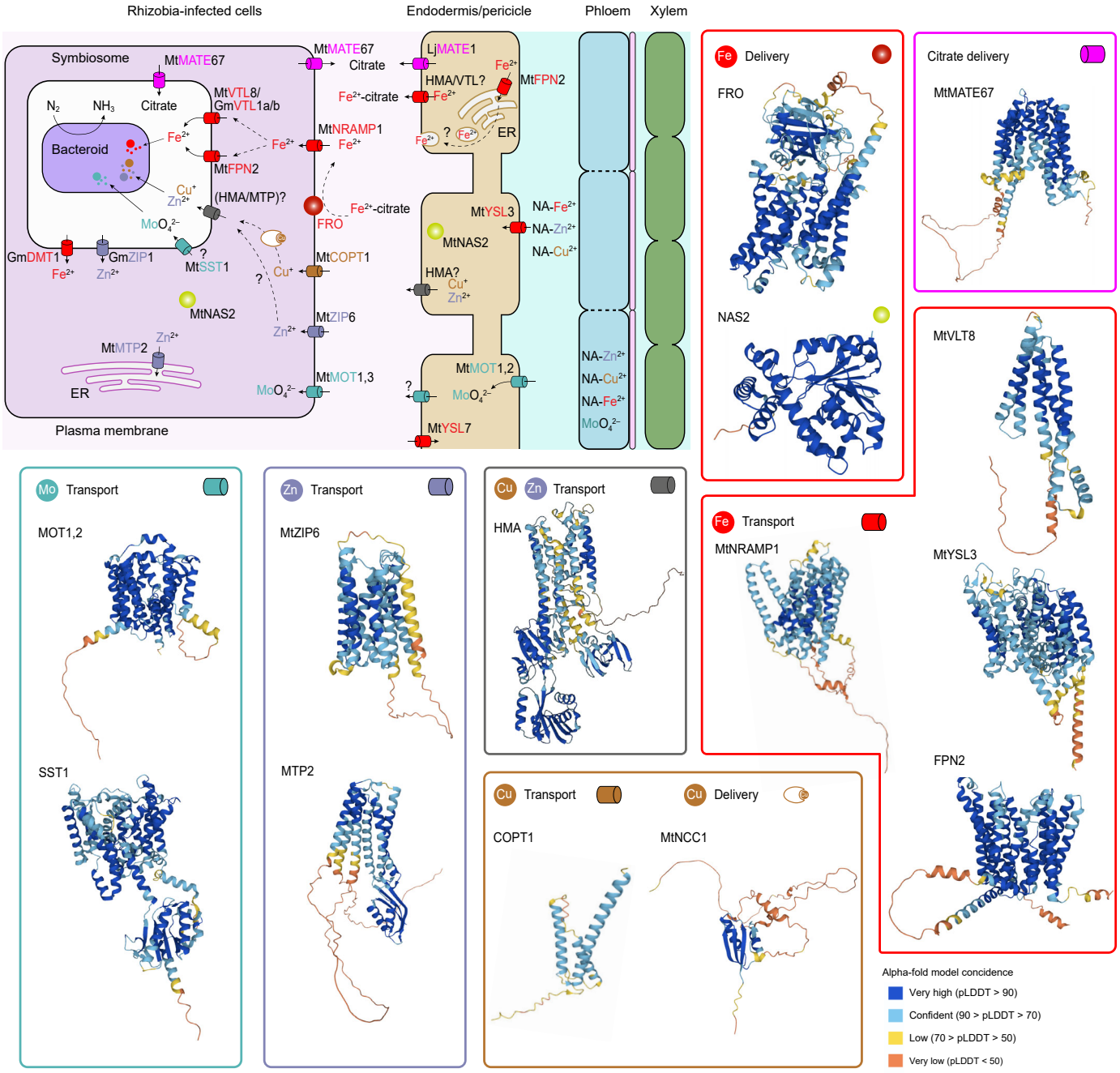

Fig. 2 Transition metal trafficking in legume nodules. The diagram illustrates the known proteins required for metal delivery from the vasculature to the symbiosomes. Interrogation marks indicate that no direct experimental evidence exists showing that specific metal substrate or that the identity of the protein in question is yet-to-be-determined although the function must exist. The different colors indicate the preferred metal substrate. The different structures have been extracted from Alpha-fold database of predicted protein structures. Gm, *Glycine max*; Lj, *Lotus japonicus*; Mt, *Medicago truncatula*.

localized in the root and nodule vasculature (Fig. 2). MOT1.2 is responsible for molybdenum delivery to nodules. Evidence for this comes from the reduction of nitrogenase activity and the lower molybdenum content of *MOT1.2* mutant nodules (Gil-Díez *et al.*, 2019).

Both YSLs and MOT1 proteins introduce their metal substrate into the cytosol (DiDonato *et al.*, 2004; Tejada-Jiménez *et al.*, 2007). Consequently, they cannot be responsible for metal release to the nodule apoplast. To date, no metal efflux transporter directly involved in metal release from nodule vasculature has been identified. No molybdate efflux transporter is currently known, and in the available transcriptomic databases, there is no evidence of any P_{1B}-ATPase induced by nodulation (Benedito *et al.*, 2010; Roux *et al.*, 2014), transporters typically responsible for zinc or copper efflux (Hussain *et al.*, 2004; Andrés-Colás *et al.*, 2006). For iron, VIT1-like (VTL) proteins and ferroportins could be carrying out this role, since they are able to extrude iron out of the cytosol as shown in biochemical assays in yeast and mammalian cells,

respectively (Li *et al.*, 2001; Drakesmith *et al.*, 2015). Recently, nodule-specific *M. truncatula* ferroportin FPN2 was observed in nodule vascular and nitrogen-fixing cells (Escudero *et al.*, 2020b). However, MtFPN2 was located in the endoplasmic reticulum of endodermal cells, and not in the plasma membrane, thus barring its participation in the direct release of iron into the nodule apoplast. Alternatively, FPN2 could mediate iron accumulation in endomembrane vesicles to be released by exocytosis to the nodule apoplast. Supporting this role is the change in iron speciation in the vasculature of mutant nodules, with a 50% decrease of Fe^{2+}-coordinated by N/O. This indicates that iron is not being bound to its usual acceptor proteins in the absence of FPN2 (Escudero *et al.*, 2020b). However, this function is not essential, since expressing *MtFPN2* only in cells from the differentiation to fixation zones of the nodule, and not in the vasculature, is sufficient to partially complement the mutant phenotype.

Regardless of the specific iron exporter implicated, apoplastic iron is ultimately bound to citrate, as indicated by the importance of citrate efflux to the nodule apoplast by multidrug and toxic compound extrusion (MATE) proteins (Fig. 2). Two nodule-specific MATE genes have been identified to date: *Lotus japonicus MATE1* and *M. truncatula MATE67* (Takanashi *et al.*, 2013; Kryvoruchko *et al.*, 2018). Both proteins are present in the nodule vasculature: LjMATE1 throughout the vasculature, while MtMATE67 only in the apical region. This different distribution pattern could be a consequence of differences in iron uptake in determinate vs indeterminate nodules. Removing these proteins results in altered iron distribution, with iron being retained in the vessels of *L. japonicus* or precipitating in the apoplast of *M. truncatula* nodules.

Metal uptake by rhizobia-infected cells

Delivered by the vasculature from the roots to the nodules, metals are released to the apoplast (specifically at the infection/differentiation zone in indeterminate-type nodules), wherefrom they must be transported into the rhizobia-infected nodule cells for nitrogen fixation to take place. In the last decade, a number of metal transporters have been identified in the plasma membrane of rhizobia-infected cells. In *M. truncatula*, NRAMP1 mediates iron uptake (Tejada-Jiménez *et al.*, 2015); COPT1, copper (Senovilla *et al.*, 2018); ZIP6, zinc (Abreu *et al.*, 2017); and MOT1.3, molybdate (Tejada-Jiménez *et al.*, 2017; Fig. 2). There is no known manganese uptake transporter described in nodules to date; however, NRAMP1 can also transport Mn^{2+} in yeast (Tejada-Jiménez *et al.*, 2015), which could reflect a dual role in iron and manganese delivery to nodules. Mutations in any of these transporters lead to a severe reduction of nitrogenase activity and altered elemental accumulation. However, since these phenotypes can be rescued with nutrient solutions fortified with the transported element, it is to be expected that other transporters are able to compensate, albeit with lower affinity. Alternatively, a symplastic route from the nodule endodermis to rhizobia-infected cells could be proposed, although this would necessarily be a suboptimal alternative, considering the strong phenotypes of many of the mutants in the transporters indicated previously.

Before uptake by the rhizobia-infected cells, a reduction step (Fe^{3+} to Fe^{2+}, for instance) would be necessary. The requirement of citrate provided by MtMATE67 to deliver iron to nodules (Kryvoruchko *et al.*, 2018) indicates that apoplastic iron is Fe^{3+}, as citrate primarily binds Fe^{3+} (Rellán-Álvarez *et al.*, 2010). However, NRAMP proteins exclusively transport divalent metals, such as Fe^{2+} (Nevo & Nelson, 2006). Therefore, to account for MtNRAMP1 function, the existence of plasma membrane ferric reductases should be expected. However, no candidate has been proposed to date.

The expression patterns of genes encoding these metal transporters also show that metal uptake occurs at specific developmental stages. MtNRAMP1, MtCOPT1, and MtZIP6 are mainly produced in the differentiation-interzone-early fixation zones of the nodules (Tejada-Jiménez *et al.*, 2015; Abreu *et al.*, 2017; Senovilla *et al.*, 2018). This is the region where apoplastic metals would be introduced into nodule-core cells, consistent with the proposed model for metal delivery to nodules (Rodríguez-Haas *et al.*, 2013). By contrast, molybdate uptake, required only for FeMo-co synthesis, occurs at a later stage, as indicated by the expression of *MtMOT1.3* in interzone and fixation zones (Tejada-Jiménez *et al.*, 2017), the region where O_2 levels drop, and nitrogenase is synthesized (Vasse *et al.*, 1990; Soupène *et al.*, 1995).

Interestingly, out of these four transporters, only MtCOPT1 and MtMOT1.3 are nodule-specific. MtZIP6 and MtNRAMP1 are also expressed in roots. This could illustrate how the plant metal homeostasis network has accommodated nodulation. Nodule cells have a higher-than-average requirement for iron and molybdenum (Johnston *et al.*, 2001). However, while iron is widely used in plant processes (Connorton *et al.*, 2017), molybdenum is part of just five plant proteins, mostly expressed in shoots (Mendel, 2011; Tejada-Jimenez *et al.*, 2013). The existence of a nodule-specific MOT1 protein might be taken to indicate that the increased demand for molybdenum could only be provided by gene duplication rather than by a simple upregulation of a preexisting transporter. Alternatively, it could also be argued that the evolution of nodule-specific transporters is still underway for iron and zinc uptake. This idea is supported by a lack of redundancy of transporter genes in nodules in contrast to roots and shoots (Waters *et al.*, 2006; Klatte *et al.*, 2009; Olsen & Palmgren, 2014; Sinclair *et al.*, 2018). In most instances, mutating just the gene of interest is enough to have a drastic effect on nitrogen fixation and nodule metal homeostasis, indicating a partial specialization to participate in symbiotic nitrogen fixation.

Metal transfer to symbiosomes

Once within a cell, metals have to be allocated to different cellular compartments and delivered to a plethora of metalloproteins. This does not only include symbiosomes, where iron, molybdenum, and copper are essential for nitrogen fixation (Preisig *et al.*, 1996; Burén *et al.*, 2020), but to host-dependent symbiosis-related and in general housekeeping reactions. Considering that there is little free, hydrated, metal in the cytosol (Outten & O'Halloran, 2001; O'Halloran & Finney, 2003; Waldron & Robinson, 2009), specific systems for sorting metals among the different cellular

compartments must be in place. Although they remain unknown, they would involve yet-to-be-characterized metallochaperones connecting the different donor and acceptor proteins (Fig. 2). Similar mechanisms are in place to control subcellular copper allocation in other bacterial and eukaryotic cells (O'Halloran & Culotta, 2000; Tottey *et al.*, 2002). Recent evidence indicates that nodules have specific Cu^{+}-chaperones that are able to metallate a subset of the cell copper proteome (Navarro *et al.*, 2023). In addition to this metal pool, transition metals would also bind to NA, considering that *NAS2* is highly expressed in all nodule cells (Escudero *et al.*, 2020a). Future work must focus on further characterization of nodule metallochaperones, on determining new metalloproteins linked to them, and on the role of the intracellular metal-NA pool to understand how intracellular metal sorting is achieved.

Iron translocation across the symbiosome membrane also requires citrate, as indicated by the localization of MtMATE67 in this membrane as well (Kryvoruchko *et al.*, 2018). This is consistent with data from rhizobia and bacteroid iron uptake that showed citrate as their preferred iron form (Moreau *et al.*, 1995; Benson *et al.*, 2005). Since MtMATE67 does not transport iron–citrate complexes, iron must be extruded by another protein. However, citrate efflux and iron efflux are linked by the activation of MATE67 by iron (Kryvoruchko *et al.*, 2018).

VTLs and ferroportins participate in iron transport across the symbiosome membrane (Fig. 2). Nodules express specific iron exporting VTLs required for nitrogenase activity (Hakoyama *et al.*, 2012; Brear *et al.*, 2020; Liu *et al.*, 2020; Walton *et al.*, 2020). Soybean produces two nodule-specific VTL (VTL1a and VTL1b) proteins, both with similar expression patterns, located in the symbiosome membrane (Brear *et al.*, 2020; Liu *et al.*, 2020). Mutation of both genes results in a severe reduction of nitrogenase activity and altered iron distribution (Liu *et al.*, 2020). However, VTL1b showed lower iron transport capabilities in yeast than VTL1a, which could indicate that they may have slightly different roles or different substrate affinities. In *M. truncatula*, the two nodule-specific VTLs have a different distribution. Only one of them, MtVTL8, is located in the symbiosomes (Walton *et al.*, 2020). However, the role that the second VTL plays in soybean symbiosomes could be played by the ferroportin MtFPN2 in *M. truncatula* (Escudero *et al.*, 2020b; Fig. 2). As with symbiosome VTL mutants, eliminating *MtFPN2* expression leads to white, nonfunctional nodules, with a lower iron content. Moreover, iron speciation in nodules is also altered. These phenotypes are not a consequence of the role of MtFPN2 in the endoplasmic reticulum of endodermal cells (described above), since expressing *MtFPN2* solely in cells in the differentiation to fixation zones is sufficient to restore nitrogenase activity (Escudero *et al.*, 2020b). The duplicity of symbiosome iron transporters is intriguing, and it illustrates the large iron demands of nitrogen fixation. It also represents another layer of control of iron nutrition in nodules, particularly in *M. truncatula*, in which different transporters could have access to different iron pools or be under different regulations. Alternatively, Chu *et al.* (2022) propose that *L. japonicus SEN1* (a VTL homolog) is a molybdate transporter responsible for molybdenum allocation to symbiosomes. However, this claim needs to be substantiated by stronger biochemical evidence, given that all previously characterized members of the family have been strongly linked to iron efflux. Furthermore, while most metal transporters can use different metal substrates, these typically have the same charge (e.g. divalent metals, not a cation vs anion), and the possibility of a molybdenum cation as a substrate is remote.

Finally, iron might also be transferred to rhizobia before the colonization of nodule cells. MtVTL4 is the second *M. truncatula* nodule-specific VTL. This protein is present in the plasma membrane of cells in the infection zone and in the infections threads (Walton *et al.*, 2020), where it could act to transport iron into the apoplast or into the infection threads that guide the rhizobia from the plant surface to the nodule cells. Although rhizobia are not able to synthesize nitrogenase at this stage of infection, mutation of *VTL4* results in a 50% reduction on nitrogenase activity. The precise cause of this is not known, but iron acquired at this stage could serve as a primer to initiate nitrogenase synthesis, or to produce some of the enzymes required for nodulation, including those involved in resistance to the reactive oxygen species produced by the plant during infection (Syska *et al.*, 2019).

In contrast to the advances in our understanding of iron transfer to symbiosomes, less is known on how other transition metals are delivered (Fig. 2). Molybdenum is provided as molybdate, as indicated by the lower nitrogen fixation rates of *mod* rhizobial mutants, and the dependence on sulfate (Delgado *et al.*, 2006). However, no molybdate efflux proteins have been identified yet, other than the proposed role for LjSEN1 (see above; Chu *et al.*, 2022). Alternatively, molybdate delivery into symbiosomes could be carried out by sulfate transporters, given the importance of sulfur for nitrogenase cofactor assembly and the similar chemical nature of molybdate and sulfate (Krusell *et al.*, 2005). For zinc and copper, no evident HMA family member can be selected based on the available transcriptomic data. Alternatively, Metal Tolerance Protein (MTP) transporters could mediate zinc or copper delivery (Kolaj-Robin *et al.*, 2015) but, so far, the one most highly expressed in nodules is located in the endoplasmic reticulum (León-Mediavilla *et al.*, 2018). Early work on metal transporters in symbiotic nitrogen fixation proposed that *Glycine max* ZIP1 protein could be introducing zinc into the symbiosomes (Moreau *et al.*, 2002). However, ZIP proteins transport metals in the opposite direction (Zhang *et al.*, 2017), and this means that GmZIP1 is likely to have an alternative role.

GmZIP1 is not the only transporter of a family mediating cytosolic metal influx located in symbiosomes (Moreau *et al.*, 2002); iron-transporting NRAMP protein GmDMT1 is also present in the same compartment (Kaiser *et al.*, 2003). Both proteins are candidates for removing metals from the symbiosome. Similarly, rhizobial transporters MbfA and Nia1 are also synthesized in nodules, where they would extrude iron out of the bacteroid (Zielazinski *et al.*, 2013; Walton *et al.*, 2020; more information on rhizobial metal transporters can be found in the recent review by Abreu *et al.*, 2019). The combination of these systems indicates that there is backwards transport of metals, contrary to increasing metal content of bacteroids for nitrogen

fixation. One possible role for this system is being a 'relief valve' to prevent metal toxicity in bacteroids. This would mean that bacteroids are under a certain risk of being overloaded with metals that would then need to be detoxified. The hypothesis of a rhizobial iron overload is also consistent with the role of NCR247 peptide in bacteroid iron regulation (Sankari *et al.*, 2022). NCRs are a large family of cysteine-rich peptides that in certain legumes are essential for bacteroid maturation (Van de Velde *et al.*, 2010). These peptides are synthesized by the host cell and transferred to the bacteroid, where they exert their role. Particularly, NCR247 has been associated with bacteroid division, translation, and protein stability (Farkas *et al.*, 2014). Furthermore, NCR247 is able to bind heme, a repressor of Irr. In the absence of heme, Irr represses RirA repressor transcription, which in turn activates the transcription of several rhizobial iron uptake genes (Chao *et al.*, 2005). As a result, NCR247 is 'forcing' the bacteroid to accept a larger amount of iron, raising the risk of toxicity, but increasing iron availability for nitrogenase cofactor synthesis. Moreover, these results illustrate a mechanism of host-controlled bacteroid metal homeostasis that would coordinate both symbionts. Other NCR peptides, rich in potential metal-coordinating cysteine residues, could conceivably work as well in metal allocation and regulation in bacteroids.

Metal recovery during nodule senescence

Nodules do not remain functional forever. As the plant enters the reproductive stage, nodules senesce (Puppo *et al.*, 2005; Van de Velde *et al.*, 2006). Considering the prevalent low metal bioavailability in soils (Chen & Barak, 1982; Alloway, 2008), the large amounts used in nodules (Johnston *et al.*, 2001), and the importance of transition metals for seed production and germination (Sancenon *et al.*, 2004; Kim *et al.*, 2006; Roschzttardtz *et al.*, 2011), a large portion of the nodule metal content should be recycled. It is estimated that around half of the nodule iron is relocalized to the seeds when nodules senesce (Burton *et al.*, 1998), and a similar recovery of other limiting transition elements can be expected.

The molecular mechanisms of metal recycling from nodules have not been fully characterized. Transporters such as GmDMT1 and GmZIP1, which remove metals from symbiosomes (Moreau *et al.*, 2002; Kaiser *et al.*, 2003), could be involved in this process. NA is likely to participate, as it does in leaves (Maillard *et al.*, 2015). In fact, *L. japonicus* expresses *NAS1* specifically in senescent nodules (Hakoyama *et al.*, 2009), indicating the existence of a dedicated mechanism of metal recovery. Metal-NA relocation to the shoot could be done through the same transporters mediating metal delivery to nodules, MtYSL3 or MtMOT1.2, since none of them has a polar localization that could prevent transport from either side of the endodermis (Gil-Díez *et al.*, 2019; Castro-Rodríguez *et al.*, 2020).

Regulation of nodule metal delivery

In contrast to most other plants, vegetatively growing legumes have two different metal sinks in distinct plant organs above and below ground. Given the importance of controlling metal homeostasis, the multiple layers of regulation, and the large number of elements involved (Bernal *et al.*, 2012; Kobayashi & Nishizawa, 2012; Yan *et al.*, 2017; Kim *et al.*, 2019; Lilay *et al.*, 2021), nodulated legumes are expected to have additional, specific levels of control. This would require synchronizing and optimizing metal delivery to photosynthesis and nitrogen-fixing organs. Unfortunately, this area of research remains largely unexplored. Transcription factors bHLH57 and bHLH300, orthologous to *A. thaliana* iron-controlling transcription factors FIT and bHLH38/39/100/101 (Riaz & Guerinot, 2021), have been shown to be involved in the regulation of iron uptake in soybean, and they are expressed in nodules (Li *et al.*, 2018). Overexpression studies of bHLH300 in soybean nodules lead to lower iron accumulation in nodules and to reduced *GmYSL7* expression (Wu *et al.*, 2023). Other regulatory factors controlling nodule metal allocation could be encoded by the BRZ and DGL genes in pea, as indicated by the increased iron content in shoots and decreased nodulation reported in mutants of that gene (Gottschalk, 1987; Grusak *et al.*, 1990b; Kneen *et al.*, 1990). Interestingly, DGL has recently been mapped to an E3 ubiquitin ligase BRUTUS homolog and BRZ to a OPT3-like transporter (Harrington *et al.*, 2023), both genes associated with metal homeostasis control in Arabidopsis (Rodríguez-Celma *et al.*, 2019; Chia *et al.*, 2023).

The nodulation signaling pathways and metal homeostasis control systems are connected from the early stages of nodulation; as the symbiosis is established, the plant triggers the iron deficiency response (Terry *et al.*, 1991). Moreover, the phenotype of plant mutants affected in metal transfer to nodules suggests the existence of metal deficiency signals originating from rhizobia-colonized cells. The plant iron deficiency response is upregulated in *MtFPN2* mutants, likely a result of bacteroids not receiving sufficient iron (Escudero *et al.*, 2020b). The existence of long-distance signaling for enhancing the supply of zinc, copper, or molybdenum nodule can be also inferred from the phenotype of *copt1-1*, *mot1.3-1* mutants and the *zip6* RNAi lines (Abreu *et al.*, 2017; Tejada-Jiménez *et al.*, 2017; Senovilla *et al.*, 2018). Reduced metal uptake capabilities of nodule cells from these mutant plants somehow lead to an oversupply of metals to these nodules, which accumulate significantly higher total concentrations of metals than healthy wild-type (WT) nodules. This metal excess is typically observed in the nodule apoplast (Abreu *et al.*, 2017; Senovilla *et al.*, 2018).

The nature of the hypothetical metal deficiency signal is not known, but YSL proteins might be involved in its transduction. In *A. thaliana*, it has been shown that functional YSL1 and YSL3 proteins participate in the long-distance signaling of iron deficiency to roots by a yet-to-be-defined mechanism (Kumar *et al.*, 2017). YSL7 is a candidate to mediate metal signaling in soybean and *M. truncatula* nodules (Castro-Rodríguez *et al.*, 2021; Gavrin *et al.*, 2021). Arabidopsis, soybean, and *M. truncatula* YSL7 proteins transport short peptides (4–12 amino acid long) when expressed in yeast. Mutation of soybean and *M. truncatula YSL7* leads to reduced nitrogen fixation rates, as well as to an altered iron homeostasis response, in addition to dysregulation of other metabolic processes. In *M. truncatula*, *ysl7* mutants accumulate more copper and iron in nodules, a likely consequence of the overexpression of the iron uptake machinery in roots (Castro-Rodríguez *et al.*, 2021). Both MtYSL7 and GmYSL7 transport a

Table 1 Transporters involved in metal delivery to nodules.

Accession no.	Name	Substrate	Nodule cell	Nodule zone	Nodule-specific	Reference
BAL46698	LjSEN1	Fe^{2+}	Rhizobia-infected cells	–	Yes	Hakoyama *et al*. (2012)
BAN59993	LjMATE1	Citrate	Rhizobia-infected cells	–	Yes	Takanashi *et al*. (2013)
Glyma.05g121600	GmVTL1a	Fe^{2+}	Rhizobia-infected cells	–	Yes	Brear *et al*. (2020); Liu *et al*. (2020)
Glyma.08g076300	GmVTL1b	Fe^{2+}?	Rhizobia-infected cells	–	Yes	Brear *et al*. (2020); Liu *et al*. (2020)
Glyma.11g203400	GmYSL7	Oligopeptides Fe-NA?	Rhizobia-infected cells	–	Yes	Gavrin *et al*. (2021); Wu *et al*. (2023)
Glyma.20g063100	GmZIP1	Zn^{2+}	Undetermined	–	Yes	Moreau *et al*. (2002)
Glyma.17g18010	GmDMT1	Fe^{2+}	Undetermined	–	No	Kaiser *et al*. (2003)
Medtr1g010270	MtMOT1.2	MoO_4^{2-}	Endodermal cells (vasculature)	–	No	Tejada-Jiménez *et al*. (2017); Gil-Díez *et al*. (2019)
Medtr3g063490	MtYSL7	Oligopeptides	Vasculature and cortical cells	–	No	Castro-Rodríguez *et al*. (2021)
Medtr3g088460	MtNRAMP1	Fe^{2+} Mn^{2+}?	Rhizobia-infected and noninfected cells	ZII-IZ	No	Tejada-Jiménez *et al*. (2015)
Medtr3g092090	MtYSL3	NA-Fe^{2+}? NA-Zn^{2+}?	Endodermal (vasculature) and cortical cells	–	No	Castro-Rodríguez *et al*. (2020)
Medtr3g464210	MtMOT1.3	MoO_4^{2-}	Rhizobia-infected and noninfected cells	ZII-ZIII	Yes	Tejada-Jiménez *et al*. (2017)
Medtr4g013240	MtFPN2	Fe^{2+}	Endodermal and parenchyma cells (vasculature); rhizobia-infected and noninfected cells	IZ-early ZIIIs	Yes	Escudero *et al*. (2020b)
Medtr4g019870	MtCOPT1	Cu^{+}	Rhizobia-infected and noninfected cells	ZII-early ZIII	Yes	Senovilla *et al*. (2018)
Medtr4g064893	MtMTP2	Zn^{2+}	Rhizobia-infected cells	ZII-ZIII	No	León-Mediavilla *et al*. (2018)
Medtr4g083570	MtZIP6	Zn^{2+}	Rhizobia-infected cells	ZII-early ZIII	No	Abreu *et al*. (2017)
Medtr4g094325	MtVTL4	Fe^{2+}	Rhizobia-infected cells	ZII-infection threads	Yes	Walton *et al*. (2020)
Medtr4g094335	MtVTL8	Fe^{2+}	Rhizobia-infected cells	IZ-early ZIIIs	Yes	Walton *et al*. (2020)
Medtr8g037170	MtMATE67	Citrate	Apical vasculature cells; rhizobia-infected and noninfected cells	ZII-ZIII	Yes	Kryvoruchko *et al*. (2018)

Lj, *Lotus japonicus*; Gm, *Glycine max*; Mt, *Medicago truncatula*. Question marks indicate those substrates that have not been confirmed or that it is not fully supported by another publication when more than one exists on the same gene. Nodule zones are indicated for those transporters expressed in indeterminate nodules.

similar substrate *in vivo*, since expressing *GmYSL7* under the *MtYSL7* promoter restores the WT phenotype in *M. truncatula ysl7* mutants (Gavrin *et al*., 2021). However, these proteins have differing localizations that suggest different roles of YSL7 in determinate and indeterminate nodules. GmYSL7 was first reported as a nodule-specific protein located in the symbiosomes (Gavrin *et al*., 2021), where it would be introducing short peptides into the cytosol, perhaps synchronizing the metabolism of the two symbionts and fine-tuning it (Gavrin *et al*., 2021). The later reported localization in the vasculature (Wu *et al*., 2023) is similar to that of the *M. truncatula* orthologue. MtYSL7 is located in the plasma membrane of nodule cortex cells and in roots, where a role in long-distance signaling is more likely (Castro-Rodríguez *et al*., 2021). Further work on the identification of the peptide(s) transported by YSL7 is critical to ascertain whether YSL7 and its substrates are involved in regulating metal allocation to symbiotic nitrogen fixation.

Conclusions

In the last decade, much has been learnt on how iron, copper, zinc, and molybdenum are delivered and used in nodules, and the main delivery routes that they use to participate in symbiotic nitrogen fixation have been identified (summarized in Fig. 2; Table 1). However, the delivery of other transition metals, such as manganese or nickel, is still largely unexplored in spite of the evidence indicating their importance for SNF (Brito *et al*., 1994; Hood *et al*., 2017). However, how those nutrients are carried to the transporters, how they are used, and how the whole process is regulated, remain largely unknown. Filling these gaps will provide a necessary step toward engineering nitrogen fixation capabilities in nonlegumes, as ensuring adequate levels of metal delivery is essential. Moreover, the available information will help in the development and selection of legume varieties with improved nitrogen fixation capabilities under prevalent low metal conditions.

This is particularly important in a context of climate change, in which sustainable alternatives to polluting and expensive nitrogen fertilizers must be found. Understanding metal homeostasis in nodules will also be relevant to our understanding of how metals are exchanged in other beneficial plant–microbe partnerships. For instance, the study of copper transport in *M. truncatula* nodules (Senovilla *et al.*, 2018) led to the identification of a specific copper transporter of arbuscular mycorrhiza (Senovilla *et al.*, 2020), highlighting the close evolutionary relationship between these two symbioses. More broadly, nodules have proven to be an excellent system to study plant metal homeostasis largely avoiding functional redundancy that might mask physiological roles. As improved methods for metal imaging and speciation, metalloproteomics, and targeted metabolomics are developed, the coming decade should deliver a wealth of new data on the molecular bases of transition metal homeostasis in plant–microbe systems.

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Competing interests

None declared.

Author contributions

The manuscript was conceptualized and written by MG-G and VE. CN-G and ER-N helped in reviewing the literature; JI in organizing the information and structuring it; CE-E was responsible for figures. All authors reviewed and contributed to the text of the manuscript.

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