



The transport of essential micronutrients in rice

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Abstract Micronutrients such as iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) are integral to living organisms for normal growth and reproduction. In plants, these minerals are involved in various cellular and molecular processes, such as chlorophyll synthesis and photosynthesis (Cu, Fe, and Mn), respiration (Cu and Fe), and stabilization of DNA and gene expression (Zn). A deficiency or an excess of these minerals severely impairs plant growth and development. When soil pH is high, these elements are often present as

oxidized compounds, making their uptake extremely difficult. Plants utilize complex mechanisms to acquire these minerals from the rhizosphere, transport them from roots to shoots, and deliver them to developing tissues and edible parts of the plants. Uptake of these metals is extremely complex and tightly regulated. Cadmium, which is toxic for all living organisms, significantly interferes with the uptake of these metals. Here, we review recent developments in understanding metal transport in plants with a particular focus on rice and discuss strategies for breeding crop plants suitable for a diverse range of soils and climates, which will contribute to the production of healthier food for human consumption.

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Introduction

Crop improvement is essential to feed the growing human population (Hickey et al. 2019). One of the main focuses of crop improvement is to improve mineral acquisition from soil and mineral transport within plants, so that plants may grow better and accumulate more minerals in their edible parts. Plants acquire minerals from the rhizosphere and efficiently utilize these minerals for various cellular processes. Among them, 14 minerals are classified as essential for plants because plants fail to complete their life cycle in the absence of these minerals (Marschner 1995). These minerals are

essential for plants not only to complete their life cycle and produce seeds but also to provide nutrition for animals and humans. Metals such as iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) are essential micronutrients for all higher organisms (Marschner 1995). Fe serves as a cofactor for various enzymes that perform diverse functions in biological systems. Fe is critical for cellular respiration, chlorophyll biosynthesis, and photosynthesis (Grotz and Guerinot 2006). Fe can quickly change its oxidative state, which makes it a good cofactor for cytochromes, catalases, and peroxidase isozymes. In Fe–sulfur (Fe–S) proteins, Fe is associated with cysteine and/or inorganic sulfur. Fe is also essential for the synthesis and stabilization of chlorophyll; thus, Fe-deficient plants turn chlorotic owing to decreased chlorophyll content, which affects plant growth and development. Subcellular organelles, such as chloroplasts and mitochondria, utilize Cu, Fe, Mn, and Zn for several activities. Micronutrient deficiency significantly inhibits the function of chloroplasts and mitochondria, ultimately hindering plant growth and development (Bashir et al. 2011a, c; Bashir et al. 2016; Mori et al. 1991; Vigani et al. 2013). Like Fe, Cu functions as a cofactor for numerous enzymes that are involved in both photosynthesis and respiration. Cu is also toxic when it accumulates at higher concentrations. In plants, Zn exists only as Zn(II) and does not take part in oxidoreduction reactions. For that reason, it is integral to enzymes involved in carbohydrate, nucleic acid, protein, and lipid metabolism (Ishimaru et al. 2011b; Broadley et al. 2007; Palmer and Guerinot 2009; Suzuki et al. 2012). Mn serves as a cofactor or activator of enzymes such as oxalate oxidase, Mn superoxide dismutase, RNA polymerase, malic enzyme, isocitrate dehydrogenase, and *phosphoenolpyruvate* carboxykinase (Marschner 1995). Mn is also required for photosynthetic oxygen evolution in chloroplasts (Rutherford and Boussac 2004). Mn deficiency makes plants more susceptible to pathogen infection and low-temperature stress (Marschner 1995).

Problems in acquiring essential mineral elements not only affect plant growth and reduce yield but also significantly affect human health, as deficiency in certain micronutrients and vitamins confers serious health problems (Nilson and Piza 1998). In recent years, the importance of micronutrient nutrition has been recognized and different strategies have been developed to enrich food crops with these micronutrients (Bashir et al. 2013c; Welch and Graham 2004, 1999). In humans, Fe

deficiency results in anemia and has pathological consequences (Hentze et al. 2004; Stoltzfus 2003). Fe is required at higher concentrations during rapid growth stages; thus, children, adolescents, and pregnant women are at increased risk of Fe deficiency (Welch and Graham 2004). Zn deficiency is a common nutritional problem associated with growth retardation, hypogonadism, immune dysfunction, and cognitive impairment (Prasad 2009). Fe and Zn deficiencies affect more than two billion people and cause more than 0.8 million deaths annually (Lopez et al. 2016; World Health Organization 2009). Although less prevalent, Mn deficiency may lead to severe birth defects and asthma. Anemia and neutropenia are the most striking hematologic abnormalities associated with Cu deficiency (Williams 1983).

Cu deficiency during pregnancy may impair development of the cardiovascular system or skeletal system, and can result in immunologic abnormalities at infancy or later stages of life (Bost et al. 2016). The combined effects of these micronutrient deficiencies pose a significant threat to human health (World Health Organization 2003).

Deficiency or excess of these metals triggers toxic symptoms in plants, and their uptake is therefore tightly regulated through extremely complex mechanisms (Kobayashi et al. 2014; Bashir et al. 2017, 2016; Bashir et al. 2014). Fe, Cu, Mn, and Zn transport overlaps in plant biological systems, and deficiency of these minerals is particularly severe when plants are grown in alkaline soils (Marschner 1995). Cadmium (Cd), which is toxic for most living organisms (Kobayashi 1978), significantly interferes with the uptake of these metals because of the broad substrate specificity of proteins responsible for their uptake and transport (Socha and Guerinot 2014). Cd is a toxic metal (World Health Organization 2003) that accumulates in the human body over time and is responsible for severe health problems such as “itai-itai” disease (Kobayashi 1978). Humans acquire Cd through the food chain. In Asia, the main source of Cd intake is from rice (Cheng et al. 2006), and reducing Cd levels in rice is essential to ensure the supply of safe food.

Developing crop plants with the ability to grow in adverse soils requires an understanding of the molecular mechanisms of metal uptake, transport, and storage under conditions of varying metal availability (Bashir et al. 2014, 2013a; Kobayashi and Nishizawa 2012). Rice serves as a model crop for investigating the

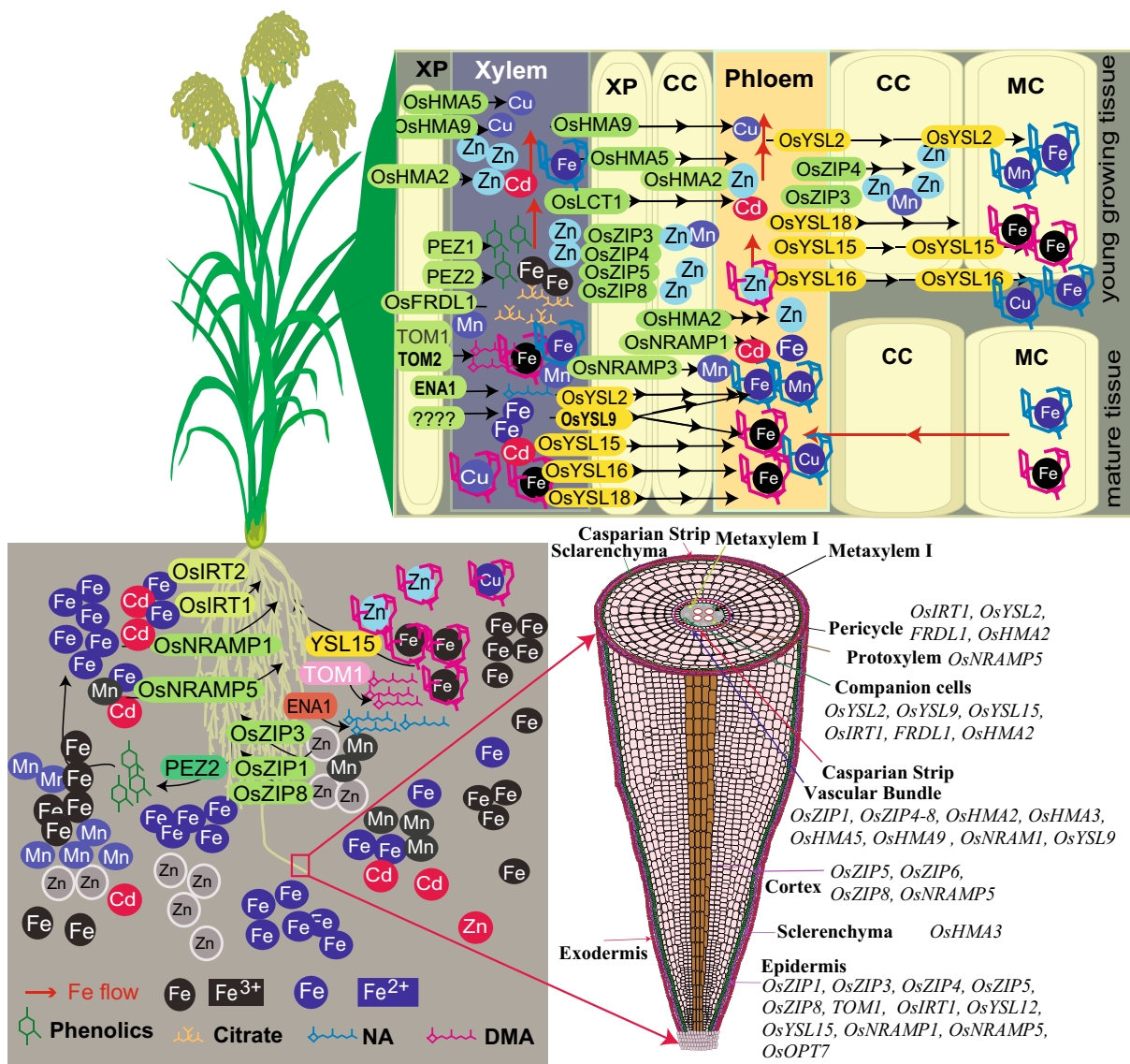
complex mechanisms of mineral uptake in plants. The mechanisms of metal uptake can be divided into five broad categories: (1) synthesis and/or secretion of metal chelators, (2) mineral uptake from soil through metal transporters, (3) metal storage in plant cells (through expression of the Fe storage protein ferritin or in vacuoles), (4) cellular metal homeostasis within plant cells and its implications for metal transport to edible parts, and (5) transcriptional control of metal uptake and distribution. Category 5 plays a regulatory role for the first four. Here, we comprehensively summarize the components involved in the uptake and translocation of Fe, Zn, Cu, Mn, and Cd in crop plants, with a particular focus on rice. We briefly discuss strategies for developing crops that efficiently regulate the uptake and storage of these metals.

Acquisition of micronutrients from the rhizosphere

Mineral soils contain sufficient micronutrients for plant cultivation. For example, the Fe concentration in soil is typically > 6%. However, Fe is not easily available to plants. Under aerobic conditions and in soils with high pH, Fe exists largely in the form of insoluble ferric chelates (Guerinot and Ying 1994). Although available Fe concentrations in the range of 10^{-9} – 10^{-4} M are considered optimal for plant growth, the free Fe^{3+} concentration in soils is estimated to be 10^{-17} M at neutral pH, which is far below plant requirements (Guerinot 2010). The absorption of Fe, Zn, and Mn significantly decreases in soils with a high pH, which comprise approximately 30% of the world's cultivated soils (Guerinot and Ying 1994). By contrast, low soil pH and anaerobic conditions, as found in lowland rice fields, trigger the reduction of Fe^{3+} to Fe^{2+} , which ultimately enhances Fe and Mn absorption (Neue et al. 1998; Zhai et al. 2014). Fe toxicity is observed at $\text{pH} < 6.5$ under anaerobic conditions and $\text{pH} < 5.8$ under aerobic conditions in flooded soils (Fageria et al. 2008; Zhai et al. 2014). Like other metals, uptake of Cd is also significantly influenced by rhizosphere conditions. In plants grown under submerged conditions, such as lowland rice, metal uptake is significantly different compared with crop species grown under aerobic conditions and is linked with the availability of other metals. Fe and Zn phytotoxicity occurs in strongly acidic soils that promote the uptake of Fe, Cd, and Zn by plants (Chaney 2015; Fageria et al. 2008). Cd accumulation

in plants may also be controlled by regulating the Cd/Zn ratio (Chaney 1993; Reeves and Chaney 2008).

The molecular mechanisms of mineral uptake from the rhizosphere have been studied extensively (Ishimaru et al. 2011c, a; Guerinot 2010; Guerinot and Ying 1994; Kobayashi and Nishizawa 2012; Kobayashi 2019) and are summarized in Fig. 1. Plants can be grouped into two broad categories based on how they take up Fe (and to some extent Zn, Mn, and Cu) from the soil—i.e., strategy I vs. strategy II plants (Marschner et al. 1986). Strategy I plants (all higher plants with the exception of graminaceous plants) first secrete protons to lower rhizosphere pH and solubilize minerals; then, ferric-chelate reductase (FRO) reduces Fe at the root surface. Finally, the resulting Fe^{2+} is transported across the root plasma membrane through IRT1 (Vert et al. 2002; Marschner and Römheld 1994). Strategy I plants also release phenolics and Fe(III)-chelating coumarins as part of their acquisition machinery (Cesco et al. 2010; Rajniak et al. 2018; Tsai and Schmidt 2017). In *Arabidopsis*, sideretin is a primary molecule secreted by roots in response to Fe deficiency. Sideretin is derived from the coumarin fraxetin, and the secretion of small-molecule reductants by roots may be a widespread and previously underappreciated component of reduction-based iron uptake (Rajniak et al. 2018). By contrast, strategy II plants (e.g., graminaceous plants) synthesize and secrete mugineic acids (MAs) that bind to and solubilize Fe, Zn, Cu, or Mn, and the resulting metal–MA complexes are readily taken up by the yellow stripe-like (YSL) family of transporters at the root surface (Curie et al. 2001; Nozoye et al. 2011). Graminaceous plants also utilize IRT transporters for metal uptake (Ishimaru et al. 2006; Pedas et al. 2008). However, the differences between strategy I and strategy II plants are not entirely distinct, as IRT transporters and *FRO* genes are present in strategy II plants, and MAs have been discovered in strategy I plants (Suzuki et al. 2016; Pedas et al. 2008; Kobayashi et al. 2014). Rice, a strategy II plant, secretes deoxymugineic acid (DMA) to acquire soil Fe (Bashir et al. 2017, 2006; Cheng et al. 2007). The genes related to MA biosynthesis and secretion, as well as uptake of metal–MA complexes, have been characterized in detail (Kobayashi et al. 2014). The conversion of three molecules of S-adenosyl methionine (SAM) to nicotianamine (NA) through NA synthase (NAS) is conserved in all plant species. In graminaceous plants, NA is further converted to a 3'-keto intermediate by NA aminotransferase, and DMA synthase reduces this 3'-keto



CC: Companion cells, CSI: Casparian strip I, CSII: Casparian strip II, MC: Mesophyll cells, MI: Metaxylem I, MII: Metaxylem II, PX: Protoxylem, ST: Sieve tube, VB, Vascular bundle

Fig. 1 Schematic diagram showing the various components of micronutrient transport in rice. The roles of different transporters participating in metal uptake from roots, root-to-shoot translocation, and vascular loading and unloading

intermediate to DMA (Bashir and Nishizawa 2006; Bashir et al. 2006). Transporter of MA family phytosiderophores 1 (TOM1) effluxes this DMA into the rhizosphere, whereas TOM2 plays a critical role in DMA efflux within the plant body (Nozoye et al. 2011, 2015). Fe and Zn deficiencies significantly enhance MA biosynthesis and secretion in barley (Suzuki et al. 2006) in a diurnal fashion, probably to avoid bacterial degradation (Römheld and Marschner 1990).

NA, an intermediate and a structural analog of MAs, is ubiquitously present in plants. It chelates Fe, Zn, Mn, and Cu (Shojima et al. 1989) and plays a significant role in Fe, Zn, Mn, and Cu homeostasis (Hell and Stephan 2003; Takahashi et al. 2003). Rice NAS2 localizes to particular vesicles which are suggested to be involved in the synthesis of MAs (Nozoye et al. 2014a, b). Although the main role of NA is related to metal homeostasis within the plant body, the Zn hyperaccumulator plant *Arabidopsis halleri*

secreted NA from roots in response to excess Zn (Tsednee et al. 2014). However, it is unclear whether rice secretes NA into the rhizosphere to chelate metals. The expression of genes related to MA biosynthesis is significantly upregulated by Fe and Zn deficiency (Suzuki et al. 2012; Kobayashi and Nishizawa 2012). Transcriptional regulation of these genes in response to Fe availability is mediated by various combinations of *cis*-acting elements and *trans*-acting factors (Kobayashi 2019).

DMA secreted by rice chelates Fe, Zn, Mn, and Cu, and its role in Fe and Zn uptake is well established in barley and rice (Suzuki et al. 2008; Inoue et al. 2009; Bashir et al. 2012). In barley, Zn(II)–DMA is preferentially taken up, whereas rice prefers Zn²⁺ over Zn(II)–DMA (Suzuki et al. 2006, 2008). Maize ZmYS1 transports Fe(III), Zn(II), Cu(II), and nickel(II) (Roberts et al. 2004). In rice, OsYSL15 takes up Fe(III)–DMA from the rhizosphere; however, the transporter participating in Zn(II)–DMA uptake has not been characterized (Ishimaru et al. 2011b).

The members of the Zn-regulated transporter/Fe-regulated transporter-related protein (ZIP) family play a significant role in Zn uptake and transport. OsZIP3 and OsZIP4 specifically transport Zn, whereas OsZIP7a and OsZIP8 transport Fe and Zn (Yang et al. 2009; Ishimaru et al. 2005; Ramesh et al. 2003). Based on their expression patterns, OsZIP1, OsZIP3, OsZIP8, and to some extent OsZIP5 seem important for Zn and/or Fe uptake from soil. Uptake of Zn by OsZIP3 is significantly inhibited in the presence of Mn, indicating that it may also transport Mn (Ramesh et al. 2003). In rice, OsNRAMP1 is a plasma membrane protein involved in Fe and Cd transport (Takahashi et al. 2011), whereas OsNRAMP5 transports Fe, Mn, and Cd (Ishimaru et al. 2012; Sasaki et al. 2012; Ishikawa et al. 2012). The rice phenolic efflux transporters phenolic efflux zero 1 and 2 (PEZ1 and PEZ2) also play a significant role in solubilizing apoplasmic Fe (Bashir et al. 2011b; Ishimaru et al. 2011a, c).

Mn uptake in rice is mediated mainly by OsNRAMP5, although its expression is not upregulated under Mn-deficient conditions (Ishimaru et al. 2012; Sasaki et al. 2012). However, the role of OsMTP9 in Mn uptake has also been discussed (Shao et al. 2017; Ueno et al. 2015). The transporters contributing to Cu uptake from the rhizosphere in rice have not been characterized yet.

Distribution of metals within the plant body

After uptake through roots, micronutrients are transported to aerial parts through the xylem and phloem. Within roots, micronutrients can move freely through the plasmodesmata (symplast) and extracellular space (apoplast); however, the latter route is blocked by Casparian strips. In *Arabidopsis*, Casparian strips are present as single-cell layers, whereas rice possesses double-cell layers of Casparian strips. As a result, rice is able to regulate the transport of minerals in roots more precisely. As noted above, excess micronutrients can be toxic; therefore, plants must strictly control their movement from roots to vegetative organs and seeds. Plants have evolved various strategies for this purpose (strictly control the movement of micronutrients from roots to vegetative organs and seeds), including the chelation of free metals and vacuolar sequestration (Briat et al. 2015; Kobayashi and Nishizawa 2012). Although the strategy I and II classifications apply only to micronutrient uptake from the rhizosphere, strategy II plants possess MAs in addition to other metal chelators such as NA, citrate, and phenolics. Strategy I plants utilize the latter three chelators. Low-molecular-weight peptides may also be utilized for the phloem transport of metals (Yoneyama et al. 2015). The presence of coumarins and other redox-active molecules has not yet been reported in rice.

Micronutrient transport to new leaves occurs mainly through the phloem, whereas micronutrient transport to old leaves occurs mainly through xylem (Yamaji and Ma 2014; Tsukamoto et al. 2009). As young leaves are more sensitive to nutrient deficiency, xylem-to-phloem transport is an extremely important step in maintaining the flow of micronutrients, and nodes play an important role in xylem-to-phloem micronutrient transport (Yamaji and Ma 2014; Sasaki et al. 2015). The transport of minerals to inflorescences and seeds is highly complex. Minerals can be directly transported from soil to flowers through the xylem, and are also relocated from leaves through the phloem (Bashir et al. 2013b, 2015; Kim et al. 2006; Tsukamoto et al. 2009; Zhang et al. 2012). At the grain-filling stage, Cd and other metals are absorbed directly by the roots. They are then transported to the panicles and accumulate in the grain (Ishikawa et al. 2011; Fujimaki et al. 2010; Tsukamoto et al. 2006, 2009). Cd translocation via the xylem is the primary physiological process determining Cd accumulation rate in shoots and grains of rice plants (Uraguchi and

Fujiwara 2012). It is suggested that at least two pathways may mediate root-to-shoot Cd translocation in rice. One depends on Zn^{2+}/Cd^{2+} loading to the xylem by rice P1B-type heavy metal ATPase 2 (OsHMA2), whereas the other may be Zn-insensitive (Fontanili et al. 2016). NA and MAs have been identified in the xylem and phloem of rice and barley, and play an important role in the long-distance transport of Fe and other metals in graminaceous plants (Kakei et al. 2009; Yoneyama et al. 2015). Concentrations of DMA and NA are similar in xylem sap and are significantly lower than citrate concentration. On the other hand, the concentration of DMA is more than two times higher in phloem sap and leaf extract compared with that of NA, whereas the concentration of citrate is negligible in leaves and phloem sap (Ando et al. 2013). In the xylem of rice plants, Fe predominantly exists as Fe citrate, but DMA–Fe(III) is also present in small amounts. Cu is predominantly bound to DMA, whereas Zn and Cd are found as free ions (Kakei et al. 2009; Yoneyama et al. 2015). In rice phloem, Fe is predominantly present as Fe(III)–DMA, and Fe-bound citrate and proteins are also present (Nishiyama et al. 2012; Yoneyama et al. 2015). In the phloem, Zn is bound to NA; Cu is bound to NA, histidine, and proteins; and Cd is bound to specific proteins and thiol compounds (Yoneyama et al. 2015). In rice, Cu-containing compounds in phloem sap comprise a mixture of high- and low-molecular-weight compounds, whereas Cu-binding compounds in the xylem sap comprise low-molecular-weight compounds (Ando et al. 2013). The concentrations of metals, MAs, citrate, and NA in xylem also vary significantly among plant species. Fe concentrations in xylem sap range from 9 to 40 μM . NA is present in the xylem sap of strategy I and strategy II plants, with higher concentrations in non-graminaceous plants. By contrast, DMA and MA are predominantly present in graminaceous plants (Ariga et al. 2014; Yoneyama et al. 2010). Recently the role of rice vacuolar phyto siderophore transporter (OsVMT) in sequestering DMA into the vacuoles has been described. *OsVMT* expresses in parenchyma cell bridges of node I and *OsVMT* knockout plants accumulate more Fe and Zn in polished rice grains as DMA increases solubilization of Fe and Zn deposited in the node (Che et al. 2019). The concentration of free citrate also varies greatly (from 4 to 2200 μM) among plant species. Fe in the xylem sap of non-graminaceous plants may form two types of Fe citrate (Ariga et al. 2014; Yoneyama et al. 2010). In dicots, Fe is transported as ferric

complexes with citrate and malate (Rellán-Álvarez et al. 2010). To solubilize these complexes, embryos efflux ascorbate to chemically reduce Fe, and this step is essential for the uptake of Fe(II). Ascorbate efflux is a novel Fe transport mechanism in plants that can play a major role in controlling Fe uptake by seeds in some plant species (Grillet et al. 2014).

DMA is secreted into the xylem and phloem through TOM1 and TOM2, where it can bind to free metals and contribute to their transport (Nozoye et al. 2011, 2015). Efflux transporter of NA (ENA1) is reported to participate in NA efflux in vascular tissue (Nozoye et al. 2019). Phenolics such as protocatechuic acid (PCA) and caffeic acid (CA) also form complexes with Fe(III), and thus play a role in Fe uptake and transport (Bashir et al. 2011b; Ishimaru et al. 2011a, c; Jin et al. 2007). PEZ1 and PEZ2 are reported to efflux PCA and CA into the xylem to remobilize precipitated apoplasmic Fe (Bashir et al. 2011b; Ishimaru et al. 2011a, c). *Arabidopsis* FRD3 and rice OsFRDL1 efflux citrate into the xylem, ensuring efficient Fe translocation to shoots (Inoue et al. 2004; Yokosho et al. 2009; Durrett et al. 2007; Roschztardt et al. 2011). The expression of *OsFRDL1* is not regulated by Fe deficiency and is observed in cells involved in long-distance transport (Kobayashi et al. 2014; Yokosho et al. 2009). *OsFRDL1* is also expressed in reproductive organs and is suggested to play a major role in Fe distribution to grains through nodes (Inoue et al. 2004; Yokosho et al. 2016).

Various transporters are involved in loading and unloading micronutrients to and from the xylem and phloem, contributing to micronutrient homeostasis in plants (Fig. 1). OsHAM2 effluxes Zn and Cd into the xylem and phloem (Yamaji et al. 2013b; Takahashi et al. 2012b), OsHMA5 and OsHMA9 play a role in xylem loading of Cu, and OsHMA9 may also play a role in Zn and Pb efflux from cells (Lee et al. 2007; Deng et al. 2013). In rice, several members of the YSL family have been characterized. OsYSL2, OsYSL9, OsYSL15, OsYSL16, and OsYSL18 regulate micronutrient transport in vegetative tissues and facilitate metal transport to seeds. The role of OsYSL13 in Fe distribution in rice has also been discussed (Zhang et al. 2018b). *OsYSL2* is expressed in the phloem under Fe-sufficient conditions, as well as in the cortex and stele under Fe-deficient conditions, whereas *OsYSL16* expression is observed in the vascular bundle of leaves (Kakei et al. 2012). Thus, *OsYSL2* facilitates phloem Fe and Mn transport (Koike et al. 2004; Ishimaru et al. 2010), which are extremely important for plant

growth. As OsYSL2 transports Fe in the form of Fe(II)–NA, and as Fe is predominantly found as Fe(III)–DMA in the phloem, OsYSL16 may play a major role in phloem Fe(III)–DMA transport (Kakei et al. 2012; Lee et al. 2012). Rice OsYSL9 transports Fe(II)–NA and Fe(III)–DMA in plants, and appears to play an important role in transporting Fe from the endosperm to the embryo in developing seeds (Senoura et al. 2017). Apart from its major role in Fe uptake from soil, *OsYSL15* expression is also observed in shoots, suggesting that it is involved in micronutrient distribution within the plant body and to developing seeds (Inoue et al. 2009; Lee et al. 2009). *OsYSL16* and *OsYSL18* play a significant role in Fe(III)–DMA distribution/translocation in plants (Aoyama et al. 2009; Kakei et al. 2012; Lee et al. 2012). Phylogenetic analysis suggests that the YSL family is composed of four subgroups. Among the 18 YSLs identified in rice to date, *OsYSL-1*, *OsYSL-3*, *OsYSL-4*, *OsYSL-7*, *OsYSL-8*, *OsYSL-17*, and *OsYSL-18* are specific to rice and may be involved in transporting metal–DMA complexes (Aoyama et al. 2009). Among the six members of this subgroup, only *OsYSL18*, which is a Fe(III)–DMA transporter, has been characterized thus far. OsYSL2 and OsYSL18 are involved in transporting Fe to developing seeds (Aoyama et al. 2009; Ishimaru et al. 2010; Koike et al. 2004). *OsYSL16* is expressed at the root epidermis and vascular bundles of roots and shoots. In the vascular bundles of non-elongated nodes, *OsYSL16* is expressed in the phloem of new leaves and the xylem of old leaves (Kakei et al. 2012). OsYSL16 also transports Cu–NA and is important for Cu distribution in rice, where it preferentially delivers Cu to floral organs (Zhang et al. 2018a; Zheng et al. 2012). OsHMA4 controls root-to-shoot translocation of Cu and its accumulation in rice grains by sequestering Cu into root vacuoles (Huang et al. 2016). A single amino-acid substitution makes a significant difference in grain Cu accumulation (Huang et al. 2016). In addition, *OsYSL2*, *OsYSL15*, *TOM1*, and *TOM2* are expressed in the vascular tissues of shoots (Inoue et al. 2009; Koike et al. 2004; Nozoye et al. 2011, 2015). OsYSL6 transports Mn–NA complexes and plays an important role in detoxifying excess Mn in rice (Sasaki et al. 2011). OsNRAMP3 is localized at nodes and is believed to serve as a switch in response to changes in Mn levels (Shao et al. 2018). Rice accumulates high levels of Mn in leaves without exhibiting phenotypic abnormality. Metal tolerance protein 8.1 (MTP8.1), a member of the Mn-cation diffusion facilitator (CDF) family, has been shown to play a central role in Mn tolerance by sequestering Mn into vacuoles (Chen et al. 2013). Furthermore, rice

MTP11 is a trans-Golgi-localized Mn transporter involved in intracellular Mn compartmentalization, leading to Mn tolerance (Tsunemitsu et al. 2018). MTP11 is also responsible for maintaining high fertility in rice (Tsunemitsu et al. 2018).

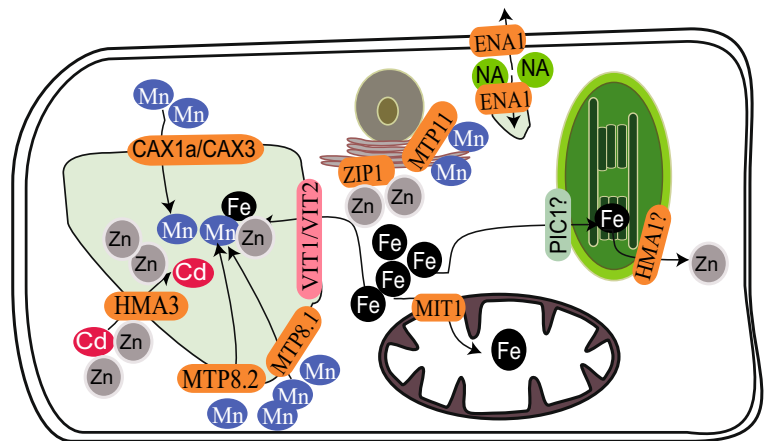
In rice, OsZIP4, OsZIP5, OsZIP7, and OsZIP8 appear to play an important role in Zn distribution in shoots and inflorescences (Ishimaru et al. 2005; Yang et al. 2009; Ricachenevsky et al. 2018; Tan et al. 2019; Lee et al. 2010a, b), while OsZIP6 transports Fe, Cd, and cobalt (PG et al. 2015). These transporters could be utilized to enhance Zn uptake and/or distribution to improve crop plants. In addition to ZIP family transporters, HMAs are also involved in Zn translocation in rice. *OsHMA2* is expressed mainly in the mature zone of the roots at the vegetative stage, and in nodes at the reproductive stage. Its expression does not change based on Zn availability. OsHMA2 contributes to Zn and Cd transport, particularly to developing tissues (Takahashi et al. 2012b; Yamaji et al. 2013b). As discussed above, OsHMA5 and OsHMA9 play a role in the xylem loading of Cu and Zn, and may also be involved in the transportation of Cu and/or Zn to the developing embryo in rice (Lee et al. 2007; Deng et al. 2013).

OsNRAMP5 contributes to Mn distribution in shoot tissue and is also involved in Mn, Fe, and Cd uptake from soil (Yang et al. 2014). OsIRT1 and OsNRAMP1 are involved in Fe and Cd distribution in shoots (Ishimaru et al. 2007a; Lee and An 2009; Takahashi et al. 2011).

Subcellular metal homeostasis

Distribution of minerals among different cellular compartments to regulate cellular metabolism is considered a key factor for crop improvement (Bashir et al. 2016). The precise control of storage to mitigate mineral deficiency or toxicity is important not only for maintaining cellular function but also for regulating mineral transport to seeds. Here, we briefly review subcellular mineral transport in rice, which remains incompletely understood (Fig. 2). Mitochondrial iron transporter (MIT) transports Fe to mitochondria, where it is essential for plant growth and development (Vigani et al. 2016; Bashir et al. 2011a, c). OsVIT1 and OsVIT2 contribute to Fe, Mn, and Zn transport to tonoplasts (Zhang et al. 2012). ENA1 is suggested to play a critical role in the intracellular trafficking of Fe (Nozoye et al. 2011, 2019). ENA1 is also thought to be involved in recycling

Fig. 2 Subcellular metal transport in rice. Various transporters participate in subcellular metal transport within plant cells



NA in the plasma membrane and cellular compartments through vesicular transport (Nozoye et al. 2019).

MTP8.1 and MTP8.2 contribute to Mn sequestration into the vacuole (Takemoto et al. 2017; Chen et al. 2013), and CAX1a and CAX3 have also been suggested to play a role in Mn sequestration into the vacuole with broader substrate specificity (Kamiya et al. 2005). Rice HMA3 deposits Cd and Zn into vacuoles (Sasaki et al. 2014), whereas HMA1 may be involved in Zn efflux from chloroplasts into the cytosol (Takahashi et al. 2012a). Thus, regulating these transporters may be a useful approach to crop improvement.

Potential of plant transporters for improvement of crop plants

Biotechnology has great potential to significantly advance the biofortification of cereals and other crop plants. In rice, efforts have been focused on increasing the Fe content of rice endosperm with different approaches, such as overexpressing ferritin and genes involved in NA and MA biosynthesis to increase metal uptake and translocation. Several reports have comprehensively summarized the progress that has been made in developing various strategies for biofortification (Bashir et al. 2013a, c; Masuda et al. 2013; Ludwig and Slamet-Loedin 2019). Therefore, we do not discuss these strategies here, and instead focus on the role of metal transporters in biofortification and crop improvement.

Plants may be improved for sustainable crop production using membrane transporters to increase the concentration of micronutrients in edible parts (Schroeder

et al. 2013), decrease toxic elements in the food chain, and extract toxic heavy metals from soil (Takahashi et al. 2014). Increased uptake and translocation of micronutrients have the potential to significantly increase seed micronutrient concentration. However, it is logistically difficult to achieve this goal owing to issues associated with the regulation of transporters at the protein level, poor discrimination of substrates by metal transporters, and the complexity of root-to-shoot transport.

Rice plants with enhanced *OsIRT1* expression are tolerant of Fe deficiency under field conditions (Lee and An 2009). Overexpression of *OsIRT1* also increases the accumulation of Fe and Zn in seeds (Lee and An 2009). However, overexpression of *OsZIP4*, *OsZIP5*, *OsZIP7*, *OsZIP8*, and *OsYSL2* results in increased accumulation of metals in roots instead of shoots (Ishimaru et al. 2007b, 2010; Lee et al. 2010a, b). This problem could be solved by regulating the expression of these transporters by a suitable promoter. For example, plants expressing *OsYSL2* under the control of the promoter of the phloem-specific sucrose transporter *OsSUT1* exhibit up to a 4.4-fold increase in seed Fe concentration compared with wild-type plants. These results indicate that gene regulation has the potential to significantly increase Fe flow to rice seeds (Ishimaru et al. 2010). Root-to-shoot Zn translocation may also be increased under optimal Zn availability. Thus, overexpression of *HvZIP7*, a low-affinity Zn transporter in barley, specifically increases Zn uptake under moderately high Zn concentrations, with no measurable increase in Fe, Mn, Cu, or Cd (Tiong et al. 2014, 2015,). Transgenic plants overexpressing *OsYSL15* accumulate up to 29% more Fe than wild-type plants (Lee et al.

2009). Expressing multiple genes regulated by different promoters such as two copies of ferritin (one under the control of the promoter of glutelin b1 and 2nd under the control of the promoter of the globulin b1) with *HvNAS1* (under the control of the promoter of actin) and *OsYSL2* (under the control of the promoter of the *OsSUT1* and globulin b1) significantly increases Fe, Zn, and Cu contents in rice seeds without any yield penalty (Masuda et al. 2012, 2013; Aung et al. 2013). These results indicate that the strict control of gene expression through tissue- and organ-specific promoters, as well as sufficient availability of micronutrients, is extremely important for successful micronutrient biofortification.

Several ZIP and NRAMP transporters exhibit very poor ability to discriminate among divalent metal cations, and as a result, they transport Cd along with essential micronutrients (Clemens et al. 2013). For example, AtNRAMP3 transports Fe, Mn, and Cd; AtNRAMP4 transports Fe, Mn, Zn, and Cd; and AtNRAMP6 specifically transports Cd (Oomen et al. 2009; Thomine et al. 2000; Cailliatte et al. 2009; Molins et al. 2013). AtNRAMP3 and AtNRAMP4 mobilize Fe stores during germination, and the failure of this process triggers Fe-deficiency responses that specifically affect plastids, but not mitochondria (Bastow et al. 2018). OsZIP1, OsIRT1, and OsIRT2 also transport Cd along with Fe (Nakanishi et al. 2006; Ramesh et al. 2003). Thus, overexpressing these transporters to achieve biofortification targets would also increase the risk of Cd accumulation in rice grains, especially when rice is grown in Cd-contaminated soils. Similarly, in *PEZ1* and *PEZ2* mutants, several genes related to metal uptake are upregulated, which lead to Cd accumulation in seeds (Ishimaru et al. 2011a, c; Bashir et al. 2011b). Plant growth is significantly compromised in *PEZ1*-overexpressing plants, likely due to increased Fe accumulation (Ishimaru et al. 2011a, c; Bashir et al. 2011b). There are only few exceptions among NRAMPs, such as rice OsNRAT1 and OsNRAMP3, which specifically transport Al^{3+} and Mn^{2+} , respectively (Xia et al. 2010; Yamaji et al. 2013a). Similarly, OsZIP3 does not transport Cd (Ramesh et al. 2003; Sasaki et al. 2015) and OsZIP4 specifically transports Zn (Ishimaru et al. 2005).

Developing mutated versions of these proteins that do not transport Cd while still transporting beneficial metals is one approach to addressing these constraints. For example, replacing specific amino acids changes the substrate specificity of IRT1 in *Arabidopsis* (Rogers

et al. 2000). The histidine-rich loop and residues within transmembrane domain 3 can influence metal selectivity in the CDF family, possibly through conformational changes induced at the cation transport site located within the membrane or at the cytoplasmic C-terminal domain (Podar et al. 2012). Despite the strong homology between AtNRAMP3 and AtNRAMP4, only AtNRAMP4 can transport Zn^{2+} , suggesting that differences of a few residues in the primary sequence modulate substrate range (Lanquar et al. 2004). *Arabidopsis* NRAMP3 and NRAMP4 also play a significant role in exporting vacuolar Fe in germinating seeds (Bastow et al. 2018). Mutations in AtNRAMP4 proteins selectively modify Cd^{2+} and Zn^{2+} accumulation without affecting Fe transport mediated by NRAMP4 in planta (Pottier et al. 2015). These results are of particular importance because NRAMP plays a major role in Cd uptake. The results also emphasize that modification of transporters could be effectively utilized for biofortification while minimizing the risk of toxic metal accumulation (Podar et al. 2012; Pottier et al. 2015).

Rice plasma membrane low-affinity cation transporter 1 (*OsLCT1*) is strongly expressed in leaf blades and nodes during the reproductive stage. In *OsLCT1*-knockdown plants, phloem-mediated Cd transport significantly decreases; as a result, these plants accumulate significantly less Cd in the grains compared with wild-type plants (Uraguchi et al. 2011). Regulating the expression of *OsHMA3*, which is a Cd and Zn transporter, significantly reduces root-to-shoot Cd translocation by sequestering Cd into the root vacuole, and the expressions of *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10* significantly increase in *OsHMA3*-overexpressing plants (Sasaki et al. 2014; Ueno et al. 2010; Cai et al. 2019). Thus, grain Cd content may be significantly reduced by regulating the expression of *OsHMA3* and *OsLCT1*, which could contribute to the elimination of Cd from the food chain.

In rice, the plasma membrane transporter OsNRAMP5 is reported to be the main pathway for Cd^{2+} influx. Complete knockout of *OsNRAMP5* significantly reduces Cd uptake from soil, enabling the development of rice lines with minimal Cd in grains (Ishikawa et al. 2012; Ishimaru et al. 2012; Sasaki et al. 2012). Ishikawa et al. (2012) produced *NRAMP5*-knockout mutants through ion-beam irradiation. When grown on Cd-contaminated soils, these non-transgenic mutants accumulated extremely low levels of Cd in their rice grains. Thus, the mutants may be easily adopted by farmers with low biosafety risk, significantly contributing to the development of healthier foods.

Although Cd uptake is significantly reduced in *OsNRAMP5* RNAi plants, root-to-shoot translocation significantly increases (Takahashi et al. 2014). Root-to-shoot translocation of Cd in knockdown rice in the cultivar Anjana Dhan was more efficient than in the corresponding wild type, which is one of the highest Cd-accumulating cultivars, suggesting that these knockdown plants are promising candidates for Cd phytoremediation. As discussed above, Cd uptake and translocation are mediated in part by Fe transporters such as *OsIRT1*, *OsIRT2*, and *OsNRAMP1* (Takahashi et al. 2011; Nakanishi et al. 2006; Ishimaru et al. 2006). The expressions of *OsIRT1*, *OsIRT2*, and *OsNRAMP1* increase significantly in *OsNRAMP5*-knockdown plants, which may be responsible for enhancing Cd translocation to shoots (Ishimaru et al. 2012; Takahashi et al. 2014). Thus, carefully regulating the expression of *NRAMP* family genes may facilitate the development of safer foods and soil improvement programs based on Cd extraction. Carefully regulating the expression of genes, such as *OsVIT1* and *OsVIT2*, and *MIT* and *MIR*, which are essential for maintaining cellular levels of available Fe and other metals, may significantly improve plant growth and support biofortification (Bashir et al. 2016).

Future prospects

Various strategies have been developed to breed crop plants able to grow on poor soils with low metal availability and/or with improved accumulation of beneficial metals in edible parts. These plants could be cultivated on a diverse range of soils. These successes have been achieved through regulating the expressions of metal chelators, enzymes, and/or transporters. There is still great potential to further exploit such strategies by devising better combinations of promoters and genes. For example, newly discovered genes could be transformed alone or in combination with other genes. In addition to the known genetic networks, several new aspects of metal translocation have been discovered in recent years. These include peptides that regulate metal accumulation by manipulating substrate specificity and gene expression in response to varying metal availability. Regulating the expression of small proteins or peptides may be a promising approach for crop improvement. *Cd accumulation in leaf 1 (CAL1)* encodes a defensin-like protein that specifically binds to Cd, facilitating Cd secretion to extracellular spaces and lowering cytosolic Cd concentration while driving long-distance Cd

transport via the xylem (Luo et al. 2018). *CAL1* regulates Cd translocation from roots to shoots in a direction opposite to that of *OsHMA3*; hence, *CAL1* does not contribute to Cd accumulation in grains (Zhao and Huang 2018). In *Arabidopsis*, the *FEP1* peptide functions in iron homeostasis by activating bHLH38 and bHLH39. Interestingly, *IRT1* and *FRO2* are activated by bHLH39 without the involvement of *FIT* (Hirayama et al. 2018). Similarly, *Arabidopsis* *IRON MAN (IMA)*, which seems to be conserved across species, plays an integral role in Fe uptake and transport by regulating the expression of related genes. *IMA1* is predominantly expressed in the phloem, preferentially in leaves. Grafting experiments revealed that the *IMA1* peptide in shoots positively regulates Fe uptake in roots (Grillet et al. 2018). In rice, the expression of several small open reading frames (sORFs; putatively encoding small functional peptides) is regulated by metal availability (Bashir et al. 2014). These results indicate that small peptides may play an important role in regulating metal metabolism in plants. Integrated efforts are required to elucidate the molecular mechanisms of these effects.

Few studies have focused on the crystal structure of metal transporters to highlight the interactions between these proteins and metals in plants. Recently, the crystal structure of *Eucalyptus grandis* (rose gum) *VIT1* was determined. *VIT1* adopts a novel protein fold, forming a dimer of five membrane-spanning domains. A second transmembrane helix protrudes from the lipid membrane and connects to a three-helix bundle within a triangular cytoplasmic domain, which binds to and solubilizes the substrate metal ions (Kato et al. 2019). Understanding the binding of proteins to metals creates unique opportunities to manipulate the selectivity of metal transport in plants. Developing mutated versions of *NRAMPs*, *HMA*s, *VIT*s, *IRT*s, and other transporters may facilitate the development of plants with increased Fe/Zn/Mn/Cu transport without affecting Cd uptake, improving food safety.

The use of new technologies such as CRISPR/Cas9 could significantly contribute to the development of crop plants with enhanced beneficial minerals while limiting Cd accumulation (Tang et al. 2017). Regulating the synthesis of phytohormones could also contribute to the mineral use efficiency along with enhanced tolerance to other stresses in plants enabling these plants to grow under diverse environmental conditions (Bashir et al. 2019; Rasheed et al. 2016; Lei et al. 2014; Zhao et al. 2014; Matsuoka et al. 2013). Although many studies have elucidated the various factors regulating plant homeostasis and metal uptake from the

rhizosphere, only a few have focused on epigenetic regulation of metals (Liu et al. 2019; Feng et al. 2016). Exposure to Cd significantly changes CG and non-CG methylation patterns in rice (Feng et al. 2016). OsZIP1 is upregulated by excess Cu, Cd, and Zn (but not by Fe or Mn), and the mechanism for this effect is DNA methylation/demethylation of histone H3K9me2 in response to Cd exposure (Liu et al. 2019). Understanding epigenomic regulation in response to metal deficiency or excess may provide additional opportunities to breed crop plants with more favorable nutritional qualities.

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Compliance with ethical standards

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