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FROM SOIL TO SEED: MICRONUTRIENT MOVEMENT INTO AND WITHIN THE PLANT

Topic Editors
Raul A. Sperotto, Felipe K. Ricachenevsky, Lorraine E. Williams, Marta W. Vasconcelos and Paloma K. Menguer

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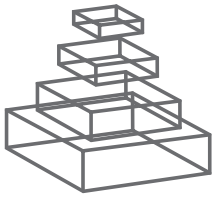

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FROM SOIL TO SEED: MICRONUTRIENT MOVEMENT INTO AND WITHIN THE PLANT

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Panicles of rice plants cultivated in greenhouse conditions - Rio Grande do Sul, Brazil (image by Felipe Klein Ricachenevsky).

In all living organisms, essential micronutrients are cofactors of many ubiquitous proteins that participate in crucial metabolic pathways, but can also be toxic when present in excessive concentrations. In order to achieve correct homeostasis, plants need to control uptake of metals from the environment, their distribution to organs and tissues, and their subcellular compartmentalization. They also have to avoid deleterious accumulation of metals and metalloids such as Cd, As and Al. These multiple steps are controlled by their transport across various membrane structures and their storage in different organelles. Thus, integration of these transport systems required for micronutrient trafficking within the plant is necessary for physiological processes to work efficiently.

To cope with the variable availability of micronutrients, plants have evolved an intricate collection of physiological and developmental processes, which are under tight control of short- and long-range signaling pathways. Understanding how plants perceive and deal with different micronutrient concentrations, from regulation to active transport, is important to completing the puzzle of plant metal homeostasis. This is an essential area of research, with several implications for plant biology, agriculture and human nutrition.

There is a rising interest in developing plants that efficiently mobilize specific metals and prosper in soils with limited micronutrient availability, as well as those that can selectively accumulate beneficial micronutrients in the edible parts while avoiding contaminants such as Cd and As. However, there is still an important gap in our understanding of how nutrients reach the seeds and the relative contribution of each step in the long pathway from the rhizosphere to the seed. Possible rate-limiting steps for micronutrient accumulation in grains should be the primary targets of biotechnological interventions aiming at biofortification.

Over the last 10 years, many micronutrient uptake- and transport-related processes have been identified at the molecular and physiological level. The systematic search for mutants and transcriptional responses has allowed analysis of micronutrient-signaling pathways at the cellular level, whereas physiological approaches have been particularly useful in describing micronutrient-signaling processes at the organ and whole-plant level. Large-scale elemental profiling using high-throughput analytical methodologies and their integration with both bioinformatics and genetic tools, along with metal speciation, have been used to decipher the functions of genes that control micronutrients homeostasis.

In this research topic, we will follow the pathway of metal movement from the soil to the seed and describe the suggested roles of identified gene products in an effort to understand how plants acquire micronutrients from the soil, how they partition among different tissues and subcellular organelles, and how they regulate their deficiency and overload responses. We also highlight the current work on heavy metals and metalloids uptake and accumulation, the studies on metal selectivity in transporters and the cross-talk between micro and macronutrients. Thus, we believe a continued dialogue and sharing of ideas amongst plant scientists is critical to a better understanding of metal movement into and within the plant.

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EDITORIAL
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From soil to seed: micronutrient movement into and within the plant

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The ability of roots to obtain micronutrients from the soil and to deliver these to the aerial tissues—including seeds—is essential to ensure that the shoot has the resources it needs to function effectively. However, plants need to control several steps during the journey from soil to seed, including uptake, transport, remobilization and storage. A better comprehension of the relative contribution of these processes, together with their overall coordination, is necessary for a more complete understanding of plant metal homeostasis and for the development of successful biofortification strategies. This Research Topic aims at addressing some of the most recent advances in micronutrient movement from soil to seed and to provide an overview of different approaches that can be used to generate micronutrient-efficient and biofortified plants. Here, we highlight some of the major points arising from these reports.

MICRONUTRIENT UPTAKE AND TRANSPORT

As the first step in the pathway of elements reaching shoots and seeds, micronutrient uptake from the soil is arguably the most studied aspect of metal and metalloid homeostasis. As such, identifying the mechanisms and proteins involved in transport into roots from the rhizosphere was key to further studies on metal movement within the plant. Iron (Fe) acquisition in plants, for example, has long been thought to be based on a reduction strategy (strategy I) in non-Poaceae but a chelation strategy (strategy II) in Poaceae species; however, recent data suggest that certain monocots, such as rice, (*Oryza sativa*) can combine characteristics of both. Ricachenevsky and Sperotto (2014) suggest two models of how these strategies were shaped through evolutionary time, and propose that the combined strategy might be common to more species than just rice. Jain et al. (2014) review the roles of Fe(III)-reductases, discussing Fe and copper (Cu) reduction at the rhizoplane, and the proposed roles for the reductases at leaf cell surfaces and in subcellular compartments.

When considering transport of micronutrients from the soil to the seed we need to understand the contribution of long distance transport, tissue and intercellular transport and the role of organelles. It is also important to consider the transport of toxic metals such as cadmium (Cd). The vacuole is a pivotal compartment in storing heavy metals (both essential and toxic), but also makes a substantial contribution to long-distance transport. Peng and Gong (2014) discuss the concept of vacuolar sequestration capacity (VSC), proposing it plays the role of a "buffering pool" not only controlling metal accumulation in cells but also dynamically mediating transport of metals over long distances. Certainly chelators such as phytochelatins and nicotianamine play an important role in regulating VSC. In rice, the membrane transporters VIT1 and VIT2 also appear to have a key role in long distance transport of zinc (Zn) and Fe between source (flag leaves) and sink organs (seeds) via the modulation of flag leaf Zn and Fe VSC. Other transporters implicated in the connection between VSC and long distance transport include AtMTP1, AtZIF1 (Zn) and AtCOPT5 (Cu). VSC is also key to the transport of toxic metals including Cd and arsenic (As). OsHMA3 sequesters Cd in root vacuoles and hence modulates shoot accumulation of Cd in rice. Heavy metal hyperaccumulator plants have reduced VSC in roots, promoting long distance metal transport and leading to metal accumulation in the shoots (Peng and Gong, 2014).

We are now starting to understand metal transport processes in a wider range of species. Palmer et al. (2014) provide a picture of the mineral transporter families and their expression over the life cycle in different tissues in switchgrass (*Panicum virgatum*), a C4 perennial grass that has great potential as a biofuel crop. Jung et al. (2014) report on the wild grass *Brachypodium distachyon* as a model for studying Cu transport in cereal crops, identifying CTR/COPT transporters and characterizing expressions patterns, subcellular localization and their function by growth complementation assay of yeast mutants under Cu deficiency. Some genes were transcriptionally up-regulated by Cu deficiency (*BdCOPT3* and *4*) and BdCOPT3, 4, and 5 conferred low affinity Cu transport. It was proposed that the increased sensitivity of some grass species to Cu deficiency could result from COPTs with lower uptake capacity and from an impaired partitioning among organs and tissues.

The uptake of other elements into roots and their transport within the plant are also discussed. Socha and Guerinot (2014) provide a comprehensive review of the transporters with

potential roles in manganese (Mn) transport including information about their localization and regulation by Mn toxicity or deficiency. Molybdenum (Mo) metabolism is presented by Bittner (2014), including transporters and Mo cofactor (Moco)-dependent proteins, as well as the yet to be fully understood crosstalk between Mo and Fe metabolism. Finally, the role of metals in nodulation and symbiotic nitrogen fixation is presented by González-Guerrero et al. (2014), providing an overview of the role of different elements, metalloproteins and metal cofactors for nodule formation and function, and discussing contributions of distinct transporters for metal uptake, accumulation and spatial distribution within the nodules.

MINERAL REMOBILIZATION

Remobilization of reserves to supply seeds with minerals has been emphasized in previous studies, but the contribution of stored minerals to total seed content is unclear, highlighting the need for a better understanding of metal remobilization to improve metal use efficiency in the context of biofortification. Sankaran and Grusak (2014) conducted a mineral partitioning study in pea to assess whole-plant growth and mineral content and the potential source-sink remobilization of different minerals. They conclude that net remobilization of some minerals from different tissues into seeds can occur, but continued uptake and translocation of minerals to source tissues during seed fill is as important, if not more important, than remobilization of previously stored minerals. Using ^{65}Zn, Impa et al. (2013) show that Zn-efficient rice genotypes have a greater ability to translocate Zn from older to actively growing tissues than genotypes sensitive to Zn deficiency. Actually, as proposed by Sperotto (2013), under Zn sufficient condition, grain Zn accumulation in rice occurs mainly through continued root uptake during grain filling stage, whereas under Zn deficient condition both continued root uptake and remobilization of Zn from source tissues contribute equally to grain Zn loading. A similar pattern is found for Fe remobilization under Fe deficient or sufficient conditions. However, Stomph et al. (2014) used ^{70}Zn applications at different times during rice development and suggested that the major barrier to enhanced Zn allocation toward grains is between stem and reproductive tissues, and that simply enhancing root to shoot transfer will not contribute proportionally to grain Zn enhancement. Pottier et al. (2014) reviewed micronutrient remobilization from leaves to seeds, and suggested that autophagy (a well-known mechanism involved in nitrogen remobilization to seeds during leaf senescence) is also involved in metal recycling and remobilization.

MICRONUTRIENT STORAGE

The ultimate goal of biofortification strategies is to develop grains with higher nutritional value and lower content of non-essential elements for human consumption or animal feed. Mineral homeostasis in plants is a tightly regulated process, depending on metal transporter activity and specificity, which directly affects mineral seed loading. Khan et al. (2014) highlight the importance of a safe nutritional enrichment of grains by means of precision breeding and transport engineering. This review reports the current understanding of the mechanisms involved in plant translocation and distribution of non-essential toxic elements like Cd and As by the same transporters that otherwise move nutrients such as Fe, Zn and Mn.

An important issue regarding micronutrient movement in plants and storage into seeds is the chemical form in which metals circulate in and between cells. There has been a number of advances in the identification of ligand and metal-ligand complexes in plant fluids (xylem and phloem sap, apoplastic fluid and embryo sac liquid) and it is now considered unlikely that metals are present in plant fluids in significant quantities as free ions. Instead it is much more likely that they occur in less reactive chemical forms and that may play a major role in controlling mineral seed loading and accumulation. Álvarez-Fernández et al. (2014) provide information on available methods for sampling plant fluids and the associated advantages and disadvantages with different techniques.

Grillet et al. (2014) review the mechanisms of Fe transport from the root to the seed, emphasizing the current knowledge on the chemical forms of Fe transported between symplastic and apoplastic compartments, including phloem loading. Also the authors show that the Fe bioavailability in seeds varies widely across species mainly depending on tissue localization and storage forms of Fe. Vasconcelos et al. (2014) approached the same subject analyzing soybean plants overexpressing the *AtFRO2* iron reductase gene and mineral accumulation in source and sink tissues to determine whether the reductase activity is a rate-limiting step for seed mineral acquisition. When exposed to high Fe supply the transgenic plants had an increase in leaf and pod wall Fe concentrations by as much as 500%. However, the seed Fe concentration only increased by 10% suggesting that factors other than plant reductase activity are limiting the translocation of Fe into the seed.

As Zn deficiency is prevalent in many parts of the world, especially where there is reliance on a plant-based diet, there is great interest in increasing the level and bioavailability of Zn in the grain of cereal crops. Olsen and Palmgren (2014) present an overview of the processes occurring in the transport of Zn from uptake at the plasma membrane of root cells to accumulation in the seed, reporting what we know in Arabidopsis to what we are starting to learn in cereals. The mechanisms involved in phloem unloading and post-phloem movement of Zn in the developing seed are discussed with respect to the apoplastic barriers found in the Arabidopsis seed.

APPLICATIONS AND OUTLOOK

Knowing the fundamental processes governing mineral uptake, transport, remobilization and storage brings fundamental scientific knowledge that is important *per se*. However, an important goal in the understanding of these processes is to devise practical strategies to solve tangible problems (either via optimized agronomical practices, novel molecular and conventional breeding strategies, or genetic transformation utilizing the most suitable candidate genes). The biofortification of plant foods is one such area of research that benefits directly from a better understating of the molecular players and their physiological functions in nutrient homeostasis. In the current issue, Borrill et al. (2014) provide an integrated review on the available molecular tools for enrichment of wheat with Fe and Zn, and show us that we

finally have the necessary molecular tools to accomplish this goal. They also remind us that when devising the best biofortification strategy, we will need to be mindful of future climate changes, such as a rise in atmospheric CO_2 (which will negatively impact Fe and Zn contents in the seeds). Also, the authors emphasize the need for better communication between breeders and other plant scientists to enhance Fe and Zn in the grain in a sustainable way. Relevant to this is the consideration of toxic metals in these strategies to achieve a safe nutritional enrichment of seeds. Two main strategies could be important here: increasing the selectivity of transporters toward essential elements, and re-routing non-essential elements to non-edible parts of the plant (Khan et al., 2014).

Another benefit of an improved understanding of mineral uptake and transport is linked with abiotic stress issues. Plants can suffer from a multitude of mineral deficiencies, with boron (B) deficiency being observed in various agricultural soils, where it severely limits crop production. Here, Uraguchi et al. (2014) present a possible solution, increasing the growth of tomato plants under B deficiency by heterologously expressing Arabidopsis BOR1. This approach could be expanded to other crop species suffering from B deficiencies or at least considered for future breeding strategies.

This special issue presents novel mechanisms, some of which have not been fully recognized thus far, but have an obvious impact on plant mineral dynamics. One of these mechanisms is ubiquitination, a post-translational modification involved in protein turnover. Recently, several publications have revealed that ubiquitination also has roles in nutrient utilization. However, the extent to which plants rely on ubiquitination for regulating nutrient transport and compartmentalization is still in its infancy. In a perspective article, Yates and Sadanandom (2013) highlight the importance of the role ubiquitin plays in a plant's ability to uptake and process nutrients, including recent advances in understanding how ubiquitin supports nutrient homeostasis by affecting the trafficking of membrane-bound transporters. It is possible that we need to become more mindful of ubiquitination processes in future strategies when further improving biofortification or abiotic stress tolerance.

Altogether, the work presented here documents recent advances in the study of membrane transporters, chelators and regulatory proteins. Now, the challenge is to move forward and to integrate this information for an improved understanding of how the individual components and processes work together in the long pathway of micronutrients from the rhizosphere to the seed.

REFERENCES

Álvarez-Fernández, A., Díaz-Benito, P., Abadía, A., López-Millán, A. F., and Abadía, J. (2014). Metal species involved in long distance metal transport in plants. *Front. Plant Sci.* 5:105. doi: 10.3389/fpls.2014.00105

Bittner, F. (2014). Molybdenum metabolism in plants and crosstalk to iron. *Front. Plant Sci.* 5:28. doi: 10.3389/fpls.2014.00028

Borrill, P., Connorton, J. M., Balk, J., Miller, A. J., Sanders, D., and Uauy, C. (2014). Biofortification of wheat grain with iron and zinc: integrating novel genomic resources and knowledge from model crops. *Front. Plant Sci.* 5:53. doi: 10.3389/fpls.2014.00053

González-Guerrero, M., Matthiadis, A., Sáez, Á., and Long, T. A. (2014). Fixating on metals: new insights into the role of metals in nodulation and symbiotic nitrogen fixation. *Front. Plant Sci.* 5:45. doi: 10.3389/fpls.2014.00045

Grillet, L., Mari, S., and Schmidt, W. (2014). Iron in seeds – loading pathways and subcellular localization. *Front. Plant Sci.* 4:535. doi: 10.3389/fpls.2013.00535

Impa, S. M., Gramlich, A., Tandy, S., Schulin, R., Frossard, E., and Johnson-Beebout, S. E. (2013). Internal Zn allocation influences Zn deficiency tolerance and grain Zn loading in rice (*Oryza sativa* L.). *Front. Plant Sci.* 4:534. doi: 10.3389/fpls.2013.00534

Jain, A., Wilson, G. T., and Connolly, E. L. (2014). The diverse roles of FRO family metalloreductases in iron and copper homeostasis. *Front. Plant Sci.* 5:100. doi: 10.3389/fpls.2014.00100

Jung, H., Gayomba, S. R., Yan, J., and Vatamaniuk, O. K. (2014). *Brachypodium distachyon* as a model system for studies of copper transport in cereal crops. *Front. Plant Sci.* 5:236. doi: 10.3389/fpls.2014.00236

Khan, M. A., Castro-Guerrero, N., and Mendoza-Cozatl, D. G. (2014). Moving toward a precise nutrition: preferential loading of seeds with essential nutrients over non-essential toxic elements. *Front. Plant Sci.* 5:51. doi: 10.3389/fpls.2014.00051

Olsen, L. I., and Palmgren, M. G. (2014). Many rivers to cross: the journey of zinc from soil to seed. *Front. Plant Sci.* 5:30. doi: 10.3389/fpls.2014.00030

Palmer, N. A., Saathoff, A. J., Waters, B. M., Donze, T., Heng-Moss, T. M., Twigg, P., et al. (2014). Global changes in mineral transporters in tetraploid switchgrasses (*Panicum virgatum* L.). *Front. Plant Sci.* 4:549. doi: 10.3389/fpls.2013.00549

Peng, J. S., and Gong, J. M. (2014). Vacuolar sequestration capacity and long-distance metal transport in plants. *Front. Plant Sci.* 5:19. doi: 10.3389/fpls.2014.00019

Pottier, M., Masclaux-Daubresse, C., Yoshimoto, K., and Thomine, S. (2014). Autophagy as a possible mechanism for micronutrient remobilization from leaves to seeds. *Front. Plant Sci.* 5:11. doi: 10.3389/fpls.2014.00011

Ricachenevsky, F. K., and Sperotto, R. A. (2014). There and back again, or always there? The evolution of rice combined strategy for Fe uptake. *Front. Plant Sci.* 5:189. doi: 10.3389/fpls.2014.00189

Sankaran, R. P., and Grusak, M. A. (2014). Whole shoot mineral partitioning and accumulation in pea (*Pisum sativum*). *Front. Plant Sci.* 5:149. doi: 10.3389/fpls.2014.00149

Socha, A. L., and Guerinot, M. L. (2014). Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants. *Front. Plant Sci.* 5:106. doi: 10.3389/fpls.2014.00106

Sperotto, R. A. (2013). Zn/Fe remobilization from vegetative tissues to rice seeds: should I stay or should I go? Ask Zn/Fe supply! *Front. Plant Sci.* 4:464. doi: 10.3389/fpls.2013.00464

Stomph, T. J., Jiang, W., Van Der Putten, P. E. L., and Struik, P. C. (2014). Zinc allocation and re-allocation in rice. *Front. Plant Sci.* 5:8. doi: 10.3389/fpls.2014.00008

Uraguchi, S., Kato, Y., Hanaoka, H., Miwa, K., and Fujiwara, T. (2014). Generation of boron-deficiency-tolerant tomato by overexpressing an *Arabidopsis thaliana* borate transporter AtBOR1. *Front. Plant Sci.* 5:125. doi: 10.3389/fpls.2014.00125

Vasconcelos, M. W., Clemente, T. E., and Grusak, M. A. (2014). Evaluation of constitutive iron reductase (*AtFRO2*) expression on mineral accumulation and distribution in soybean (*Glycine max* L.). *Front. Plant Sci.* 5:112. doi: 10.3389/fpls.2014.00112

Yates, G., and Sadanandom, A. (2013). Ubiquitination in plant nutrient utilization. *Front. Plant Sci.* 4:452. doi: 10.3389/fpls.2013.00452

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Ubiquitination in plant nutrient utilization

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Ubiquitin (Ub) is well-established as a major modifier of signaling in eukaryotes. However, the extent to which plants rely on Ub for regulating nutrient uptake is still in its infancy. The main characteristic of ubiquitination is the conjugation of Ub onto lysine residues of acceptor proteins. In most cases the targeted protein is rapidly degraded by the 26S proteasome, the major proteolysis machinery in eukaryotic cells. The Ub-proteasome system is responsible for removing most abnormal peptides and short-lived cellular regulators, which, in turn, control many processes. This allows cells to respond rapidly to intracellular signals and changing environmental conditions. This perspective will discuss how plants utilize Ub conjugation for sensing environmental nutrient levels. We will highlight recent advances in understanding how Ub aids nutrient homeostasis by affecting the trafficking of membrane bound transporters. Given the overrepresentation of genes encoding Ub-metabolizing enzymes in plants, intracellular signaling events regulated by Ub that lead to transcriptional responses due to nutrient starvation is an under explored area ripe for new discoveries. We provide new insight into how Ub based biochemical tools can be exploited to reveal new molecular components that affect nutrient signaling. The mechanistic nature of Ub signaling indicates that dominant form of any new molecular components can be readily generated and are likely shed new light on how plants cope with nutrient limiting conditions. Finally as part of future challenges in this research area we introduce the newly discovered roles of Ub-like proteins in nutrient homeostasis.

Keywords: ubiquitin, plants, nutrients, abiotic stress, signaling

INTRODUCTION

Our understanding of the layers of regulation that control the cell is deepening at a rapid rate. Much like how the discovery of microRNAs and epigenetics caused major rethinking of well-established gene control systems, protein modification processes are proving to have significant roles in the control of protein function. An example of this is ubiquitination, as a post-translational modifier it is well-known as a system involved in protein turnover, and to a lesser extent is known for its roles in membrane trafficking, DNA repair, chromatin remodeling, and hormone synthesis. However, recent publications have revealed that in addition to its extensive role is stress signaling, ubiquitination also has roles in nutrients utilization. We highlight the importance of the role ubiquitin (Ub) plays in plants ability to uptake and process nutrients using recent examples. It is clear that this area of research is in its infancy and much work has to be done in order to understand the extent to which ubiquitination influences this field.

UBIQUITINATION

Ubiquitin is a small seventy-six amino acid peptide that is highly conserved throughout eukaryotes. Ub is conjugated to the target protein through linkage between a C-terminal glycine and one or more of its seven possible lysine residues. Which of the seven lysine residue forms the attachment and the topology of the subsequent Ub chain directs the fate of the protein. Polyubiquitination via lysine 48 is usually associated with proteasomal degradation and in yeast and mammalian cells mono-ubiquitination and multiubiquitination are precursors to endocytic sorting and degradation via the lysosome and vacuole (Mukhopadhyay and Riezman, 2007). Once the target and the proteasome are connected, deubiquitinating enzymes remove the poly-Ub chain from the target protein, the Ub molecules are recycled, and the target is unfolded and fed into the 26S proteasome for proteolysis (Hartmann-Petersen et al., 2003). Opposed to lysine 48, attachment at lysine 63 is linked to endosomal degradation and trafficking (Duncan et al., 2006). Ub has five other lysines which can also take part in target conjugation, however, the precise physiological implications of these lysine linkages are yet to be discovered.

There are three main steps to Ub attachment to target proteins, each requiring a different enzyme type categorized as E1, E2, and E3. The first of these, E1, is an Ub activating enzyme. At a conserved cysteine residue, E1 forms a high-energy thioester bond with a C-terminal glycine of the Ub molecule, a step that requires ATP (Hatfield et al., 1997). Ub, in its active form, is passed from the E1 to a cysteine residue in the E2 Ub-conjugation enzyme (UBC). which forms an intermediate complex. From here Ub is transferred to the lysine on the target protein, facilitated by the Ub ligase, E3. The E3 protein, of which there are two major subclasses in *Arabidopsis*; containing either a Really Interesting New Gene (RING)- or homologus to E6-AP carboxyl terminus (HECT)-domains, has the specificity to get the Ub to the appropriate target. This is achieved either by direct transfer from E2 to the substrate (RING domain E3) or by the formation of an Ub-E3 intermediate complex (HECT domain E3, Vierstra, 2009). In the *Arabidopsis* genome there are at least 16 genes encoding the Ub molecule itself, two genes for E1's, at least 45 genes for E2's, and over 1400

genes encode E3's. In combination, the elements of the Ub system (UbS) can give specificity to thousands of proteins each targeted by its unique combination of UbS components. This allows very tight control of protein levels and regulation of numerous cellular processes.

Ubiquitin system mechanisms are involved in most areas of plant life from embryogenesis to senescence (Sadanandom et al., 2012). It is postulated that the UbS enables plants to respond rapidly to changes in the environmental conditions by flexible modification of key regulators of cellular physiology.

NUTRIENTS AND UBIQUITIN

Iron is central to many cellular processes in plants including but not limited to photosynthesis, respiration, DNA and hormone synthesis (Curie and Briat, 2003). The uptake of iron by plant roots is dependent on the protein IRON-REGULATED TRANSPORTER 1 (IRT1), which acts at the surface of root-hairs in *Arabidopsis*. A lack of available iron has severe detrimental effects on plants (Briat and Lebrun, 1999). Over accumulation of iron is detrimental in large amounts and can lead to accumulation of reactive oxygen species (Briat and Lebrun, 1999), therefore tight control of IRT1 levels is essential for maintaining iron homeostasis. During iron deprivation the IRT1 transcription is up-regulated, and in *irt1* knockout mutant lines chlorosis forms and is quickly followed by death (Vert, 2002). This shows that IRT1 is essential for iron uptake and interestingly the endocytosis processing of IRT1 is dependent upon ubiquitination. IRT1 shuttles from the plasma membrane to the *trans*-Golgi network/early endosomes (tGN-EE), and undergoes Ub mediated vacuolar degradation (Barberon et al., 2011). Mono-ubiquitination of various lysine residues on IRT1 marks the protein for vacuole sorting; mutation of these residues renders the protein unable to transport, stabilizing it at the plasma membrane (Barberon et al., 2011). This suggests that IRT1 controls iron uptake at the transcriptional level (Vert, 2002) and also post-transcriptionally via Ub. Recently a gene has been isolated that encodes an E3-ligase which facilitates IRT1 degradation. The *IRT1 DEGRADATION FACTOR 1* (IDF1) is a RING-type E3-ligase which interacts with IRT1 and when mutated, results in reduced IRT1 degradation and hence increased iron accumulation (Shin et al., 2013).

Phosphorus is a major element essential to plant life. When active, it is crucial in both dark and light reactions of photosynthesis, as well as playing a vital role in energy metabolism and carbon assimilation. Organic phosphate is abundant in soil but inaccessible to plants. Inorganic phosphate (Pi) is generally of low availability in soils, and because of this, plants have evolved mechanisms to cope with the lack of Pi. In Pi-limited conditions plants have been shown to undergo changes in the distribution and morphology of roots, increased Pi uptake, and secretion of phosphatases and nucleases which enable the mobilization of organic phosphate (Ticconi and Abel, 2004). A major response to phosphorus deprivation is the up-regulation of a microRNA; miR399 (Chiou et al., 2006). In phosphorus poor environments miR399 regulates expression of an E2 enzyme (Fujii et al., 2005). miR399 is found at six loci in the *Arabidopsis* genome, all six transcripts are up-regulated significantly when Pi levels are low. The miR399 nucleotide sequence shows strong similarity to regions of the 5′ UTR of E2 ubiquitin-conjugation enzyme (*UBC24*), making the UBC24 transcript a likely target for decay by miR399 interaction (Allen et al., 2005). This is evident as the up-regulation of miR399 results in cleavage of the UBC24 transcript causing down regulation or even silencing of the UBC24 protein. When overexpressing miR399 in plants, accumulation of phosphate is observed and results in both increased uptake and increased translocation from the roots to the shoots (Chiou et al., 2006). Therefore given that there is interaction between miR399 and the UBC24 transcript, miR399 is most likely controlling phosphorus homeostasis by regulating both the uptake and trafficking of phosphorus, via control of the UBC24 protein level. The only suggested target of UBC24 is PHOSPHATE1 (PHO1), which is involved in the loading of phosphate to the xylem (Liu et al., 2012). Interestingly, the mammalian homolog of UBC24 is Apollon (Bartke et al., 2004) which acts as an inhibitor of cell death localized to the tGN and vesicles. Apollon has been shown to monoubiquitinate substrates in the presence of an E1 enzyme alone. Taken together, it is likely that the UBC24 also functions as an E2 with specific targets found in the tGN and vesicles.

Boron is another essential nutrient for plant life but is highly toxic in large quantities (Shorrocks, 1997). Plants depend on Borate to form a crosslinking dimer for proper formation of the cell wall component pectic polysaccharide rhamnogalacturonan II, without which plants do not develop normally and are largely non-viable (O'Neill et al., 2001). The uptake of boron depends on the transporter protein BORONTRANSPORTER1 (BOR1), and the boric acid channel protein NIP5;1. NIP5;1 is found on the soil facing surface of root cells and is essential for boric acid uptake in boron limited conditions (Takano et al., 2006). BOR1 is an efflux channel and is responsible for boron transport in low boron conditions (Takano et al., 2002, 2010). The BOR1 transporter utilizes the endocytic degradation pathway in high boron conditions where the excess is trafficked to the vacuole. This sorting of the loaded BOR1 transporter is made possible by its mono-ubiquitination or di-ubiquitination at lysine 590 and this is induced by high boron conditions. Mutation of BOR1 at lysine 590 abolishes both the ubiquitination and the degradation via the endocytic pathway (Takano et al., 2010). However, although ubiquitination is necessary for the sorting into multivesicular bodies, it is not required for BOR1 endocytosis, this process is very similar to the sorting of epidermal growth factor receptors (EGFR) in mammals (Huang et al., 2007). The E3 ligase responsible for targeting BOR1 is not yet known (Kasai et al., 2011). However, the EGFR undergoes ubiquitination by a WW (has two highly conserved tryptophans) containing domain E3 ligase which is homologous to HECT-type E3 ligase in *Arabidopsis* (Kraft et al., 2005).

There are similarities in the way BOR1 and IRT1 exploit the endocytic pathway for regulating their respective minerals, however, there is no current examples of plasma membrane transport proteins that involve processing by HECT-type E3 ligases (Mulet et al., 2013). However, it is probable, given the example of the Apollon, that there may be E2 enzymes involved in processing these membrane transporters without the need for a separate E3.

Plants need nitrogen in high abundance, as it is a building block for proteins and nucleic acids. Nitrogen limiting conditions are widespread and as such plants have evolved a range of adaptive

responses to cope with the low availability. Amino acids are the major source of nitrogen transported in the xylem and phloem and they provide an important link between the nitrogen status of the roots and the rest of the plant (Paungfoo-Lonhienne et al., 2008). Amino acids can impede nitrogen uptake by roots and may play a major role in the regulation of transporters, providing part of a feedback system in sensing nitrogen (Miller et al., 2007). The import and export of amino acids in and out of the plant cells involve various membrane proteins and proton-gradient dependant transporters. The importing mechanisms are well-studied but little insight to the export processes exists (for review see Okumoto and Pilot, 2011). Despite the lack of understanding of the export mechanisms however, *GLUTAMINE DUMPER1 (GUD1)* overexpression in *Arabidopsis* leads to excess amino acid export. It is not understood exactly how this functions but mutant screens revealed it is the essential component for secretion of amino acids. Yeast-two-hybrid screens and glutathione S-transferase pull-down experiments shows that GUD1 interacts with a RING-type Ub E3 ligase; LOSSOFGUD2 (LOG2). LOG2 is required for the export of amino acids induced by GUD1 (Pratelli et al., 2012). This interaction does not appear to be negatively regulating GUD1 by Ub based degradation but instead may serve as an activation step or perhaps GUD1 and LOG2 form part of a complex for another Ub target which could then act as a repressor or activator of that substrate (Léon and Haguenauer-Tsapis, 2009; Pratelli et al., 2012). Either way this work highlights a significant role for ubiquitination in the homeostasis of nitrogen, via amino acid regulation/secretion.

NITROGEN LIMITATION ADAPTION (NLA) mutants in *Arabidopsis* are unable to respond at a transcriptional level to nitrogen limiting conditions. NLA is the only identified component of the plants response to nitrogen limitation and is another RING-type E3 Ub ligase. Mutations within the RING motif of *NLA*, are unable to initiate a response to low nitrogen conditions and they show the same phenotype as low nitrogen grown plants and induce early senescence. NLA is a positive regulator of the plants adaptability to nitrogen limiting conditions (Kant et al., 2011a). This identifies a major role for ubiquitination in the plants response to limited nitrogen and perhaps hints at a universal role for Ub in other areas of metabolism. This claim is reinforced by the fact that two E3 ligases have been shown to play a role in the carbon/nitrogen response (Sato et al., 2009). In addition to this, phosphate homeostasis is in part controlled by NLA in a nitrate dependant manner, and Pi shows an antagonistic cross-talk with nitrate in terms of both their accumulation and influence on the onset of flowering (Kant et al., 2011b). The PHOSPHATE TRANSPORTER1 (PHT1) group of proteins are crucial for Pi homeostasis and these are also regulated by NLA. PHOSPHATE2 (PHO2 also called UBC24), works in harmony with NLA at the plasma membrane, however, data suggest they act independently and both become targets of microRNAs (miR827 and miR399, respectively) under Pi deprivation (Huang et al., 2013). Interestingly PHO2 also induces PHT1 degradation under high Pi (Lin et al., 2013).

The Ub ligases ATL31 and ATL6 have been identified as crucial components of the proper recognition of the balance between carbon and nitrogen during germinative growth. The ratio of carbon to nitrogen is sensed and responded to by *Arabidopsis* and if the peripheral ratio is too high growth is arrested. The *atl31* mutant was found to be insensitive to carbon/nitrogen stress, while the overexpression of *ATL31* resulted in the stress response but under normal conditions (Sato et al., 2009). ATL31 is therefore likely to negatively regulate proteins which detect the ratio of carbon to nitrogen under stress conditions. This underlines the importance ubiquitination has on the various stages of growth and development and again suggests that ubiquitination has a role in a plethora of functions in relation to nutrient homeostasis.

UBIQUITIN-LIKE PROTEINS AND NUTRIENT STARVATION

In addition to ubiquitination there are other small protein modifiers categorized as Ub-like proteins (Ubls) that can also be conjugated to target proteins. An example of this is the small Ub-related modifier (SUMO). The addition of SUMO to its target can have an effect on its targets ability to bind with the substrate. There are examples of SUMO preventing linkage between the target protein and its binding partner, and also examples of where the SUMOylation enables the binding of the target and its binding partner (for review see Geiss-Fridelander and Melchior, 2007; Miura and Hasegawa, 2010). These mechanisms allow the process of SUMOylation to regulate some of the cells systems by acting as on and/or off switches for rapid responses to various changes in the cell and the environment. SUMOylation has also been linked to the area of nutrient regulation. Phosphate starvation dependant responses have been shown to be under the control of a SUMO E3 ligase; SIZ1 (Miura et al., 2005). SIZ1 controls the activation of the transcription factor PHOSPHATE STARVATION RESPONSE 1(PHR1), which has been shown to bind to the promoter region of the majority of genes which are either up-regulated or down-regulated in response to phosphate starvation (Bustos et al., 2010). Mutant plants with altered SIZ1 show typical phosphate starvation responses when grown on normal conditions, this includes; discontinued primary root development, exaggerated lateral root growth, increased root hair development and excess anthocyanin accumulation, despite the internal phosphate levels being normal (Miura et al., 2005). Further to this deubiquitination has also been shown to play part in the plants response to limited Pi. UBP14 is an Ub-specific protease that acts by modifying root morphology in Pi limited conditions (Li et al., 2010).

Copper is needed by plants in small amounts but is essential for normal development. It is heavily involved in many aspects of growth and development including electron transport, redox reactions and as a cofactor for many metalloproteins. Copper is also toxic is surplus quantities, with excess build-up leading to chlorosis, root growth cessation, and even reduced iron uptake (Burkhead et al., 2009). In high copper conditions, SUMOylation is induced and *SIZ1* mutant plants grown in excess copper conditions tend to show underdeveloped shoot growth compared to wild-type. *SIZ1* mutants also accumulate more copper than any other metal relative to normal amounts. Further to this, there is a stark difference in the shoot-to-root ratio of copper in mutant plants compared to wild-type in high copper conditions and accumulation of SUMO1 conjugates is not observed in *SIZ1* plants whereas it is stimulated by copper in the wild-type (Chen et al., 2011). SIZ1 also regulates nitrogen levels by its E3 SUMO ligase activity. A reduction of nitrate reductase activity is observed

in *siz1-2* plants. Data show this is due to SIZ1 targeting nitrate reductases (NIA1 and NIA2), which become highly active when SUMOylated (Park et al., 2011).

TOOLS FOR TARGETING UBIQUITIN AND UBIQUITIN-LIKE PROTEINS AND COMPONENTS

Its clear that target identification is key to revealing the influence of UbS in nutrient use efficiency in plants. More sensitive mass spectrometry methods are now available to identify targets for ubiquitination and SUMOylation in planta (Elrouby and Coupland, 2010; Miller et al., 2010). Refined pull-down assays and *in vitro* ubiquitination assays now make finding targets of the process much more straight forward. One method uses detection of thioester bonds between either the E2 and a subclass of E3, or E3 and its substrate (Zhao et al., 2012). This allows for identifying the target protein and the residue to which the thioester bond forms enabling the generation of dominant forms of acute signaling molecules. It can also be used to work back toward identification of the E3 and E2 if the target is already known.

CONCLUSION

From the evidence here, it is clear that ubiquitination and Ubls play essential roles in uptake, trafficking and maintenance of many of the essential nutrients for plants. However, it is also clear that this area of research in ripe for new discoveries. It is highly likely that understanding the role of Ub in nutrient uptake and processing will provide much needed insight for the development of crops better suited to nutrient deprived land.

REFERENCES

Allen, E., Xie, Z., Gustafson, A. M., and Carrington, J. C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121, 207–221. doi: 10.1016/j.cell.2005.04.004

Barberon, M., Zelazny, E., Robert, S., Conejero, G., Curie, C., Friml, J., et al. (2011). Monoubiquitin-dependent endocytosis of the iron-regulated transporter 1 (IRT1) transporter controls iron uptake in plants. *Proc. Natl. Acad. Sci. U.S.A.* 108, E450–E458. doi: 10.1073/pnas.1100659108

Bartke, T., Pohl, C., Pyrowolakis, G., and Jentsch, S. (2004). Dual role of BRUCE as an antiapoptotic IAP and a chimeric E2/E3 ubiquitin ligase. *Mol. Cell* 14, 801–811. doi: 10.1016/j.molcel.2004.05.018

Briat, J. F., and Lebrun, M. (1999). Plant responses to metal toxicity. *C. R. Acad. Sci. III* 322, 43–54. doi: 10.1016/S0764-4469(99)80016-X

Burkhead, J. L., Reynolds, K. A. G., Abdel-Ghany, S. E., Cohu, C. M., and Pilon, M. (2009). Copper homeostasis. *New Phytol.* 182, 799–816. doi: 10.1111/j.1469-8137.2009.02846.x

Bustos, R., Castrillo, G., Linhares, F., Puga, M. I., Rubio, V., Pérez-Pérez, J., et al. (2010). A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in *Arabidopsis*. *PLoS Genet.* 6:e1001102. doi: 10.1371/journal.pgen.1001102

Chen, C. C., Chen, Y. Y., Tang, I. C., Liang, H. M., Lai, C. C., Chiou, J. M., et al. (2011). *Arabidopsis* SUMO E3 ligase SIZ1 is involved in excess copper tolerance. *Plant Physiol.* 156, 2225–2234. doi: 10.1104/pp.111.178996

Chiou, T. J., Aung, K., Lin, S. I., Wu, C. C., Chiang, S. F., and Su, C. L. (2006). Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell* 18, 412–421. doi: 10.1105/tpc.105.038943

Curie, C., and Briat, J. F. (2003). Iron transport and signaling in plants. *Annu. Rev. Plant Biol.* 54, 183–206. doi: 10.1146/annurev.arplant.54.031902.135018

Duncan, L. M., Piper, S., Dodd, R. B., Saville, M. K., Sanderson, C. M., Luzio, J. P., et al. (2006). Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J.* 25, 1635–1645. doi: 10.1038/sj.emboj.7601056

Elrouby, N., and Coupland, G. (2010). Proteome-wide screens for small ubiquitin-like modifier (SUMO) substrates identify *Arabidopsis* proteins implicated in diverse biological processes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17415–17420. doi: 10.1073/pnas.1005452107

Fujii, H., Chiou, T. J., Lin, S. I., Aung, K., and Zhu, J. K. (2005). A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* 15, 2038–2043. doi: 10.1016/j.cub.2005.10.016

Geiss-Fridelander, R., and Melchior, F. (2007). Concepts in SUMOylation: a decade on. *Nat. Rev. Mol. Cell Biol.* 8, 947–956. doi: 10.1038/nrm2293

Hartmann-Petersen, R., Seeger, M., and Gordon, C. (2003). Transferring substrates to the 26S proteasome. *Trends Biochem. Sci.* 28, 26–31. doi: 10.1016/S0968-0004(02)00002-6

Hatfield, P. M., Gosink, M. M., Carpenter, T. B., and Vierstra, R. D. (1997). The ubiquitin-activating enzyme (E1) gene family in *Arabidopsis thaliana*. *Plant J.* 11, 213–226. doi: 10.1046/j.1365-313X.1997.11020213.x

Huang, F., Goh, L. K., and Sorkin, A. (2007). EGF receptor ubiquitination is not necessary for its internalization. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16904–16909. doi: 10.1073/pnas.0707416104

Huang, T.-K., Han, C.-L., Lin, S.-I., Chen, Y.-J., Tsai, Y.-C., Chen, Y.-R., et al. (2013). Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in *Arabidopsis* Roots. *Plant Cell* doi: 10.1105/tpc.113.115998 [Epub ahead of print].

Kant, S., Peng, M., and Rothstein, S. J. (2011a). A mutation in NLA, which encodes a RING-type ubiquitin ligase, disrupts the adaptability of *Arabidopsis* to nitrogen limitation. *PLoS Genet.* 7:e1002021. doi: 10.1111/j.1365-313X.2007.03050.x

Kant, S., Peng, M., and Rothstein, S. J. (2011b). Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in *Arabidopsis*. *PLoS Genet.* 7:e1002021. doi: 10.1371/journal.pgen.1002021

Kasai, K., Takano, J., Miwa, K., Toyoda, A., and Fujiwara, T. (2011). High boron-induced ubiquitination regulates vacuolar sorting of the BOR1 borate transporter in *Arabidopsis thaliana*. *J. Biol. Chem.* 286, 6175–6183. doi: 10.1074/jbc.M110.184929

Kraft, E., Stone, S. L., Ma, L., Su, N., Gao, Y., Lau, O. S., et al. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of *Arabidopsis*. *Plant Physiol.* 139, 1597–1611. doi: 10.1104/pp.105.067983

Léon, S., and Haguenauer-Tsapis, R. (2009). Ubiquitin ligase adaptors: regulators of ubiquitylation and endocytosis of plasma membrane proteins. *Exp. Cell Res.* 315, 1574–1583. doi: 10.1016/j.yexcr.2008.11.014

Li, W.-F., Perry, P. J., Prafulla, N. N., and Schmidt, W. (2010). Ubiquitin-Specific Protease 14 (UBP14) is involved in root responses to phosphate deficiency in *Arabidopsis*. *Mol. Plant* 3, 212–223. doi: 10.1093/mp/ssp086

Lin, W.-Y., Huang, T.-K., and Chiou, T.-J. (2013). NITROGEN LIMITATION ADAPTATION, a target of microRNA827, mediates degradation of plasma membrane–localized phosphate transporters to maintain phosphate homeostasis in *Arabidopsis*. *Plant Cell* doi: 10.1105/tpc.113.116012 [Epub ahead of print].

Liu, T.-Y., Huang, T.-K., Tseng, C.-Y., Lai, Y.-S., Lin, S.-I., Lin, W.-Y., et al. (2012). PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in *Arabidopsis*. *Plant Cell* 24, 2168–2183. doi: 10.1105/tpc.112.096636

Miller, A. J., Fan, X., Shen, Q., and Smith, S. J. (2007). Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *J. Exp. Bot.* 59, 111–119. doi: 10.1093/jxb/erm208

Miller, M. J., Barrett-Wilt, G. A., Hua, Z., and Vierstra, R. D. (2010). Proteomic analyses identify a diverse array of nuclear processes affected by small ubiquitin-like modifier conjugation in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16512–16517. doi: 10.1073/pnas.1004181107

Miura, K., and Hasegawa, P. M. (2010). Sumoylation and other ubiquitin-like post-translational modifications in plants. *Trends Cell Biol.* 20, 223–232. doi: 10.1016/j.tcb.2010.01.007

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A. S., Raghothama, K. G., et al. (2005). The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7760–7765. doi: 10.1073/pnas.0500778102

Mukhopadhyay, D., and Riezman, H. (2007). Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* 315, 201–205. doi: 10.1126/science.1127085

Mulet, M. J., Llopis-Torregrosa, V., Primo, C., and Marqués, C., Yenush, L. (2013). Endocytic regulation of alkali metal transport proteins in mammals, yeast and plants. *Curr. Genet.* 59, 207–230. doi: 10.1007/s00294-013-0401-2

Okumoto, S., and Pilot, G. (2011). Amino acid export in plants: a missing link in nitrogen cycling. *Mol. Plant* 4, 453–463. doi: 10.1093/mp/ssr003

O'Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001). Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* 294, 846–849. doi: 10.1126/science.1062319

Park, B. S., Song, J. T., and Seo, H. S. (2011). *Arabidopsis* nitrate reductase activity is stimulated by the E3 SUMO ligase AtSIZ1. *Nat. Commun.* 2, 400. doi: 10.1038/ncomms1408

Paungfoo-Lonhienne, C., Lonhienne, T. G. A., and Rentsch, D. (2008). Plants can use protein as a nitrogen source without assistance from other organisms. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4524–4529. doi: 10.1073/pnas.0712078105

Pratelli, R., Guerra, D. D., Yu, S., Wogulis, M., Kraft, E., Frommer, W. B., et al. (2012). The ubiquitin E3 ligase LOSS OF GDU2 is required for GLUTAMINE DUMPER1-induced amino acid secretion in *Arabidopsis*. *Plant Physiol.* 158, 1628–1642. doi: 10.1104/pp.111.191965

Sadanandom, A., Bailey, M., Ewan, R., Lee, J., and Nelis, S. (2012). The ubiquitin-proteasome system: central modifier of plant signalling. *New Phytol.* 196, 13–28. doi: 10.1111/j.1469-8137.2012.04266

Sato, T., Maekawa, S., Yasuda, S., Sonoda, Y., Katoh, E., Ichikawa, T., et al. (2009). CNI1/ATL31, a RING-type ubiquitin ligase that functions in the carbon/nitrogen response for growth phase transition in *Arabidopsis* seedlings. *Plant J.* 60, 852–864. doi: 10.1111/j.1365-313X.2009.04006.x

Shin, L., Lo, J., Chen, G., Callis, J., Fu, H., and Yeh, K. (2013). IRT1 DEGRADATION FACTOR1, a RING E3 ubiquitin ligase, regulates the degradation of IRON-REGULATED TRANSPORTER1 in *Arabidopsis*. *Plant Cell* 25, 3039–3051. doi: 10.1105/tpc.113.115212

Shorrocks, V. M. (1997). The occurrence and correction of boron deficiency. *Plant Soil* 193, 121–148. doi: 10.1007/978-94-011-5580-9_9

Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., et al. (2002). *Arabidopsis* boron transporter for xylem loading. *Nature* 420, 337–340. doi: 10.1038/nature01139

Takano, J., Tanaka, M., Toyoda, A., Miwa, K., Kasai, K., Fuji, K., et al. (2010). Polar localization and degradation of *Arabidopsis* boron transporters through distinct trafficking pathways. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5220–5225. doi: 10.1073/pnas.0910744107

Takano, J., Wada, M., Ludewig, U., Schaaf, G., von Wirén, N., and Fujiwara, T. (2006). The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell* 18, 1498–1509. doi: 10.1105/tpc.106.041640

Ticconi, C. A., and Abel, S. (2004). Short on phosphate: plant surveillance and countermeasures. *Trends Plant Sci.* 9, 548–555. doi: 10.1016/j.tplants.2004.09.003

Vert, G. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

Vierstra, R. D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat. Rev. Mol. Cell Biol.* 10, 385–397. doi: 10.1038/nrm2688

Zhao, Q., Liu, L., and Xie, Q. (2012). In vitro protein ubiquitination assay. *Methods Mol. Biol.* 876, 163–172. doi: 10.1007/978-1-61779-809-2_13

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Zn/Fe remobilization from vegetative tissues to rice seeds: should I stay or should I go? Ask Zn/Fe supply!

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Rice is a staple food for at least 50% of the world's population. Therefore, it is one of the most important crop plants on Earth (Lucca et al., 2002). However, milled rice is a poor source of essential micronutrients such as Fe and Zn (Bouis and Welch, 2010), whose deficiencies affect over three billion people worldwide, mostly in developing countries (Welch and Graham, 2004). Fe and Zn malnutrition are leading risk factors for disability and death, especially to children eating cereal-based diets, resulting in impaired functions of the brain, the immune and reproductive systems and energy metabolism (Graham et al., 2001). The development of new cultivars with elevated concentrations of Fe and Zn would be extremely relevant to alleviate malnutrition, but the lack of knowledge about how nutrients are translocated from vegetative tissues to the seeds is one of the barriers to rice biofortification (Colangelo and Guerinot, 2006; Sperotto et al., 2012a).

There are evidences in literature demonstrating that foliar applied Zn and Fe can be absorbed by leaf epidermis, remobilized, and transferred into the rice grains through the phloem (Wu et al., 2010; Wei et al., 2012; Yuan et al., 2012), presumably using several Zn- and Fe-regulated transporters (Schaaf et al., 2005; Bashir et al., 2012; Zhang et al., 2012). However, restrictions to the mobility of Zn and Fe supplied as cations can be expected due to the abundance of negative charges in the apoplastic space, which may limit their translocation to other plant compartments and/or organs (Fernández and Brown, 2013). Meanwhile, the translocation and redistribution of these minerals after its root uptake and passage to the transpiration stream have been extensively studied in the last years (for a comprehensive review see Ishimaru et al., 2011). Distribution of Fe and Zn within rice plants largely occurs through transport in the xylem, transfer from the xylem to the phloem, and retranslocation in the phloem (Ishimaru et al., 2011). Xylem transport is simply directed from roots to shoots in the transpiration stream, whereas phloem transport from old to new leaves is more selective, and is largely dependent on the phloem mobility of each element. In relation to their phloem mobility, Zn and Fe are considered intermediate or conditionally mobile (Fernández and Brown, 2013).

Remobilization of reserves to supply rice seeds with minerals has been emphasized in previous studies, but the contribution of stored minerals to total seed mineral content is unclear. During rice grain filling, Zn remobilization from leaves is not as important as Zn uptake by roots (Jiang et al., 2007). At the same time, increased root uptake does not necessarily result in enhanced Zn accumulation in rice grains (Jiang et al., 2008). None of the Zn foliar application treatments in rice showed that the main portion of Zn loaded in grain was remobilized from leaves (Jiang et al., 2008; Stomph et al., 2009). Wu et al. (2010) showed that large amounts of the Zn in rice seeds at maturity had been retranslocated from other plant parts and not directly acquired by the roots. Recently, Yoneyama et al. (2010) reported that Zn (and Fe) in the rice grains may be actively supplied via the phloem after mobilization from the leaf blades.

Iron stored in the flag and upper leaves may also be transported to the grains via the phloem. However, probably due to its limited mobility in the phloem, it seems difficult to improve the Fe nutrition of rice grain by Fe spray. Fang et al. (2008) were able to increase Fe content of rice grains by foliar application of Zn and Selenium. According to Yuan et al. (2012), after being taken up by the leaves, low-molecular-weight amino acids might chelate with Fe, which would increase the mobility of Fe and enhance its translocation to the sink during the development of rice grains. Grain Fe and Zn may share similar protein-dependent mechanisms for translocation to or storage in the grain, and several reports indicate a positive correlation between Fe and Zn grain concentrations (for a comprehensive review see Sperotto et al., 2012a). Such similarities between Fe and Zn raise the possibility of simultaneously biofortifying crops with more than one micronutrient, as previously found through the increase of nicotianamine concentration, a chelator of transition metals that plays important roles in long- and short-distance transport of metal cations (Johnson et al., 2011).

Flag leaf plays important roles in rice plants. It is already known that removal of the rice flag leaf at any stage after panicle emergence can cause significant reduction in grain yield (Abou-Khalifa et al., 2008), being the major component for yield losses due to the impaired synthesis and translocation of photoassimilates. On the other hand, it seems that minerals do not have the same behavior of photoassimilates, since no single report has pointed flag leaves as the major source of Fe and Zn to the rice developing seeds. As it is hypothesized that flag leaves could have a role in Fe and Zn remobilization to rice

seeds, several reports tried to find a relationship between gene expression in flag leaves with concentration of mineral nutrients in rice seeds (Narayanan et al., 2007; Sperotto et al., 2009, 2010; Zhang et al., 2012).

To address this question, Sperotto et al. (2013) conducted field experiments to evaluate the effect of flag leaf removal (at anthesis) on seed Fe and Zn concentration and content. Seed Fe and Zn accumulation were not affected by flag leaf removal, suggesting that the flag leaf is not absolutely required for metal remobilization to the seeds of rice plants growing in field conditions. These authors also removed the second upper leaf and found similar results. Probably, the lack of flag leaves is compensated by other leaves and stem/sheath remobilization and/or continuous uptake by roots. In *Arabidopsis*, continuous uptake and translocation of minerals to source tissues during seed fill is as important, if not more important, than remobilization of previously stored minerals (Waters and Grusak, 2008).

In another experiment, Sperotto et al. (2012b) showed that mineral remobilization from green tissues can be severely affected by Fe status. Rice plants watered with a high Fe concentration showed no Fe remobilization from flag leaves, non-flag leaves and stems/sheaths. On the other hand, plants watered with low Fe concentration showed the highest Fe remobilization from stems/sheaths, probably due to reduced uptake from the roots during seed fill. Plants watered with a sufficient Fe concentration showed high levels of mineral remobilization (including Fe and Zn) mostly from flag leaves but also from stems/sheaths. However, as the flag leaves mineral content is much lower than the stems/sheaths content, the maximum possible contribution to seed mineral content is, in general, higher from stems/sheaths than from flag leaves (Sperotto et al., 2012b). It seems that abundant Fe supply at the root level promotes continued uptake during seed fill, which may have reduced the need for remobilization to serve as a source of Fe for seeds. A similar pattern was observed for Zn (Jiang et al., 2007, 2008; Impa et al., 2013). According to Jiang et al. (2007), in rice plants grown under sufficient or surplus Zn supply, most of the Zn accumulated in the grain originates from uptake by roots after flowering and not from Zn remobilization from leaves. It was also shown that, at lower Zn supply levels, the Zn taken up by the roots after flowering seems to accumulate mostly in the grain, which is accompanied by net Zn remobilization from the leaves and transport to the grain. However, at higher Zn supply levels, Zn content in all non-grain organs remained constant (roots, leaves and sheaths) or continued to increase after flowering, and grain Zn accumulation could be fully accounted for by Zn uptake during grain filling (Jiang et al., 2008).

Evidences show that Zn and Fe remobilization from vegetative tissues can occur in rice plants; however, remobilization is not required for seeds to acquire minerals. Contrasting results may be due to different rice genotypes behaving differently. Thus, there may not be only one way for rice to load Zn/Fe into grain, but genotype-specific variations. Such useful variations should be used as targets for biofortification breeding strategies. Probably, the xylem discontinuity at the base of the rice grain (Stomph et al., 2009) can contribute to the fact that continued uptake and translocation of minerals to source tissues during seed fill is as or more important than remobilization of previously stored minerals from vegetative tissues. Remobilization results found for *Arabidopsis* plants by Waters and Grusak (2008) were not exactly consistent between experiments, as differences were found in elements and in amounts remobilized. Also, mineral remobilization from vegetative tissues in rice seems to be modified by plant Zn and Fe nutrition, because different Zn/Fe supplies alter the remobilization levels. Probably, rice genotypes with different efficiencies in the use of Zn and Fe and also with different levels of Zn and Fe in the seeds can show different remobilization patterns, since mineral transport can be influenced by minimal changes in source-sink communication (Jiang et al., 2008; Wu et al., 2010; Impa et al., 2013).

Based on the findings of different studies (Jiang et al., 2007, 2008; Wu et al., 2010; Yoneyama et al., 2010; Sperotto et al., 2012b; Impa et al., 2013), a proposed model for Zn and Fe allocation to the rice grains is shown in **Figure 1**. Under Zn-sufficient conditions (**Figure 1A**), Zn in the rice grain is mostly supplied by continued root uptake during grain-filling stage. To a lesser extent, Zn from stems

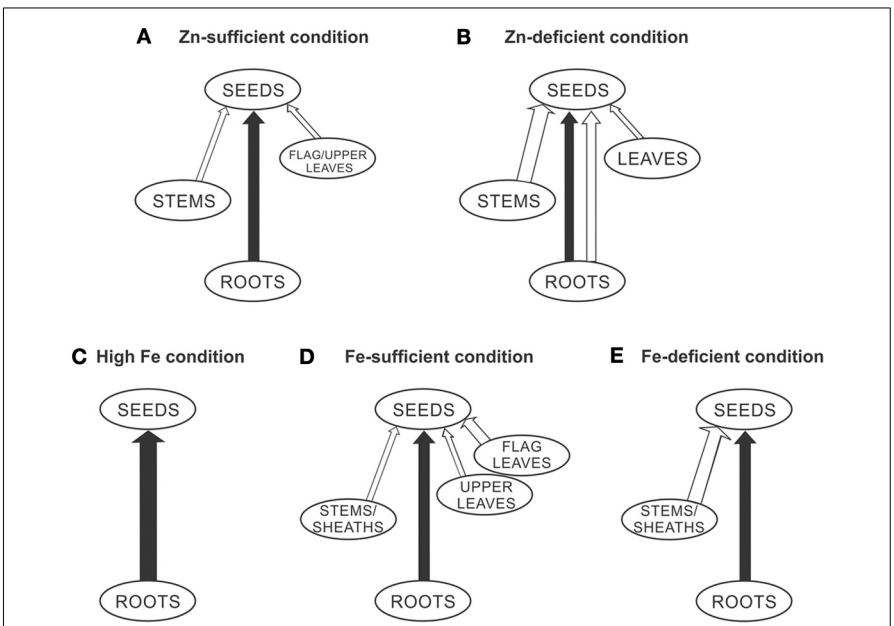

FIGURE 1 | Proposed model for Zn and Fe allocation to the rice seeds under different Zn and Fe supplies (A, Zn-sufficient condition; B, Zn-deficient condition; C, High Fe condition; D, Fe-sufficient condition; E, Fe-deficient condition), through the xylem (continued root uptake, black lines) and through the phloem (mineral remobilization, white lines). The thicknesses of the lines represent more or less active processes.

and flag/upper leaves can be remobilized. Under low Zn supply (**Figure 1B**), both continued root uptake and also remobilization from roots, stems, and leaves can occur. It is important to note that high Zn availability is rarely found in field conditions, even with adequate fertilizer application, being most common in artificial growth, under laboratory conditions. Under high Fe conditions (**Figure 1C**), continued root uptake can fully account for seed Fe allocation. Under control (or sufficient) Fe supply (**Figure 1D**), continued root uptake and also remobilization from stems/sheaths and flag/upper leaves contribute to total seed Fe. Under Fe-deficient conditions (**Figure 1E**), seed Fe can be fully addressed by remobilization from stems/sheaths and continued root uptake. It is important to highlight that this model only summarize previous studies, and cannot be used *per se* to help rice researchers to increase seed mineral concentrations. For this purpose, we would have to discover how to "trick" rice plants through manipulation of transport and accumulation processes, in order to efficiently increase Zn and Fe concentrations into grains.

Loading and bioavailability of minerals in the rice grains, mainly in milled rice, not only follows the transport and remobilization pathway. It also depends on the mechanism of entry from grain aleurone layer to the inner endosperm. Several barriers to Fe entry (and probably to Zn) were identified, as transport proteins which only internalize Fe during germination, chelating molecules such as nicotianamine and deoximugineic acid, low levels of ferritin protein in the endosperm tissue and mostly high levels of phytic acid (Paul et al., 2013), which is a potent inhibitor of Fe absorption (also called anti-nutrient factor). Phytic acid localizes almost exclusively to the bran of brown rice (Prom-u-thai et al., 2008), acting as a barrier to endosperm internalization. As biofortification efforts should focus primarily on increasing endosperm Zn/Fe bioavailability, reductions of phytic acid content would be important for rice biofortification, and several promising biotechnological approaches have been studied. Anyway, understanding the mechanisms of Zn and Fe remobilization from vegetative tissues and seed loading and bioavailability in rice is crucial to the enrichment of rice with Zn and Fe, which is a way to generate major health benefits for a large number of susceptible people.

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REFERENCES

Abou-Khalifa, A. A. B., Misra, A. N., and Salem, A. E. A. K. M. (2008). Effect of leaf cutting on physiological traits and yield of two rice cultivars. *Afric. J. Plant Sci.* 2, 147–150.

Bashir, K., Ishimaru, Y., and Nishizawa, N. K. (2012). Molecular mechanisms of zinc uptake and translocation in rice. *Plant Soil* 361, 189–201. doi: 10.1007/s11104-012-1240-5

Bouis, H. E., and Welch, R. M. (2010). Biofortification - a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Sci.* 50, S20–S32. doi: 10.2135/cropsci2009.09.0531

Colangelo, E. P., and Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Curr. Opin. Plant Biol.* 9, 322–330. doi: 10.1016/j.pbi.2006.03.015

Fang, Y., Wang, L., Xin, Z., Zhao, L., An, X., and Hu, Q. (2008). Effect of foliar application of zinc, selenium, and iron fertilizers on nutrients concentration and yield of rice grain in China. *J. Agric. Food Chem.* 56, 2079–2084. doi: 10.1021/jf800150z

Fernández, V., and Brown, P. H. (2013). From plant surface to plant metabolism: the uncertain fate of foliar-applied nutrients. *Front. Plant Sci.* 4:289. doi: 10.3389/fpls.2013.00289

Graham, R. D., Welch, R. M., and Bouis, H. E. (2001). Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. *Adv. Agron.* 70, 77–142. doi: 10.1016/S0065-2113(01)70004-1

Impa, S. M., Morete, M. J., Ismail, A. M., Schulin, R., and Johnson-Beebout, S. E. (2013). Zn uptake, translocation, and grain Zn loading in rice (*Oryza sativa* L.) genotypes selected for Zn deficiency tolerance and high grain Zn. *J. Exp. Bot.* 64, 2739–2751. doi: 10.1093/jxb/ert118

Ishimaru, Y., Bashir, K., and Nishizawa, N. K. (2011). Zn uptake and translocation in rice plants. *Rice* 4, 21–27. doi: 10.1007/s12284-011-9061-3

Jiang, W., Struik, P. C., Lingna, J., van, K. H., Zhao, M., and Stomph, T. J. (2007). Uptake and distribution of root-applied or foliar applied ^{65}Zn after flowering in aerobic rice. *Ann. Appl. Biol.* 150, 383–391. doi: 10.1111/j.1744-7348.2007.00138.x

Jiang, W., Struik, P. C., van, K. H., Zhao, M., Jin, L. N., and Stomph, T. J. (2008). Does increased Zn uptake enhance grain Zn mass concentration in rice? *Ann. Appl. Biol.* 153, 135–147. doi: 10.1111/j.1744-7348.2008.00243.x

Johnson, A. A. T., Kyriacou, B., Callahan, D. L., Carruthers, L., Stangoulis, J., Lombi, E., et al. (2011). Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS ONE* 6:e24476. doi: 10.1371/journal.pone.0024476

Lucca, P., Hurrell, R., and Potrykus, I. (2002). Fighting iron deficiency anemia with iron-rich rice. *J. Am. Coll. Nutr.* 21, 184S–190S. doi: 10.1080/07315724.2002.10719264

Narayanan, N. N., Vasconcelos, M. W., and Grusak, M. A. (2007). Expression profiling of *Oryza sativa* metal homeostasis genes in different rice cultivars using a cDNA macroarray. *Plant Physiol. Biochem.* 45, 277–286. doi: 10.1016/j.plaphy.2007.03.021

Paul, S., Ali, N., Sarkar, S. N., Datta, S. K., and Datta, K. (2013). Loading and bioavailability of iron in cereal grains. *Plant Cell Tiss. Organ Cult.* 113, 363–373. doi: 10.1007/s11240-012-0286-7

Prom-u-thai, C., Huang, L., Rerkasem, B., Thompson, G., Kuo, J., Saunders, M., et al. (2008). Distribution of protein bodies and phytate-rich inclusions in grain tissues of low and high iron rice genotypes. *Cereal Chem.* 85, 257–265. doi: 10.1094/CCHEM-85-2-0257

Schaaf, G., Schikora, A., Häberle, J., Vert, G., Ludewig, U., Briat, J. F., et al. (2005). A putative function for the *Arabidopsis* Fe-phytosiderophore transporter homolog AtYSL2 in Fe and Zn homeostasis. *Plant Cell Physiol.* 46, 762–774. doi: 10.1093/pcp/pci081

Sperotto, R. A., Boff, T., Duarte, G. L., Santos, L. S., Grusak, M. A., and Fett, J. P. (2010). Identification of putative target genes to manipulate Fe and Zn concentrations in rice grains. *J. Plant Physiol.* 167, 1500–1506. doi: 10.1016/j.jplph.2010.05.003

Sperotto, R. A., Ricachenevsky, F. K., Duarte, G. L., Boff, T., Lopes, K. L., Sperb, E. R., et al. (2009). Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. *Planta* 230, 985–1002. doi: 10.1007/s00425-009-1000-9

Sperotto, R. A., Ricachenevsky, F. K., Waldow, V. A., and Fett, J. P. (2012a). Iron biofortification in rice: it's a long way to the top. *Plant Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Sperotto, R. A., Vasconcelos, M. W., Grusak, M. A., and Fett, J. P. (2012b). Effects of different Fe supplies on mineral partitioning and remobilization during the reproductive development of rice (*Oryza sativa* L.). *Rice* 5, 27. doi: 10.1186/1939-8433-5-27

Sperotto, R. A., Ricachenevsky, F. K., Waldow, V. A., Müller, A. L. H., Dressler, V. L., and Fett, J. P. (2013). Rice grain Fe, Mn and Zn accumulation: how important are flag leaves and seed number? *Plant Soil Environ.* 59, 262–266.

Stomph, T. J., Jiang, W., and Struik, P. C. (2009). Zinc biofortification of cereals: rice differs from wheat and barley. *Trends Plant Sci.* 14, 123–124. doi: 10.1016/j.tplants.2009.01.001

Waters, B. M., and Grusak, M. A. (2008). Whole-plant mineral partitioning throughout the life cycle in *Arabidopsis thaliana* ecotypes Columbia, Landsberg erecta, Cape Verde Islands, and the mutant line ysl*1ysl3*. *New Phytol.* 177, 389–405. doi: 10.1111/j.1469-8137.2007.02288.x

Wei, Y., Shohag, M. J. I., and Yang, X. (2012). Biofortification and bioavailability of rice grain

zinc as affected by different forms of foliar zinc fertilization. *PLoS ONE* 7:e45428. doi: 10.1371/journal.pone.0045428

Welch, R. M., and Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot*. 55, 353–364. doi: 10.1093/jxb/erh064

Wu, C., Lu, L., Yang, X., Feng, Y., Wei, Y., Hao, H. L., et al. (2010). Uptake, translocation, and remobilization of zinc absorbed at different growth stages by rice genotypes of different Zn densities. *J. Agric. Food Chem*. 58, 6767–6773. doi: 10.1021/jf100017e

Yoneyama, T., Gosho, T., Kato, M., Goto, S., and Hayashi, H. (2010). Xylem and phloem transport of Cd, Zn and Fe into the grains of rice plants (*Oryza sativa* L.) grown in continuously flooded Cd-contaminated soil. *Soil Sci. Plant Nutr.* 56, 445–453. doi: 10.1111/j.1747-0765.2010.00481.x

Yuan, L., Wu, L., Yang, C., and Lv, Q. (2012). Effects of iron and zinc foliar applications on rice plants and their grain accumulation and grain nutritional quality. *J. Sci. Food Agric*. 93, 254–261. doi: 10.1002/jsfa.5749

Zhang, Y., Xu, Y. H., Yi, H. Y., and Gong, J. M. (2012). Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. *Plant J.* 72, 400–410. doi: 10.1111/j.1365-313X.2012.05088.x

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Internal Zn allocation influences Zn deficiency tolerance and grain Zn loading in rice (*Oryza sativa* L.)

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One of the important factors that influences Zn deficiency tolerance and grain Zn loading in crops is the within-plant allocation of Zn. Three independent experiments were carried out to understand the internal Zn distribution patterns in rice genotypes grown in Zn-sufficient and Zn-deficient agar nutrient solution (ANS). In one of the experiments, two rice genotypes (IR55179 and KP) contrasting in Zn deficiency tolerance were leaf-labeled with ^{65}Zn. In the other two experiments, two Zn biofortification breeding lines (IR69428 and SWHOO) were either root- or leaf-labeled with ^{65}Zn. Rice genotype IR55179 showed significantly higher Zn deficiency tolerance than KP at 21 and 42 days after planting. When KP was Zn-deficient, it failed to translocate ^{65}Zn from the labeled leaf to newly emerging leaves. Similarly, the root-to-shoot translocation of unlabeled Zn was lower in KP than in IR55179. These results suggest that some Zn-efficient rice genotypes have greater ability to translocate Zn from older to actively growing tissues than genotypes sensitive to Zn deficiency. Among the two Zn biofortication breeding lines that were leaf-labeled with ^{65}Zn at 10 days before panicle initiation stage, ^{65}Zn distribution in the grains at maturity was similar between both genotypes in Zn-sufficient conditions. However, under Zn-deficient conditions, SWHOO accumulated significantly higher ^{65}Zn in grains than IR69428, indicating that SWHOO is a better remobilizer than IR69428. When the roots of these two Zn biofortication breeding lines were exposed to ^{65}Zn solution at 10 days after flowering, IR69428 showed higher root uptake of ^{65}Zn than SWHOO in Zn-sufficient conditions, but ^{65}Zn allocation in the aerial parts of the plant was similar between both genotypes.

Keywords: rice, continued root uptake, grain Zn, grain Zn loading, Zn biofortification, Zn deficiency tolerance, Zn remobilization

INTRODUCTION

Enriching brown rice Zn concentration to the target of 30 mg kg^{-1} set by the HarvestPlus program would provide 40% of the estimated average requirement for preschool children and non-pregnant and non-lactating women (Saltzman et al., 2013). Achieving the target grain Zn concentration can be particularly challenging under conditions of low available soil Zn, which often occur with reduced conditions developed after flooding in paddy fields (Johnson-Beebout et al., 2009). Therefore, understanding the plant uptake, translocation, and loading of Zn to rice grains is crucial, as this influences not only grain Zn but also the ability of a plant to grow and yield in Zn-deficient soil. Zn deficiency tolerance (Zn-efficiency) of a rice genotype is known to be influenced by several root- and shoot-related processes such as higher root uptake of Zn, high root-to-shoot translocation of Zn, biochemical use of Zn, subcellular compartmentation and enhanced translocation of Zn from older to new tissues under Zn-deficient conditions (Hacisalihoglu and Kochian, 2003; Impa and Johnson-Beebout, 2012). Higher Zn uptake is often strongly related to Zn deficiency tolerance and is known to be influenced by several root related processes, such as efflux of phytosiderophores and low molecular weight organic acids, proton exudation, mycorhizal colonization, and formation of iron plaques on roots (Graham and Rengel, 1993; Rose et al., 2013). The effectiveness of each of these mechanisms is likely to vary depending on the soil environment, genotype, and Zn status (Impa and Johnson-Beebout, 2012).

Accumulation of Zn in rice grains can occur through continued root uptake during grain filling stage and/or through remobilization of earlier taken up and stored Zn from sources tissues to grain. Unlike wheat, in rice the presence of continuous xylem stream implies that remobilization of Zn may not contribute significantly to grain Zn accumulation, provided there is sufficient Zn supply to roots (Stomph et al., 2009). Jiang et al. (2007) found that in aerobic rice genotypes grown in Zn sufficient nutrient solution continued root uptake was the predominant source of grain Zn accumulation. Contrastingly, according to Wu et al. (2010) in a high grain Zn rice genotype most of the Zn accumulated in grain was through remobilization from sources tissues rather than continued root uptake during grain

filling. Moreover, in rice Zn was found to be supplied directly via phloem to grains and husks (Yoneyama et al., 2010). Our previous results using a set of rice genotypes contrasting for grain Zn, indicated that under sufficient Zn supply at the grain-filling stage, plants predominantly take up Zn through roots and transport it toward grain (Impa et al., 2013). Remobilization of Zn from source tissues to grain usually occurs under either Zn-deficient conditions or in plants with an accelerated rate of senescence or high phloem mobility for Zn (Bukovac and Wittwer, 1957; Kochian, 1991; Sperotto et al., 2009; Impa et al., 2013). Moreover several transporters involved in enhancing grain Zn accumulation in rice through either of these sources have been identified (Sperotto et al., 2009, 2010; Johnson et al., 2011; Lee et al., 2011; Bashir et al., 2012). Recently, Sperotto (2013), proposed a model for grain Zn loading according to which under Zn sufficient condition, grain Zn accumulation in rice occurs mainly through continued root uptake during grain filling stage with very little contribution from remobilization of Zn from stem and flag/upper leaf reserves. Whereas, under Zn deficient condition both continued root uptake and remobilization of Zn from source tissues contribute equally to grain Zn loading.

The identification of the predominant modes of grain Zn loading in rice genotypes would help breeders not only to identify donors for targeted Zn biofortification breeding but also to optimize the time and method of Zn fertilizer application. If a genotype predominantly depends on continued root uptake for grain Zn loading, it would be important to make soil Zn more available at later growth stages through Zn fertilization combined with terminal drying prior to harvest in flooded paddy fields. If a genotype has remobilization of Zn from source tissues as the main source of grain Zn loading, it would be important to get sufficient Zn into the plant early in the season through either soil Zn fertilization during the vegetative stage or foliar Zn application at heading or the early grain-filling stage (Boonchuay et al., 2013; Mabesa et al., 2013). Genotypes with both efficient root uptake of Zn during grain filling and remobilization of Zn from source tissues would be the most preferred ones for enriching grain Zn, because they would be expected to perform better across a variety of environments in which soil Zn can be available at different times of the season.

From our previous study, it was predicted through mass balance calculations that under Zn-sufficient conditions continued root uptake during grain filling was the predominant source of grain Zn loading in most rice genotypes, whereas, in Zn-deficient conditions, genotypes varied in their predominant source of grain Zn loading, with some genotypes showing continued root uptake as the predominant source while in others remobilization was predominant (Impa et al., 2013). One of the major limitations in predicting the predominant grain Zn loading sources through mass balance calculation is the difficulty in tracking the actual movement of Zn within different plant tissues. So, in the present study, two Zn biofortification breeding lines with high grain-Zn, namely, SWHOO and IR69428, contrasting in their sources of grain Zn loading as predicted from mass balance data, were selected for tracking the actual movement of Zn using ^{65}Zn labeling to either leaves or roots. In addition, two other genotypes contrasting in Zn deficiency tolerance, namely, KP and IR55179, were selected to assess the movement of Zn from older leaves to new leaves at the vegetative stage.

The two specific objectives investigated in the present study were (1) to evaluate the effect of remobilization of Zn from old to new leaves on Zn deficiency tolerance and (2) to determine the predominant source of grain Zn loading (remobilization vs. continued root uptake) in high-grain-Zn genotypes. In particular, the following hypotheses were tested: (H1) Remobilization of Zn from old to new leaves would be higher in a Zn-deficiency-tolerant genotype (IR55179) than in a Zn-deficiency-sensitive genotype (KP) in Zn-deficient conditions. (H2.1) In some high-grain-Zn genotypes, remobilization of Zn from leaves could be the major source of Zn to grains whether in Zn-deficient or Zn-sufficient conditions. (H2.2) Less Zn remobilization from leaves to grain during grain filling in some of the high-grain-Zn genotypes is associated with continued Zn uptake through roots under Zn-sufficient conditions.

MATERIALS AND METHODS

PLANT MATERIAL

The seeds were obtained from the Plant Breeding, Genetics, and Biotechnology (PBGB) Division of the International Rice Research Institute (IRRI), Philippines. The full names of the genotypes, their designations and their descriptions are given in **Table 1**.

PLANT GROWTH

The experiments were carried out in the Eidgenössische Technische Hochschule (ETH) growth chamber facility, at Eschikon, near Zürich, Switzerland. The plants were grown in an 11 h/13 h day/night cycle at a temperature of 30 and 23°C and relative humidity of 60 and 70% during day and night, respectively.

Table 1 | Initial seed Zn concentration (mg kg^{-1}) and descriptions of the rice genotypes used in the experiments.

Genotype name	Designation	Seed Zn concentration before sowing (mg kg^{-1})	Description
SWHOO	SWHOO	30	Zn biofortification donor
IR69428-6-1-1-3-3 (IR68150 × IR65600-1-3-2)	IR69428	23	Zn biofortification breeding line
IR55179-3B-11-3 (IR4630-22-2-5-1-3 × Nona Bokra)	IR55179	18	Tolerant of Zn deficiency
Kinandang Patong	KP	20	Sensitive to Zn deficiency

Seed includes hull and brown rice. Parents of breeding lines are given in parentheses.

All the plasticware and glassware were thoroughly washed with soap solution in a dishwasher, soaked in 10% HNO_3 for 2 h and then rinsed twice with deionized water to make them Zn-free prior to the experiment. Initially, the seeds were kept for germination on a moist filter paper in petri dishes kept in the dark at 23 ± 1°C for 5 days. The sprouted seeds were floated on 0.5 mM $CaCl_2$ solution with 10 μM iron sodium ethylene-diamine-tetra-acetate (FeNaEDTA) for 1 week. The seedlings were then transferred to pots filled with half-strength modified Yoshida nutrient solution (YNS) without Zn for 1 week and later on transferred to half-strength YNS with the respective Zn treatments for a week. The composition of modified YNS at full strength is as follows: 1.77 mM NH_4NO_3, 0.32 mM $NaH_2PO_4 \cdot 2H_2O$, 0.5 mM K_2SO_4, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 9 μM $MnCl_2 \cdot 4H_2O$, 0.5 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 18.5 μM H_3BO_3, 0.16 μM $CuSO_4 \cdot 5H_2O$, 36 μM FeNaEDTA (Impa et al., 2013). Zn was supplied at 0.005 μM and 1.5 μM $ZnSO_4 \cdot 7H_2O$ to establish Zn-deficient and Zn-sufficient conditions, respectively. The pots were replenished with fresh YNS once every 3 or 4 days. Further, the seedlings were transferred to agar nutrient solution (ANS) containing 0.1% agar in modified full-strength YNS with Zn supplied to establish Zn-deficient and Zn-sufficient conditions. The day when the plants were first transferred to ANS medium is considered as day 0 (0 days after planting in ANS) for all the reported data. The pH of the ANS was adjusted to 8 with NaOH. The pots were arranged in a randomized complete block design and ANS in these pots was replenished once every 14 days. The lids of the 10-L-capacity plastic pots used in the experiment had eight openings to fit the plants.

EXPERIMENT-1

Two rice genotypes, IR55179 (Zn-deficiency-tolerant) and KP (Zn-deficiency-sensitive), were grown in ANS with sufficient and deficient Zn as described above. Before leaf labeling with ^{65}Zn, 10 plants or replications from each genotype were destructively sampled to estimate the total biomass and Zn concentrations in different plant tissues. Leaf labeling was done at 21 days after growing the plants in ANS (DAP), wherein the top fully expanded leaf on main tiller was dipped in 5 mL of 600 kBq ^{65}Zn solution in an Eppendorf tube for 10 s. The ^{65}Zn solution contained around 13.5 μCi ^{65}Zn taken in an eppendorf tube, along with 10% Tween 80 and volume made up to 5 mL using sterile water. The same procedure was repeated the next day for 20 s. Leaf labeling was done in three plants or replications for each treatment and genotype. The plants were harvested at 3 weeks after labeling. Another set of plants with three replications each in Zn-sufficient and Zn-deficient conditions was maintained unlabeled and harvested at 42 DAP. After harvest, the unlabeled plants were oven-dried at 80°C until constant weight was obtained. The dry plant tissue samples were digested in HNO_3, followed by H_2O_2 to extract Zn (Huang and Schulte, 1985), and the digests were analyzed for Zn by inductively coupled plasma-optical emission spectrometry analysis (VISTA-MPX, CCD, and Simultaneous ICP-OES). For the wet digestion of plant tissues, around 200 mg of dry powder of unlabeled plant tissues was weighed in digestion tubes, to which 15 mL of 65% HNO_3 was added, and the tubes were kept in heating blocks with a temperature of 120°C for 90 min. After this, the digestion tubes were allowed to cool for 30 min under a fume hood and around 3 mL of 30% H_2O_2 was added to the digestion tubes. After another cycle of heating for 90 min at 120°C and cooling for 30 min, the digests were analyzed as the acid digests for Zn by ICP-OES. The labeled plants after harvest were oven-dried at 50°C for 3 days, weighed and analyzed for ^{65}Zn in roots, stems and leaves using γ-spectrometry (high purity germanium detectors, ORTEC, USA, with adjusted calibration for the geometry for the plant samples).

EXPERIMENT-2

Two high-grain-Zn genotypes (IR69428 and SWHOO) were grown in ANS with sufficient Zn until 30 DAP. Before leaf labeling, initial destructive biomass sampling of 10 plants or replications each from both genotypes was done to estimate the total biomass and Zn concentration in different plant tissues. Leaf labeling was done at 30 DAP (which is approximately 2 weeks before panicle initiation) by dipping the top fully expanded leaf on main tiller for 10 s into an Eppendorf tube filled with 5 mL of 600 kBq ^{65}Zn solution. The same procedure was repeated the next day for 20 s. After labeling, a set of plants (three replications for each genotype) was grown in Zn-deficient ANS and another set (three replications for each genotype) in Zn-sufficient ANS until maturity. In addition, three replicate plants per genotype were also maintained unlabeled in each Zn treatment. All the plants were harvested at physiological maturity (around 122 and 143 DAP for SWHOO and IR69428, respectively). The processing of harvested plants for dry weight, Zn concentration, and ^{65}Zn distribution was similar to Experiment-1 except that the harvested plants were separated into roots, stems, leaves, rachis, and grains.

EXPERIMENT-3

Two high-grain-Zn genotypes (IR69428 and SWHOO) were grown in ANS with sufficient Zn until the early grain filling stage. Before initiating root labeling with ^{65}Zn, initial destructive biomass sampling of five plants or replications each from both genotypes was done to estimate the total biomass and initial Zn concentration in different plant tissues. Three plants or replications from both the genotypes were root labeled with ^{65}Zn at the early grain-filling stage, i.e., 10 days after 50% flowering (93 and 123 DAP for SWHOO and IR69428, respectively), during which the plant roots were exposed to 185 kBq ^{65}Zn in 500 mL of YNS with 1.5 μM $ZnSO_4 \cdot 7H_2O$ for 24 h. After exposure to ^{65}Zn for 24 h, the labeled roots were washed with deionized water for 1 min, followed by washing in ice-cold desorption solution containing 100 μM $ZnSO_4 \cdot 7H_2O$ for 15 min. The roots were again washed with deionized water for 1 min before putting the plants back in ANS with sufficient Zn until maturity. The plants were always (before and after labeling) maintained under Zn-sufficient conditions so that there was enough Zn for root uptake and to make sure that the contribution of remobilization to grain Zn loading was minimal. In addition, three replicate plants per genotype were also maintained unlabeled until maturity. The plants were harvested at physiological maturity. The processing of harvested plants for dry weight, Zn concentration, and ^{65}Zn distribution was similar to Experiment-1 except that the harvested plants were separated into roots, stems, leaves, rachis, and grains.

The grains of unlabeled plants were manually dehulled and the Zn concentration in both brown rice and hull was quantified through ICP-OES.

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was performed using R/aov [R version 2.11.0 (2010-04-22)]. Within a data set, means were compared by the least significant difference (LSD) method. Zn efficiency was calculated by the ratio of shoot dry weight under Zn-deficient conditions to shoot dry weight under Zn-sufficient conditions and was expressed in percentages (Rengel and Graham, 1996). Total Zn content in a specific plant tissue was calculated as the product of the tissue's Zn concentration and its dry weight. Root-to-shoot Zn translocation index was calculated as the ratio of total shoot Zn content to total Zn content per plant (Rengel and Graham, 1996). The percent distribution of ^{65}Zn accumulated among different plant parts was calculated excluding the labeled part of the leaf (the part of the leaf that was dipped in 5 mL of 600 kBq ^{65}Zn solution) as it retained 80–90% of the total ^{65}Zn in plants.

RESULTS

EXPERIMENT-1

The relative distribution of accumulated ^{65}Zn in new leaves was similar in both genotypes under Zn-sufficient conditions, whereas, under Zn-deficient conditions, IR55179 showed significantly higher accumulation of ^{65}Zn than KP (**Figure 1**). There was no significant difference between the genotypes for percent distribution of accumulated ^{65}Zn in the remainder of the labeled tiller in both Zn-sufficient and Zn-deficient conditions (**Figure 1**). In labeled plants, ^{65}Zn activity was seen in new leaves that emerged on the same tiller after labeling and also in the remainder of the labeled tiller but not in other shoots and roots.

Genotypes differed significantly in their Zn efficiency (indicator of Zn deficiency tolerance) at 21 and 42 DAP in ANS (**Figure 2**), with IR55179 showing significantly higher Zn efficiency than KP at both growth stages. Zn efficiencies of IR55179 and KP at 21DAP was around 65 ± 2.7 and 50 ± 2.4 respectively and at 41 DAP was around 60 ± 2.3 and 20 ± 2.2 respectively. At 21 DAP, both genotypes showed significantly lower root and shoot Zn concentration and root-to-shoot Zn translocation index in Zn-deficient conditions than in Zn-sufficient conditions (**Table 2**). At this growth stage, both genotypes showed similar tissue Zn concentration in Zn-deficient conditions, whereas, in Zn-sufficient conditions, IR55179 showed higher root and shoot Zn concentration than KP. At 42 DAP, root Zn concentration in IR55179 did not vary between the Zn treatments, whereas KP showed a significantly lower root Zn concentration in Zn-deficient conditions than in Zn-sufficient conditions (**Table 2**). Significant treatment differences were noticed for shoot Zn concentration in both genotypes at 42 DAP. In Zn-deficient conditions, IR55179 showed significantly higher root-to-shoot Zn translocation index than KP at 42 DAP.

EXPERIMENT-2

Stem + leaves of labeled tillers showed significantly higher percentages of ^{65}Zn accumulation than the other plant parts,

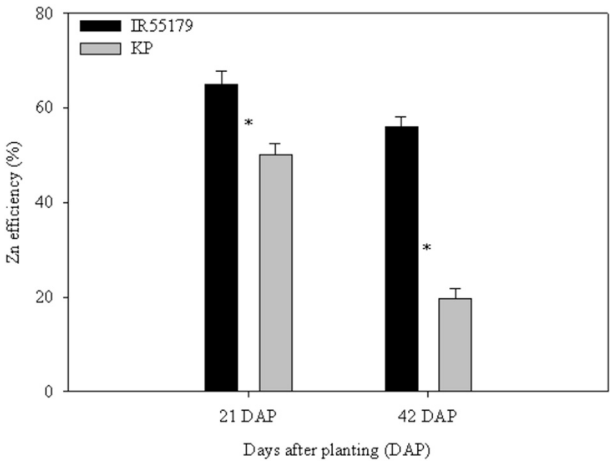

FIGURE 2 | Zn efficiency (%) of unlabeled rice genotypes at different growth stages in Experiment-1. Note: "*" indicates significant difference between the genotypes within a growth stage at $p \leq 0.05$. Error bars indicate ± SE ($n = 10$ at 21 DAP and $n = 3$ at 42 DAP). Zn efficiency was calculated by the ratio of shoot dry weight under Zn-deficient conditions to shoot dry weight under Zn-sufficient conditions and is expressed in percentages.

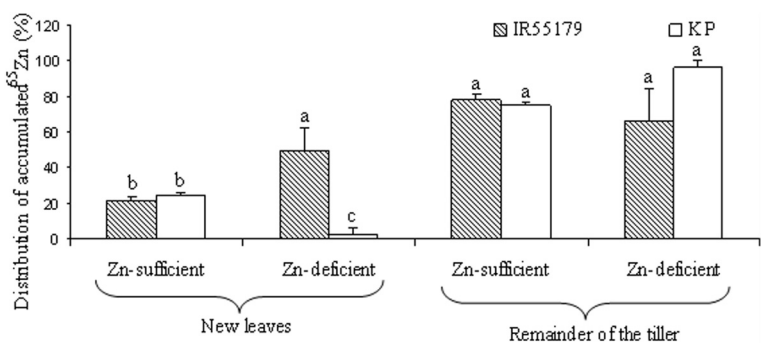

FIGURE 1 | Distribution of ^{65}Zn at 3 weeks after vegetative-stage leaf labeling in different plant parts of rice genotypes grown under Zn-sufficient and Zn-deficient conditions in Experiment-1. Bars with different letters are significantly different between the two genotypes for a given plant tissue at 5% LSD. Error bars indicate ± SE ($n = 3$).

Table 2 | Zn concentrations of different plant parts and root-to-shoot Zn translocation of unlabeled rice genotypes at 21 and 42 days after planting (DAP) under Zn-sufficient and Zn-deficient ANS in Experiment-1.

Growth stage	Genotype	Zn treatment	Root Zn concentration (mg kg^{-1})	Shoot Zn concentration (mg kg^{-1})	Root-to-shoot Zn translocation index (%)
21 DAP	IR55179	Zn-sufficient	$52^{a} \pm 4.0$	$76^{a} \pm 7.5$	$77.7^{a} \pm 2$
		Zn-deficient	$26^{c} \pm 0.4$	$11^{c} \pm 0.4$	$52.4^{b} \pm 1$
	KP	Zn-sufficient	$40^{b} \pm 2.0$	$54^{b} \pm 1.7$	$78.9^{a} \pm 1$
		Zn-deficient	$26^{c} \pm 0.5$	$12^{c} \pm 0.4$	$54.0^{b} \pm 1$
	Genotype		$p = 0.01$	$p = 0.03$	NS
	Treatment		$p < 0.001$	$p < 0.001$	$p < 0.001$
	Genotype × treatment		$p = 0.015$	$p = 0.003$	NS
	5% LSD (Zn × G)		6	10	4.5
42 DAP	IR55179	Zn-sufficient	$24.3^{a} \pm 1.3$	$23.1^{a} \pm 1$	$65.8^{b,c} \pm 3$
		Zn-deficient	$23.4^{a} \pm 3.4$	$16.0^{b,c} \pm 3$	$89.0^{a} \pm 1$
	KP	Zn-sufficient	$22.0^{a} \pm 1.0$	$22.0^{a,b} \pm 3$	$73.4^{b} \pm 2$
		Zn-deficient	$17.0^{b} \pm 0.3$	$9.4^{c} \pm 1$	$57.2^{c} \pm 4$
	Genotype		$p = 0.009$	NS	$p = 0.03$
	Treatment		$p = 0.03$	$p = 0.002$	NS
	Genotype × treatment		NS	NS	$p = 0.001$
	5% LSD (Zn × G)		3.9	6.7	11.5

Zn × G, Zn treatments × genotype. Values with different letters in a column within a growth stage are significantly different at 5% LSD. Shoot includes leaf blade, sheath and stem. The values given are means ± SE (n = 10 for 21 DAP and n = 3 for 42 DAP).

followed by grains of labeled tillers (**Figure 3**). The rachis and grains of other tillers showed significantly lower percentages of ^{65}Zn accumulation than other plant parts. In both Zn treatments, SWHOO showed higher ^{65}Zn accumulation in grains of labeled tillers than IR69428 (**Figure 3**). SWHOO also showed significantly lower ^{65}Zn distribution in shoots of labeled tillers. Percent ^{65}Zn accumulation in grains did not differ between the two Zn treatments in IR69428, whereas SWHOO exhibited significantly higher ^{65}Zn accumulation in grains under Zn-sufficient conditions than in Zn-deficient conditions. Apart from the leaves, stems, grains, and rachis of labeled tillers, ^{65}Zn activity was found in the grains of unlabeled tillers but not in the roots, leaves, and stems of unlabeled tillers. The labeled part of the leaf retained 80–90% of the total ^{65}Zn in plants, so it was excluded while calculating the percent ^{65}Zn distribution in different parts of the plant.

At 30 DAP, there was no significant difference between the genotypes in root, stem + sheath, and leaf blade Zn concentration in Zn-sufficient conditions (**Table 3**). At maturity, both genotypes showed significantly lower root, stem + sheath, and leaf blade Zn concentration in Zn-deficient conditions than in Zn-sufficient conditions. Leaf blade Zn concentration at maturity differed significantly between the genotypes in Zn-sufficient conditions, but not in Zn-deficient conditions. IR69428 showed a significantly lower brown rice Zn concentration in Zn-deficient conditions than in Zn-sufficient conditions, whereas brown rice Zn concentration in SWHOO did not differ significantly between the Zn treatments (**Table 3**). The rachis and hull showed lower Zn concentration than brown rice in both genotypes in both Zn treatments.

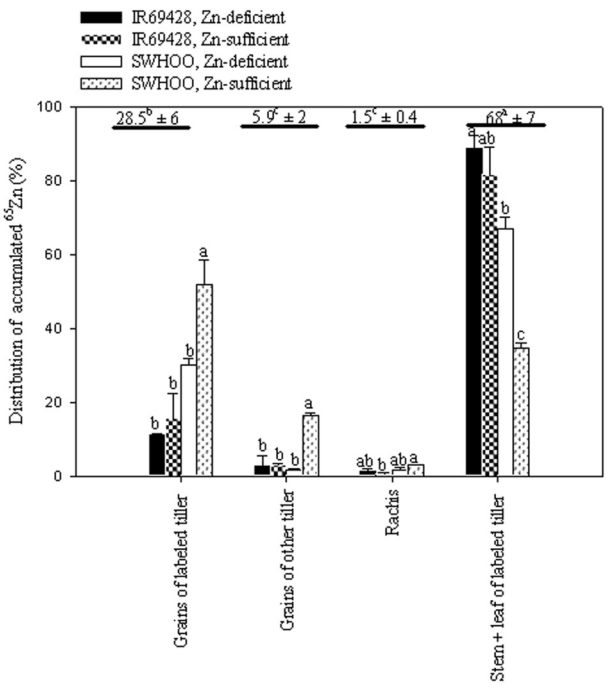

FIGURE 3 | Distribution of leaf-labeled ^{65}Zn among different plant parts measured at maturity in Experiment-2. Bars with different letters represent significant differences between genotypes for each trait at 5% LSD. The values above the horizontal lines represent the average of relative ^{65}Zn accumulation by different plant parts and different letters indicate significant difference at 5% LSD. Grains include brown rice and hull.

Table 3 | Zn concentration in different plant parts of unlabeled rice genotypes at different growth stages under Zn-sufficient and Zn-deficient ANS in Experiment-2.

			Zn concentration in individual plant parts (mg kg^{-1})					
Growth stage	**Genotype**	**Zn treatment**	**Root**	**Stem + sheath**	**Leaf blade**	**Rachis**	**Hull**	**Brown rice**
30 DAP	IR69428	Zn-sufficient	$20.8^{a} \pm 1$	$26.7^{a} \pm 2$	$21.5^{a} \pm 1.0$	–	–	–
	SWHOO		$20.7^{a} \pm 1$	$21.9^{a} \pm 2$	$21.5^{a} \pm 0.4$	–	–	–
	Genotype		NS	NS	NS			
	5% LSD (G)		2.4	4.9	3.4			
Maturity	IR69428	Zn-sufficient	$66.2^{a} \pm 9$	$19.5^{a} \pm 3$	$19.0^{a} \pm 0.5$	11^{a}	12^{a}	$31^{a} \pm 1$
		Zn-deficient	$33.5^{b} \pm 6$	$10.5^{b} \pm 0.7$	$14.5^{c} \pm 1$	17^{a}	8^{a}	$18^{b} \pm 1$
	SWHOO	Zn-sufficient	$52.4^{a,b} \pm 10$	$19.4^{a} \pm 2$	$13.0^{b} \pm 1$	$17^{a} \pm 5$	$15^{a} \pm 3$	$35^{a} \pm 5$
		Zn-deficient	$31.6^{b} \pm 3$	$10.0^{b} \pm 1$	$12.0^{c} \pm 1$	$8^{a} \pm 0.4$	$7^{a} \pm 0.4$	$28^{a,b} \pm 1$
	Genotype		NS	NS	$p < 0.001$	NS	NS	$p = 0.029$
	Treatment		$p = 0.01$	NS	$p = 0.006$	NS	NS	$p = 0.02$
	Genotype × treatment		NS	$p = 0.001$	NS	NS	NS	NS
	5% LSD (G × Zn)		26.5	6	2.3	13	13	10

DAP, days after planting in ANS. Values given are averages of 10 replications at 30 DAP and three replications at maturity. Values with different letters are significantly different between genotypes within a growth stage for each trait at 5% LSD (G × Zn, genotype × Zn treatment).

EXPERIMENT-3

The roots of IR69428 accumulated significantly higher amounts of ^{65}Zn than those of SWHOO, but both genotypes accumulated similar amounts of ^{65}Zn in the aerial parts, including grains (**Figure 4**). Dry leaves did not have any ^{65}Zn, whereas the green leaves and rachis had very little ^{65}Zn in both genotypes (**Figure 4**). There was no significant difference between the ^{65}Zn content of distal and apical grains. In unlabeled plants, both genotypes showed similar root Zn concentration at 10 days after 50% flowering (**Table 4**). IR69428 showed higher Zn concentration and % Zn allocation in the leaf blade than SWHOO, while the opposite was found in the panicles (**Table 4**). At maturity, IR69428 showed lower root Zn concentration than SWHOO, which is contrasting to the root Zn concentration between these two genotypes in Experiment-2, but % Zn allocation in roots was similar between both genotypes (**Table 4**). SWHOO showed lower Zn concentration and % Zn allocation in green leaf blades than IR69428, while the Zn concentration and % Zn allocation in brown rice were higher in SWHOO than in IR69428 at maturity. There was no difference between distal and apical brown rice Zn concentration and % Zn allocation in either of the genotypes (**Table 4**).

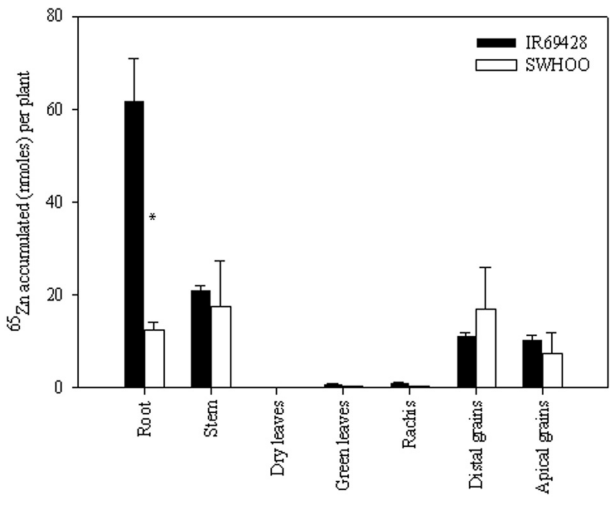

FIGURE 4 | Distribution of root-labeled ^{65}Zn among different plant parts at maturity in Experiment-3. Note: "*" indicates significant difference between the genotypes for a tissue at 5% LSD.

DISCUSSION

Zn DEFICIENCY TOLERANCE

KP showed significantly lower Zn efficiency than IR55179 at the vegetative stage, which is consistent with the results of our previous study (Impa et al., 2013). The higher root and shoot Zn concentration of IR55179 than of KP in Zn-sufficient conditions and a similar root and shoot Zn concentration between these genotypes under Zn-deficient conditions at 21 DAP (**Table 2**) could be due to the overall higher biomass accumulation in KP (2.1 and 9.3 g $plant^{-1}$ at 21 and 42 DAP respectively) than in IR55179 (1.6 and 7.2 g $plant^{-1}$ at 21 and 42 DAP respectively) under Zn-sufficient conditions and a greater reduction in biomass of KP under Zn-deficient conditions than in Zn-sufficient conditions (**Table A1**), resulting in lower Zn efficiency of KP than of IR55179. Wide variation among rice genotypes in their Zn deficiency tolerance has been noticed in several studies (Quijano-Guerta et al., 2002; Wissuwa et al., 2006; Impa et al., 2013). The higher distribution of ^{65}Zn in new leaves of IR55179 than of KP under Zn-deficient conditions indicates a higher remobilization of Zn from labeled leaves to new leaves in IR55179 than in KP (**Figure 1**), although both genotypes had a similar shoot Zn concentration within a treatment at the time of leaf labeling, i.e., 21 DAP (**Table 2**). Moreover, KP showed lower remobilization of ^{65}Zn from labeled leaves to new emerging leaves under

Table 4 | Zn concentration and percent Zn allocation in different plant parts of unlabeled plants at different growth stages in Experiment-3.

Growth stage	Plant parts	Zn concentration (mg kg^{-1})		% Zn allocation in different plant parts	
		IR69428	SWHOO	IR69428	SWHOO
Ten days after 50% flowering	Root	50.3 ± 8	48.8 ± 6	30.6	25.9
	Stem + sheath	20.0 ± 2	26.7 ± 4	45.5	45.9
	Leaf balde	22.5 ± 1	16.6 ± 2	17.2	9.0
	Panicle	30.6 ± 2	41.4 ± 3	6.7	19.0
Maturity	Root	16.6 ± 3.0	30.2 ± 4	12.6	14.0
	Stem + sheath	14.9 ± 0.3	19.6 ± 4	50.7	46.2
	Dry leaves (blade)	19.2 ± 0.5	22.8 ± 3	14.3	4.8
	Green leaves (blade)	30.0 ± 6.0	13.8 ± 1	6.8	3.6
	Rachis	15.4 ± 2.3	19.0 ± 6	0.8	1.0
	Hull	17.5 ± 2.5	14.0 ± 2	1.4	2.7
	Distal brown rice	23.8 ± 1.0	28.3 ± 5	6.2	14.6
	Apical brown rice	23.8 ± 0.5	30.2 ± 5	7.0	13.0

Values are averages of five replications at 10 days after 50% flowering and three replications at maturity.

Zn-deficient conditions than in Zn-sufficient conditions. This could be due to the very low Zn in labeled leaves in Zn-deficient conditions and most of the absorbed ^{65}Zn is probably strongly bound to cell constituents that could not be remobilized, and similar results were also noticed in wheat by Kutman et al. (2011). Such differences in remobilization of Zn from old leaves to new leaves between the genotypes was not noticed under Zn-sufficient conditions, as there was enough unlabeled Zn in the growth medium for plant uptake and translocation. The inability of KP to remobilize ^{65}Zn from labeled leaves to newly emerging leaves could be one of the reasons for its higher sensitivity to Zn deficiency. In IR55179, in addition to higher remobilization of Zn from older to new leaves (H1), a higher root-to-shoot translocation of Zn than in KP (**Table 2**) resulted in higher tolerance of Zn deficiency.

GRAIN Zn LOADING

Remobilization of Zn from source tissues to grain during grain filling

The lower ^{65}Zn distribution in shoots and higher distribution in grains of labeled tillers upon leaf labeling with ^{65}Zn in SWHOO than in IR69428 (**Figure 3**) indicates greater remobilization of Zn from stems or leaves to grains in SWHOO than in IR69428 in both Zn treatments. Remobilization of Zn from source tissue to grains was also found to be the predominant source of grain Zn loading in high grain Zn rice genotype IR68144 (Wu et al., 2010). In this experiment, the plants were grown under Zn-sufficient conditions until 30 DAP in ANS and then transferred to Zn-sufficient or Zn-deficient treatments. The similar brown rice Zn concentration between SWHOO and IR69428 in Zn-sufficient conditions and a lower brown rice Zn concentration in IR69428 than in SWHOO under Zn-deficient conditions (**Table 3**) indicates that, in Zn-deficient conditions, SWHOO was able to remobilize Zn taken up and stored earlier in leaves or tillers when the plants were grown in Zn-sufficient conditions until 2 weeks before panicle initiation. Moreover, SWHOO maintained a similar brown rice Zn concentration between Zn-sufficient and Zn-deficient conditions, unlike IR69428, which showed significantly lower brown rice Zn concentration in Zn-deficient conditions, indicating that IR69428 was unable to efficiently remobilize Zn taken up earlier and stored in source tissues, even when the plants were grown in Zn-deficient conditions. These results suggest that, in SWHOO, remobilization is the predominant source of grain Zn loading (H2.1), but that IR69428 has only limited ability to remobilize Zn from leaves to grains. On the contrary, Jiang et al. (2007) found that in aerobic rice genotypes continued root uptake contributed significantly to grain Zn accumulation and remobilization of Zn from leaves was not that important.

Continued root uptake of Zn during grain filling

Both genotypes accumulated ^{65}Zn when roots were exposed to ^{65}Zn at 10 days after 50% flowering, indicating that the roots continued to take up Zn even after flowering (**Figure 4**). Similarly, continued root uptake of Zn even after flowering stage has been observed in aerobic rice genotypes irrespective of Zn status of plants (Jiang et al., 2007). Even though both genotypes had similar root dry weight and root length at the time of root labeling (**Table A2**), IR69428 roots absorbed around 5-fold higher ^{65}Zn than SWHOO, indicating a higher root uptake of Zn during the grain-filling period in IR69428 than in SWHOO (H2.2). Most of the Zn taken up by IR69428 accumulated in roots rather than being transported to aerial parts, indicating that, in spite of increased root Zn uptake in IR69428 during grain filling, it did not readily translocate it from roots to shoots. In Experiment-3, we observed contradictory results for root Zn concentration between labeled and unlabeled Zn at maturity. Unlabeled root Zn concentration in IR69428 was lower than in SWHOO (**Table 4**), whereas ^{65}Zn concentration in roots was significantly higher in IR69428 than in SWHOO. And, this difference could be due to the fact that ^{65}Zn uptake began only at the grain-filling stage but unlabeled Zn uptake started from the seedling stage. The genotypes were apparently similar in their root Zn uptake at the earlier

stage, with the genotype difference appearing only at later growth stages.

In conclusion, Zn-efficient line IR55179 showed significantly higher remobilization of ^{65}Zn from older to new leaves and root-to-shoot Zn translocation than KP, suggesting that Zn distribution to active growing parts enhanced Zn efficiency. High-grain-Zn line SWHOO exhibited higher remobilization of Zn from source tissue to grain than IR69428 in both Zn-deficient and Zn-sufficient conditions. This indicates that rice genotypes vary in their phloem mobility of Zn from leaves to grain. There was a higher root uptake of Zn in IR69428 than in SWHOO at the grain-filling stage, but the Zn taken up by IR69428 accumulated in roots rather than being transported to grains. The findings of this paper would help in identifying donors for location specific breeding for Zn deficiency tolerance and Zn biofortication and thereby further crop improvement.

AUTHOR CONTRIBUTIONS

Sarah E. Johnson-Beebout, Somayanda M. Impa, Anja Gramlich, Rainer Schulin, and Susan Tandy planned the experiments. Somayanda M. Impa and Anja Gramlich carried out the experiments under the supervision of Rainer Schulin and Emmanuel Frossard. Somayanda M. Impa and Sarah E. Johnson-Beebout wrote the paper with inputs from Rainer Schulin, Anja Gramlich, Susan Tandy, and Emmanuel Frossard.

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REFERENCES

Bashir, K., Ishimaru, Y., and Nishizawa, N. K. (2012). Molecular mechanisms of zinc uptake and translocation in rice. *Plant Soil* 361, 189–201. doi: 10.1007/s11104-012-1240-5

Boonchuay, P., Cakmak, I., Rerkasem, B., and Prom-U-Thai, C. (2013). Effect of different foliar zinc application at different growth stages on seed zinc concentration and its impact on seedling vigor in rice. *Soil Sci. Plant Nutr.* 59, 180–188. doi: 10.1080/00380768.2013.763382

Bukovac, M. J., and Wittwer, S. H. (1957). Absorption and mobility of foliar applied nutrients. *Plant Physiol.* 32, 428–435. doi: 10.1104/pp.32.5.428

Graham, R. D., and Rengel, Z. (1993). "Genotypic variation in zinc uptake and utilization by plants," in *Zinc in Soils and Plants*, ed A. D. Robson (Dordrecht: Kluwer Academic Publishers), 107–118. doi: 10.1007/978-94-011-0878-2_8

Hacisalihoglu, G., and Kochian, L. V. (2003). How do some plants tolerate low levels of soil zinc? Mechanisms of zinc efficiency in crop plants. *New Phytol.* 159, 341–350. doi: 10.1046/j.1469-8137.2003.00826.x

Huang, C. Y. L., and Schulte, E. E. (1985). Digestion of plant tissue for analysis by ICP emission spectroscopy. *Commun. Soil Sci. Plant Anal.* 16, 943–958. doi: 10.1080/00103628509367657

Impa, S. M., and Johnson-Beebout, S. E. (2012). Mitigating zinc deficiency and achieving high grain Zn in rice through integration of soil chemistry and plant physiology research. *Plant Soil* 361, 3–41. doi: 10.1007/s11104-012-1315-3

Impa, S. M., Morete, M. J., Ismail, A. M., Schulin, R., and Johnson-Beebout, S. E. (2013). Zn uptake, translocation and grain Zn loading in rice (*Oryza sativa* L.) genotypes selected for Zn deficiency tolerance and high grain Zn. *J. Exp. Bot.* 64, 2739–2751. doi: 10.1093/jxb/ert118

Jiang, W., Struik, P. C., Lingna, J., van Keulen, H., Ming, Z., and Stomph, T. J. (2007). Uptake and distribution of root-applied or foliar-applied ^{65}Zn after flowering in aerobic rice. *Ann. Appl. Biol.* 150, 383–391. doi: 10.1111/j.1744-7348.2007.00138.x

Johnson, A. A. T., Kyriacou, B., Callahan, D. L., Carruthers, L., Stangoulis, J., Lombi, E., et al. (2011). Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron and zinc-biofortification of rice endosperm. *PLoS ONE* 6:e24476. doi: 10.1371/journal.pone.0024476

Johnson-Beebout, S. E., Lauren, J. G., and Duxbury, J. M. (2009). Immobilization of zinc fer-tilizer in flooded soils monitored by adapted DTPA soil test. *Commun. Soil Sci. Plant Anal.* 40, 1842–1861. doi: 10.1080/00103620902896738

Kochian, L. V. (1991). "Mechanisms of micronutrient uptake and translocation in plants," in *Micronutrients in Agriculture*, ed J. J. Mortvedt (Madison, WI: Soil Sci. Soc. Am., Inc), 229–296.

Kutman, U. B., Yildiz, B., and Cakmak, I. (2011). Effect of nitrogen on uptake, remobilization and partitioning of zinc and iron throughout the development of durum wheat. *Plant Soil* 342, 149–164. doi: 10.1007/s11104-010-0679-5

Lee, S., Persson, D. P., Thomas, H. Hansen, T. H., Husted, S., Schjoerring, J. K., et al. (2011). Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. *Plant Biotechnol. J.* 9, 865–873. doi: 10.1111/j.1467-7652.2011.00606.x

Mabesa, R. L., Impa, S. M., Grewal, D., and Johnson-Beebout, S. E. (2013). Contrasting grain-Zn response of biofortification rice (*Oryza sativa* L.) breeding lines to foliar Zn application. *Field Crops Res.* 149, 223–233. doi: 10.1016/j.fcr.2013.05.012

Quijano-Guerta, C., Kirk, G. J. D., Portugal, A. M., Bartolome, V. I., and McLaren, G. C. (2002). Tolerance of rice germplasm to zinc deficiency. *Field Crop Res.* 76, 123–130. doi: 10.1016/S0378-4290(02)00034-5

Rengel, Z., and Graham, R. D. (1996). Uptake of zinc from chelate buffered nutrient solutions by wheat genotypes differing in Zn efficiency. *J. Exp. Bot.* 47, 217–226. doi: 10.1093/jxb/47.2.217

Rose, T. J., Impa, S. M., Rose, M. T., Pariasca-Tanaka, J., Mori, A., Heuer, S., et al. (2013). Enhancing phosphorous and zinc acquisition efficiency in rice: a critical review of root traits and their potential utility in rice breeding. *Ann. Bot.* 112, 331–345. doi: 10.1093/aob/mcs217

Saltzman, A., Birol, E., Bouis, H. E., Boy, E., de Moura, F. F., Islam, Y., et al. (2013). Biofortification: progress toward a more nourishing future. *Glob. Food Secur.* 2, 9–17. doi: 10.1016/j.gfs.2012.12.003

Sperotto, R. A. (2013). Zn/Fe remobilization from vegetative tissues to rice seeds: should I stay or should I go? Ask Zn/Fe supply. *Front. Plant Sci.* 4:464. doi: 10.3389/fpls.2013.00464

Sperotto, R. A., Boff, T., Duarte, G. L., Santos, L. S., Grusak, M. A., and Fett, J. P. (2010). Identification of putative target genes to manipulate Fe and Zn concentrations in rice grains. *J. Plant Physiol.* 167, 1500–1506. doi: 10.1016/j.jplph.2010.05.003

Sperotto, R. A., Ricachenevsky, F. K., Duarte, G. L., Boff, T., Lopes, K. L., Sperb, E. R., et al. (2009). Identification of up-regulated genes in flag leaves durin g rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. *Planta* 230, 985–1002. doi: 10.1007/s00425-009-1000-9

Stomph, T. J., Jiang, W., and Struik, P. C. (2009). Zinc biofortification of cereals: rice differs from wheat and barley. *Trends Plant Sci.* 14, 123–124. doi: 10.1016/j.tplants.2009.01.001

Wissuwa, M., Ismail, A. M., and Yanagihara, S. (2006). Effects of zinc deficiency on rice growth and genetic factors contributing to tolerance. *Plant Physiol.* 142, 731–741. doi: 10.1104/pp.106.085225

Wu, C., Lu, L., Yang, X., Feng, Y., Wei, Y., Hao, H., et al. (2010). Uptake, translocation, and remobilization of zinc absorbed at different growth stages by rice genotypes of different Zn densities. *J. Agric. Food Chem.* 58, 6767–6773. doi: 10.1021/jf100017e

Yoneyama, T., Gosho, T., Kato, M., Goto, S., and Hayashi, H. (2010). Xylem and phloem transport of Cd, Zn and Fe into grains of rice plants (*Oryza sativa* L.) grown in continuously flooded Cd-contaminated soil. *Soil Sci. Plant Nutr*. 56, 445–453. doi: 10.1111/j.1747-0765.2010.00481.x

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APPENDIX

Table A1 | Growth parameters of unlabeled rice genotypes at different growth stages and Zn treatments in experiment-1.

Growth stages	Genotype	Treatment	Plant height (cm)	Root length (cm)	Shoot dry weight (g plant^{-1})	Root dry (g plant^{-1})	Total dry matter (g plant^{-1})
21 DAP	IR55179	Zn-sufficient	$50^{b} \pm 1.6$	$22^{b} \pm 0.7$	$1.1^{b} \pm 0.14$	$0.5^{b} \pm 0.05$	$1.6^{b} \pm 0.18$
		Zn-deficient	$40^{c} \pm 0.6$	$18^{c} \pm 0.6$	$0.7^{c} \pm 0.05$	$0.3^{c} \pm 0.03$	$1.0^{c} \pm 0.07$
	KP	Zn-sufficient	$68^{a} \pm 1.2$	$26^{a} \pm 0.5$	$1.6^{a} \pm 0.09$	$0.6^{a} \pm 0.04$	$2.1^{a} \pm 0.12$
		Zn-deficient	$52^{b} \pm 1.0$	$23^{b} \pm 0.7$	$0.8^{c} \pm 0.04$	$0.3^{c} \pm 0.02$	$1.0^{c} \pm 0.06$
	Genotype		$p < 0.001$	$p < 0.001$	$p = 0.002$	$p = 0.05$	$p = 0.005$
	Treatment		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	Genotype × treatment		$p = 0.01$	NS	NS	NS	NS
	5% LSD (Zn × G)		3	2	0.2	0.1	0.3
42 DAP	IR55179	Zn-sufficient	$54^{b} \pm 0.3$	$34^{a} \pm 0.6$	$4.8^{b} \pm 0.2$	$2.4^{a} \pm 0.06$	$7.2^{b} \pm 0.2$
		Zn-deficient	$39^{c} \pm 1.2$	$17^{b} \pm 1.2$	$2.7^{c} \pm 0.1$	$0.2^{c} \pm 0.02$	$2.9^{c} \pm 0.1$
	KP	Zn-sufficient	$72^{a} \pm 2.0$	$43^{a} \pm 5.2$	$6.9^{a} \pm 0.2$	$2.4^{a} \pm 0.08$	$9.3^{a} \pm 0.2$
		Zn-deficient	$54^{b} \pm 2.5$	$40^{a} \pm 0.7$	$1.3^{d} \pm 0.2$	$0.6^{b} \pm 0.08$	$1.9^{d} \pm 0.2$
	Genotype		$p < 0.001$	$p = 0.001$	$p = 0.04$	$p = 0.02$	$p = 0.008$
	Treatment		$p < 0.001$	$p = 0.009$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	Genotype × treatment		NS	$p = 0.05$	$p < 0.001$	NS	$p < 0.001$
	5% LSD (Zn × G)		6	10	0.5	0.3	0.5

DAP, days after planting in ANS. Zn × G, Zn treatment × genotype. Values with different letters in a column within a growth stage are significantly different at 5% LSD. Shoot = leaf lamina + sheath + stem. The values given are means ± SE (n = 10 for 21 DAP and n = 3 for 42 DAP).

Table A2 | Growth parameters of unlabeled rice genotypes in Zn-sufficient conditions in experiment-3.

Trait	Ten days after 50% flowering		Maturity	
	SWHOO	IR69428	SWHOO	IR69428
Plant height (cm)	77 ± 3	78 ± 6	80 ± 3	79 ± 3
Root length (cm)	43 ± 2	48 ± 2	41 ± 0.9	54 ± 5
Tiller number (plant^{-1})	8 ± 0.7	4 ± 0.4	6 ± 1	5 ± 0.6
Root dry weight (g plant^{-1})	4 ± 1	4 ± 0.8	5 ± 1	4 ± 1.6
Stem dry weight (g plant^{-1})	12 ± 0.6	16.4 ± 2	13 ± 1	19 ± 0.4
Dry leaf dry weight (g plant^{-1})	–	–	1 ± 0.3	4 ± 0.2
Green leaf dry weight (g plant^{-1})	4 ± 0.4	6 ± 0.7	2 ± 0.5	2 ± 0.8
Panicle dry weight (g plant^{-1})	3 ± 0.7	2 ± 0.1	–	–
Rachis dry weight (g plant^{-1})	–	–	0.3 ± 0.03	0.3 ± 0.06
Hull dry wieght (g plant^{-1})	–	–	1 ± 0.2	0.5 ± 0.09
Apical grain weight (g plant^{-1})	–	–	3 ± 0.5	2 ± 0.2
Distal grain weight (g plant^{-1})	–	–	3 ± 0.4	2 ± 0.04

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Global changes in mineral transporters in tetraploid switchgrasses (*Panicum virgatum* L.)

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Switchgrass (*Panicum virgatum L*) is perennial, C_4 grass with great potential as a biofuel crop. An in-depth understanding of the mechanisms that control mineral uptake, distribution and remobilization will benefit sustainable production. Nutrients are mobilized from aerial portions to below-ground crowns and rhizomes as a natural accompaniment to above-ground senescence post seed-set. Mineral uptake and remobilization is dependent on transporters, however, little if any information is available about the specific transporters that are needed and how their relative expression changes over a growing season. Using well-defined classes of mineral transporters, we identified 520 genes belonging to 40 different transporter classes in the tetraploid switchgrass genome. Expression patterns were determined for many of these genes using publically available transcriptomic datasets obtained from both greenhouse and field grown plants. Certain transporters showed strong temporal patterns of expression in distinct developmental stages of the plant. Gene-expression was verified for selected transporters using qRT-PCR. By and large these analyses confirmed the developmental stage-specific expression of these genes. Mineral analyses indicated that K, Fe, Mg, Co, and As had a similar pattern of accumulation with apparent limited remobilization at the end of the growing season. These initial analyses will serve as a foundation for more detailed examination of the nutrient biology of switchgrass.

Keywords: bioenergy, crowns and rhizomes, growing-season, mineral transporters, nutrients, *Panicum virgatum*, switchgrass, qPCR

INTRODUCTION

Plant mineral composition depends on uptake and translocation of minerals from the rhizosphere, through the root-shoot junction (crown), and into the aboveground tissues. These processes are influenced by both environmental and genotypic factors. In the perennial plant growth cycle, certain minerals can be recycled or remobilized from senescing tissues in the autumn, stored in perennial tissues during winter dormancy, and then remobilized and translocated to growing tissues in the spring. In switchgrass (*Panicum virgatum* L.), the stems and leaves survive for 1 year, while perennial tissues including the crowns, rhizomes, and older roots survive for much longer periods of time. The crown and rhizome tissues connect the root system to the shoot system, thus, minerals that are remobilized from annual or perennial tissues must pass through the crown tissue. These tissues can also serve as repository for remobilized nutrients at the end of the growing season. Thus, mineral uptake and recycling are cornerstones for the sustainable production of biomass from switchgrass and other perennial herbaceous bioenergy crops.

In *Arabidopsis thaliana*, concentrations of several minerals were highest in young tissues (Waters and Grusak, 2008), suggesting that stage of harvest may be important for mineral concentration in plant tissues. In switchgrass, harvests during late vegetative stage or during summer had substantially higher P, Cl, K, and S than at post-senescence stage (Reynolds et al., 2000; Vogel et al., 2002; Dien et al., 2006; Lemus et al., 2009; Yang et al., 2009), demonstrating that these compounds are remobilized from leaf and stem tissue. Genotypic differences in remobilization were shown to be present (Reynolds et al., 2000; El-Nashaar et al., 2009; Yang et al., 2009).

The most abundant minerals in above ground switchgrass tissues are Si, K, Cl, Ca, and P (Monti et al., 2008; El-Nashaar et al., 2009). While several minerals are remobilized from aboveground biomass during senescence in switchgrass, some abundant minerals are not, such as Si, Ca, and Mg (Dien et al., 2006; Lemus et al., 2009; Yang et al., 2009). Feedstock quality requirements depend on the conversion platform (Sarath et al., 2008; Vogel et al., 2011) and pyrolysis and other thermochemical platforms will benefit from feedstocks that contain high lignin and low levels of N

and alkali metals (Patwardhan et al., 2010). Reducing minerals such as Si will lower ash content as well. A number of genes that contribute to root uptake of Ca and Mg (Karley and White, 2009; Waters, 2011), and transporters for Si uptake and distribution (Ma et al., 2006; Yamaji et al., 2008; Yamaji and Ma, 2009) have been identified in grasses. Currently, no whole genome-scale annotation and transcriptomic information for mineral and related transporter genes are available in switchgrass, however, this data would be useful to correlate specific genes with mineral accumulation.

Increased understanding of genes that impact mineral acquisition, transport and recycling in switchgrass can be used to improve both the genetics and management of switchgrass as a high-yielding biomass crop. Mineral transporters will be key players in these processes, as transport into and out of cells and organelles are the molecular events that underlie cellular storage and whole-plant translocation or recycling of minerals. Mineral transporter families have been studied extensively in species such as Arabidopsis and rice, providing gene sequence data to predict identity and function of unknown transcripts from other species. In switchgrass, no molecular studies of transporter genes have been conducted. Understanding of the interactions in uptake and remobilization between different minerals over the course of a growing season is limited for switchgrass. The release of the switchgrass genome (PviDraft0.0) by the Joint Genomes Institute (www.phytozome.org) (Goodstein et al., 2012) has greatly facilitated the discovery and annotation of genes and gene families in switchgrass (Saathoff et al., 2013).

The long-term goal of our research is to develop and utilize genotyping and phenotyping tools that can significantly enhance the breeding of switchgrass (*Panicum virgatum* L.) as a sustainable bioenergy crop for marginal crop lands (Vogel et al., 2011). Our objectives in this study were to use next-generation sequencing data to discover, annotate and quantify expression of switchgrass genes that are potentially involved with mineral transport in switchgrass. Here, we have combined bioinformatics and real-time qRT-PCR to classify transporter gene families in switchgrass and to identify specific genes that show altered expression over the growing season. We also used mineral analysis to quantify seasonal concentration changes in crown tissues.

MATERIALS AND METHODS

GENE DISCOVERY

Known mineral and nutrient transporters in *Arabidopsis thaliana* were used to identify putative homologs in *Panicum virgatum*, *Sorghum bicolor*, and *Setaria italica* based on protein similarity using BLASTp (Altschul et al., 1990, 1997) and the respective reference genomes for each plant (www.phytozome.org) (Paterson et al., 2009; Bennetzen et al., 2012; Goodstein et al., 2012). A maximum *e*-value of 1×10^{-25} and minimum alignment of 50% were used as thresholds in filtering the BLASTp results for putative homolog identification.

PHYLOGENETIC ANALYSES

Cladograms were generated for genes of selected Arabidopsis and putative switchgrass transporter families. Sequences were analyzed for phylogenetic relationships using Phylogeny.fr (Dereeper et al., 2008).

Publically available 454 transcriptome sequencing datasets were used to generate expression profiles for identified putative mineral and nutrient transporters in switchgrass as described previously (Saathoff et al., 2013). 454 sequence reads were aligned to the draft switchgrass transcriptome using Bowtie2 (Langmead and Salzberg, 2012) and gene counts calculated using HTSeq-Count version 0.5.1p2 (https://pypi.python.org/pypi/HTSeq). Expression counts were normalized through conversion from raw counts to RPKM (reads per kilo base exon per million mapped reads).

HEAT MAPS AND CLUSTERING

Heat maps were generated using estimated 454 expression data and two-way hierarchical clustering with JMP 9.0 (SAS Institute Inc., Cary, NC). RPKM expression values were converted to standardized values (z-scores) for each gene, and hierarchical clustering using Ward's method was performed to yield heat maps and clusters of coexpressing transporters.

PLANT MATERIAL

Crowns and rhizomes were collected, cleaned and flash-frozen from field-established plants of cv Summer, as described earlier (Palmer et al., 2012). At each harvest date tissues were obtained from three individual plants. Flash-frozen tissues were stored at −80°C until needed. Tissues were ground in a cryogenic grinder (Palmer et al., 2012). Aliquots (0.1 g) of ground materials were used for isolating RNA as previously described (Chomczynski and Sacchi, 1987). RNA samples were subsequently purified using RNeasy columns (Qiagen; Valencia, CA, USA) according to manufacturer's instructions.

qRT-PCR AND PRIMERS

DNase treated RNA samples were used to synthesize first strand cDNA by using SuperScript III reverse transcriptase (Invitrogen; Carlsbad, CA, USA) and random primers according to the manufacturer's protocol. qRT-PCR reactions were set up in a total volume of 15 uL using 7.5 uL master mix (Bio-Rad), 0.2 uL cDNA template, 0.75 uL primers, and 6.55 uL 18 MΩ water and conducted on a BioRAD CFX Connect Real Time PCR instrument. Each reaction was performed in quadruplicate and the experimental design blocked plate with amplicon (a single amplicon per plate). Primers were designed using Primer3Plus (Untergasser et al., 2012). Data was efficiency corrected using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009), and geNORM was used to screen for effective normalization genes and calculate relative quantities for each gene of interest (Hellemans et al., 2007). Primers used are shown in Table A1.

MINERAL ANALYSES

Tissues were dried at 60°C for at least 72 h and weighed. Samples (typically 25–50 mg) were digested as described previously (Waters et al., 2012). In brief, samples were digested with 3 ml of concentrated HNO_3 (VWR, West Chester, PA, USA, Trace metal grade) at room temperature overnight, then at 100°C for 1.5 h, followed by addition of 2 ml of 30% H_2O_2 (Fisher Scientific, Fair Lawn, NJ, USA) and digestion for 1 h each at steps of 125°C,

150°C, 165°C, and finally were heated to dryness at 180°C. Dried samples were then resuspended in 5 ml of 1% HNO3, and minerals were quantified by inductively coupled plasma mass spectrometry (ICP-MS) at the University of Nebraska Redox Biology Center Spectroscopy and Biophysics Core Facility.

STATISTICAL ANALYSES

Transcript levels were investigated by utilizing cDNA that originated from three individual genotypes (biological replicates) at each time point with four technical replicates per genotype. The cDNA was not pooled prior to qRT-PCR analysis. Thus, for any given harvest date, there were 12 total reactions that were conducted which included both true biological as well as technical replicates. The GeNORM program (Hellemans et al., 2007) within the qbase+ software package was used to analyze reference genes in order to find suitably stable ones with a *M*-value below 1.5. In this way, Pavirv00026367m (a ubiquitin protein ligase) was selected as the stable reference gene for generation of relative quantities. The relative quantities were then statistically analyzed using PROC GLM in SAS (SAS Institute, Cary, NC) and Tukey's multiple comparison procedure was utilized to conduct pairwise comparisons of different harvest dates. Familywise error rate was controlled at $\alpha = 0.05$.

Data for the mineral analyses were subjected to single-factor ANOVA analysis of each mineral, error bars are standard deviations are from 3 biological replicates, with 2 technical replicates each. Of the 16 minerals analyzed, the 10 minerals showed statistically significant variation for at least two time points. *P*-values for the mineral analysis were calculated by Single Factor ANOVAs (in Excel).

RESULTS AND DISCUSSION

Little is known about the identities of specific genes that contribute to remobilization of minerals from senescing tissues. Some genes are known to be important for remobilization and/or translocation of minerals from source to sink tissues in Arabidopsis, for example *YSL1*, *YSL3*, and *OPT3* for iron, zinc, and copper (Waters et al., 2006; Stacey et al., 2008; Waters and Grusak, 2008), *NRT1.7* for nitrate (Fan et al., 2009), *Sultr1;3* for sulfate (Yoshimoto et al., 2003), and *Pht1;5* for phosphate (Nagarajan et al., 2011). These genes have usually been discovered by analysis of mutants. A transcriptomic approach can reveal new insights to help understand nutrient deficiency signaling pathways (Maruyama-Nakashita et al., 2003; Hermans et al., 2010; Waters et al., 2012). Likewise, transcriptomic studies in Arabidopsis during senescence have identified many transporters, transcription factors, and other senescence associated genes that are up or down regulated (Buchanan-Wollaston et al., 2003, 2005; Van Der Graaff et al., 2006; Balazadeh et al., 2008). However, their correlation with specific changes in minerals or N remobilization is still incomplete. Our overall goal in this study was to identify and classify switchgrass transcripts into mineral transporter gene families and quantify their expression over the life cycle in different tissues as a first step to finding correlations between gene expression and mineral translocation through tissues. This will allow focused future studies to pinpoint the specific roles of individual genes during plant development.

DISCOVERY OF POTENTIAL SWITCHGRASS TRANSPORTERS

In mining the switchgrass genome for mineral transporter gene family members we found a total of 520 genes belonging to 40 different classes in the current annotation of the switchgrass genome. The number of switchgrass genes was approximately twice as many as identified in the annotated genomes of *Sorghum bicolor* (274) and *Setaria italica* (281) (**Table 1**). Our results indicate that for the most part, the switchgrass genome (tetraploid, A and B genomes) contained about twice as many genes in each class (**Table 1**), although some exceptions were noticed. For example, four putative copper transporters (*COPT*) (Pilon et al., 2009) were identified in the switchgrass genome, as compared to 1 each in sorghum and *Setaria*. Likewise, 7 potassium transporters (*HAKs*) (Grabov, 2007) were found in Sorghum and *Setaria*, whereas 9 putative *HAKs* were identified in the switchgrass genome. As anticipated, the switchgrass genome contained large numbers (>10 genes per genome) for many classes of transporters including those for nitrate, phosphate, S, K, Mg, and putative peptide/nitrate transporters.

DIFFERENTIAL REGULATION OF TRANSPORTER GENES IN SWITCHGRASS TISSUES OVER DEVELOPMENT

Both tissue and temporal specificity in the expression of putative transporter genes in switchgrass was observed. Transcriptome datasets generated for greenhouse grown switchgrass cv Alamo (**Figure 1**) were mined for the relative abundance of transcripts for transporters shown in **Table 1**. There appeared to be both tissue and stage specific expression for many transporter genes at the three stages of harvest, early vegetative, shoot elongation and reproductive (**Figure 1**). Most transporter transcripts had different apparent abundances over plant developmental stages for the roots and shoots, and in flowers at reproductive stage (**Figure 1**). In roots, a cluster of transporters with high transcript counts were observed at the early vegetative stage of harvest (cluster A1). Several genes associated with this cluster were downregulated at the shoot elongation stage, and a larger cluster of transcripts were upregulated in roots at the shoot elongation stage (**Figure 1**; cluster A2). At the reproductive stage of plant growth, a new set of transporter genes was more abundant in the roots (cluster A6), and there was an apparent downregulation of many of the genes present in greater abundance at the early vegetative and shoot elongation stages of plant growth. A few genes appeared to be upregulated at the early vegetative stage in shoots as compared to roots. At the shoot elongation stage of plant development, most transporter genes were less abundant in shoots as compared to roots, and also less abundant than in shoots at the early vegetative stage (**Figure 1**). Interestingly, transcripts for a cluster of transporter genes had higher abundance in reproductive stage shoots (cluster A5). These included genes that appeared to be primarily expressed in shoots and some that overlapped with roots and reproductive structures. Reproductive tissues contained greater levels of transcripts for a cluster of transporter genes that were less abundant in roots or shoots (**Figure 1**, cluster A4).

We next evaluated expression profiles of several transporter gene families in field grown crown and rhizome tissues from cv Summer plants at different stages during a growing season. Of the 520 total mineral transporter genes identified in the

Table 1 | Bioinformatic analysis of switchgrass genome for mineral transporter classes.

	Identifed transporters from genome mining				
Class	**Descriptions**	**In ref**	**Pvi0**	**Sb**	**Si**
ACA	Ca2+-transporting ATPase	8	22	13	13
AKT/KAT	Shaker family K+ ion channel	9	18	12	11
AMT	Ammonium transporter	6	13	8	8
CAX	Cation/proton exchanger	7	16	7	8
CHX	Cation/H(+) antiporter	28	26	17	18
COPT	Copper transporter	5	4	1	1
FRO	Ferric reduction oxidase	8	1	1	1
FRU	ER-like iron deficiency-induced TF	1	2	1	2
HKT	Sodium transporter	1	5	3	4
HMA	Heavy metal ATPase	8	18	8	8
IREG	Iron regulated protein	3	6	3	3
IRT	Fe(2+) transport protein	3	1	0	0
KEA	K(+) efflux antiporter	6	9	4	4
KUP	Potassium transporter	13	33	22	21
LSI	Silicon transporter (from Hv)	2	5	2	2
MHX	Magnesium/proton exchanger	1	4	1	1
MOT	Molybdate transporter	2	5	1	2
MRS	Magnesium transporter	11	20	10	9
MTP	Metal tolerance protein	1	2	1	1
NAS	Nicotianamine synthase 1	4	4	3	3
NAXT	Nitrate excretion transporter	1	3	1	3
NHX	Sodium/hydrogen exchanger	8	15	7	7
NRAMP	Metal transporter	6	16	6	6
NRT	Nitrate transporter	16	35	18	19
NTRm	Misc NTR-class (major facilitator)	16	32	15	17
OPT	Oligopeptide transporter	9	15	9	8
PHO	Phosphate transporter	11	9	4	4
PHT	Inorganic phosphate transporter	19	45	22	24
PNT	Putative peptide/nitrate transporters	23	48	25	23
PTR	Peptide transporter	5	11	8	9
SULTR	Sulfate transporter	12	23	11	11
TPC	Two pore calcium channel protein	1	1	1	1
TPK	Ca-activated outward-rectifying K channel	1	7	2	3
VIT	Vacuolar iron transporter	1	4	2	2
YSL	Metal-nicotianamine transporter	8	15	11	13
ZIP	Zinc transporter	13	27	14	11
	Total	277	520	274	281

In ref, Genes annotated as having mineral transporter function in the Arabidopsis thaliana genome; Pvi0, Draft 0.0 switchgrass genome; Sb, Sorghum bicolor; Si, Setaria italica.

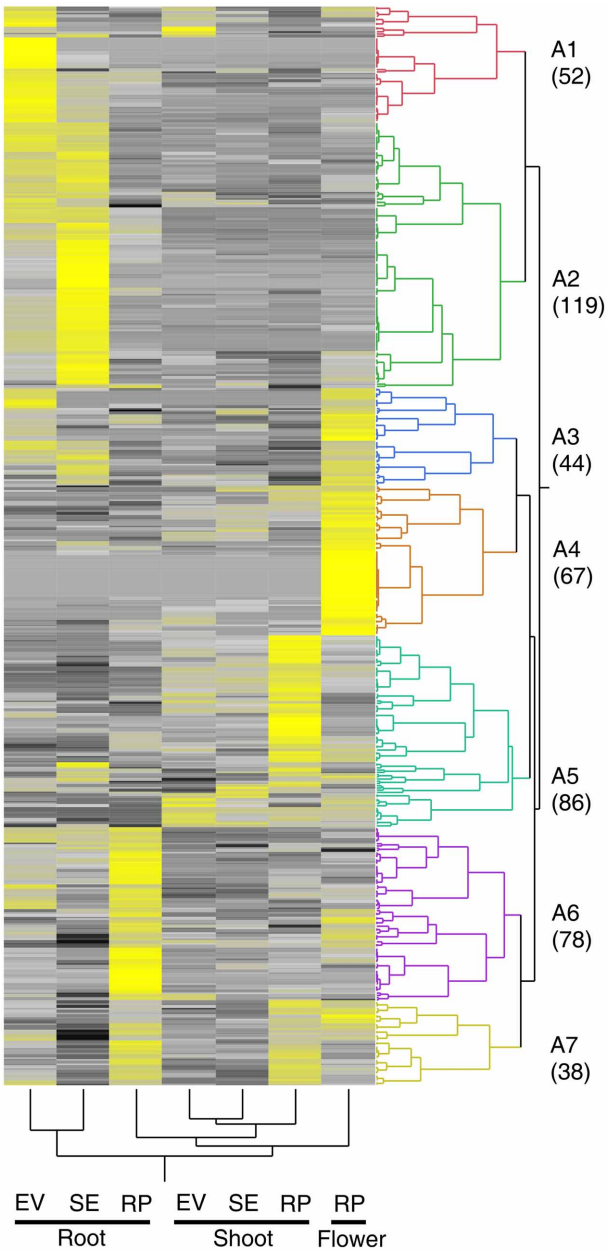

FIGURE 1 | Two-Way clustered expression profile map of genes of switchgrass nutrient transporters present in different tissues of cv Alamo plants grown in a greenhouse. Stages of plant development are early vegetative (EV), stem elongation (SE), and reproductive (RP) are as described in these datasets. Yellow indicates high abundance, gray is intermediate and black is low or negligible abundance. The clusters are labeled as A1 through A7. The numbers in parentheses under each cluster indicate the total number of transporter identified within each cluster. The appropriate SRA identification numbers for these individual NGS files are SRX057826, SRX057827, SRX057828, SRX057829, SRX057830, SRX057831, and SRX057834.

switchgrass genome, transcripts for 401 mineral transporter genes were detected in the crown and rhizome datasets (see **Table 1**). As observed for the greenhouse grown cv Alamo datasets, some gene clusters were up- or downregulated at certain harvest dates (**Figure 2**). Some transcripts that were abundant early in the growing season (spring green up; May cluster C5) were less abundant later, suggesting that these genes are important for rapid growth in the spring. Enrichment of specific transporter classes (GO-terms) was not observed in these clusters. Other subsets of transporter genes were strongly upregulated during the periods of active shoot and rhizome growth (June and July, clusters C2 and C3), suggesting that genes in this cluster are important

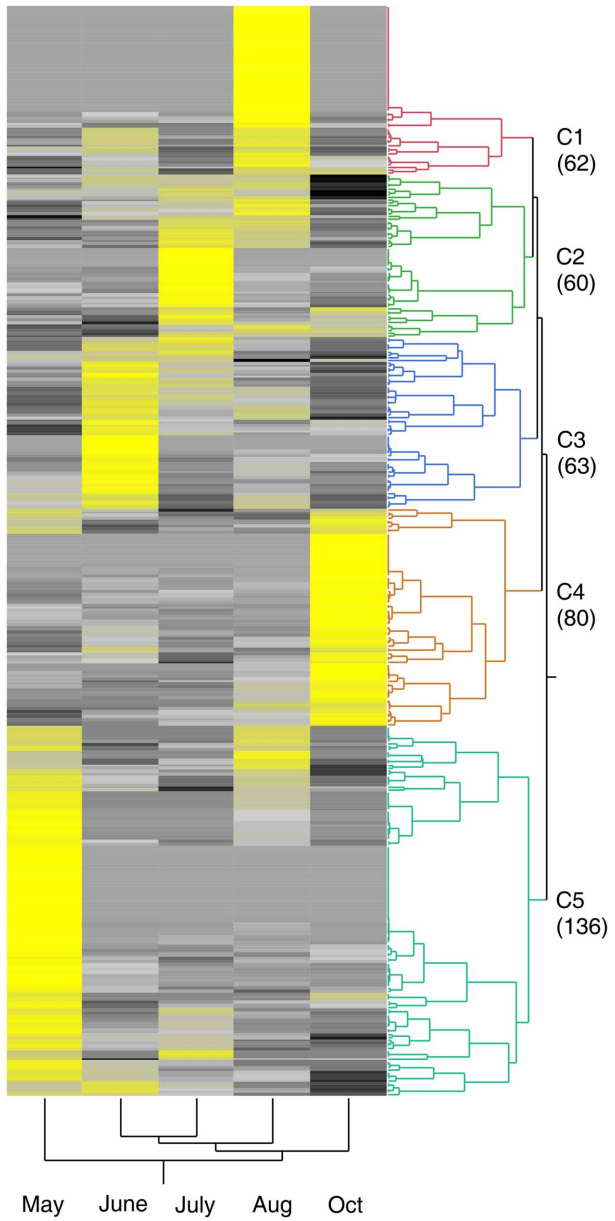

FIGURE 2 | Two-Way clustered expression profile map of genes of switchgrass nutrient transporters present in crown and rhizome tissues of field grown cv Summer plants harvested at different times during the growing season. Yellow indicates high abundance, gray is intermediate and black is low or negligible abundance. The clusters are labeled as C1 through C5. The numbers in parentheses under each cluster indicate the total number of transporter identified within each cluster. The appropriate SRA identification numbers for these individual NGS files are SRX257007, SRX257030, SRX257031, SRX102934, and SRX257032.

as plants continue to grow and progress to the reproductive development stage. At the July harvest plants were heading, with inflorescences visible at the top of the shoots. Although some of these genes were apparently being transcribed at continued high rates, a new cluster of transporter genes was upregulated at the August harvest date (**Figure 2**, cluster C1), when the plants were nearing physiological maturity, suggesting that these genes could be important for moving minerals to developing seeds. In crowns and rhizomes obtained from plants after a leaf killing frost in October, many of the transporter genes that were upregulated at the earlier harvest dates had decreased, whereas a new cluster of genes had increased transcript counts (cluster C4). This is a particularly interesting pattern, as these transporters are likely to be important for mineral storage or translocation of minerals to perennial storage tissues such as roots and rhizomes.

Comparison of the gene members for each cluster in the two 454 transporter datasets showed significant overlap. Approximately 40% of the genes expressed early in the growing season in crown and rhizomes (clusters C5 and C3) are also found expressed in roots during the early vegetative and shoot elongation stages in Alamo (clusters A1, A2, and A3). Similarly, approximately 30% of the genes expressed during the reproductive period in crowns and rhizomes (clusters C2 and C1) are expressed in Alamo roots during the reproductive stage (clusters A6 and A7). These metadata analyses from both greenhouse grown cv Alamo and field grown cv Summer plants indicated that nutrient transporters were transcriptionally controlled at the tissue level and expression was influenced by the developmental stage of the plant. It will be interesting and useful to compare the gene expression data to changes in mineral concentrations over the seasonal growth and senescence of switchgrass.

EXPRESSION PROFILES OF SELECTED MINERAL TRANSPORTER GENE FAMILIES

Phylogenetic relationships and expression levels for different classes of transporter genes were analyzed in crown and rhizome datasets. *HAK/KUP/KT* genes encode K^+/H^+ symporters (Szczerba et al., 2009) and are associated with the uptake of K^+ into roots and efflux from vacuoles (Rodriguez-Navarro and Rubio, 2006). *KUP* genes are involved with a number of different aspects of plant development (Grabov, 2007), and expression of *KUP* genes throughout the plant (Szczerba et al., 2009) indicates roles in many tissue and cell types. A total of 33 *KUP* genes were found in our scan of the switchgrass genome, and transcripts ascribable to 29 of these genes were expressed in the crowns and rhizomes of field grown cv Summer plants (**Figure 3A**). Switchgrass and *Arabidopsis thaliana* annotated *KUP* sequences were separated into six clades. Two genes that share similarities to *AtKUP4*, *Pavirv00030241* and *Pavirv00010539*, were most highly expressed in the August harvest. Transcripts for switchgrass *KUPs* falling in the clade with *AtKUP2* were all overrepresented at the onset of spring growth. For the other *KUPs*, gene expression within clades was variable, although patterns associated with specific harvest dates were evident. For example, all the switchgrass *KUPs* with sequence similarities to *AtKUP7*/13 proteins were overrepresented in the August or November harvests, whereas transcript abundances for the larger clade of switchgrass *KUPs* with protein sequence similarities to *AtKUP5/10* were more variable (**Figure 3A**).

Magnesium is transported by members of the MRS2/MGT family, with expression in Arabidopsis noted in roots, leaves and senescing leaves, flowers, and pollen (Waters, 2011), and subcellular localizations in plasma membrane, tonoplast,

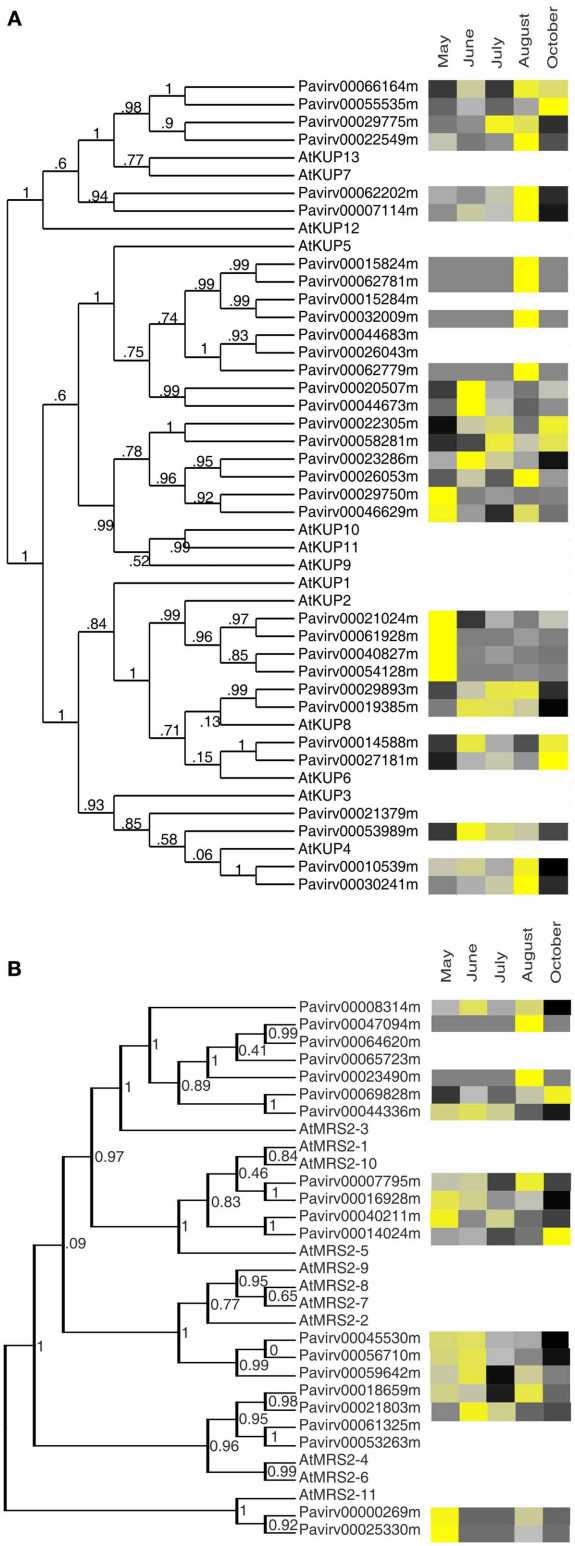

FIGURE 3 | Cladogram of the phylogentic relationships between switchgrass transporter genes to *Arabidopsis thaliana* orthologs and their expression profiles in crowns and rhizomes of field grown cv Summer plants. (A) Potassium transporters (KUP) **(B)** Magnesium transporters (MRS). Other details are as described for **Figure 2**.

mitochondria, ER and chloroplast. Most of the Mg in leaves is associated with ribosomes involved in protein synthesis, with the majority of the remaining fraction associated with chlorophyll (Karley and White, 2009). As such, it is not surprising that transcripts of several *MRS2* magnesium transporters were most abundant during the active shoot growth phase (May–July) (**Figure 3B**). However, four and two genes were highly expressed in the August and October harvests, respectively, suggesting that Mg or related mineral transport was active in the crowns and rhizomes at a time when the above ground parts of the plants were senescing (August) or fully senescent (October). These changes could arise potentially from Mg transported from roots and/or sequestration of Mg into different cellular compartments of the rhizome. It is conceivable that tiller buds and other meristematic tissues present on these rhizomes are metabolic sinks, and transport processes are associated with the continued delivery of nutrients to these critical organs.

Recycling of N in switchgrass is a major factor in sustainability and environmental impacts of production, and a number of studies have addressed this issue from production and genetic perspectives (reviewed by Schwartz and Amasino, 2013). Nitrate is a major source of N for plant roots, which is taken up by in part by nitrate transporters of the *NRT* family. Once nitrate is inside the plant, NRT transporters are also involved in xylem loading and unloading, phloem loading, and storage in vacuoles (Dechorgnat et al., 2011; Wang et al., 2012) and are essential for the translocation of plant defense compounds to the seeds (Nour-Eldin et al., 2012). Transcripts were detected for 22 putative switchgrass nitrogen transporters from a total of 35 identified in the switchgrass genome (see **Table 1**). The Arabidopsis *NRT2.4* gene is expressed in both the shoots and roots of nitrogen-starved plants (Kiba et al., 2012) and functions as a high affinity N transporter. Five switchgrass proteins with strong homology (*e*-values of 0 to 6×10^{-170}) to Arabidopsis NRT2.4 were identified. Of these, transcripts for *Pavirv00019393* (**Figure 4A**) were most abundant in the July harvest when the plants had reached anthesis, whereas *Pavirv00068021* was overexpressed at the August harvest, when seeds were nearing physiological maturity. A majority of the other switchgrass *NRTs* (14) were most abundantly expressed during the active phase of shoot and rhizome growth (May–July harvests; **Figure 4A**). The other 6 *NRTs* were overexpressed in the August and October harvests. *Pavirv00010339* had higher expression in crowns and rhizomes of cv Summer plants at the October harvest date, and appears to be orthologous to the Arabidopsis NRT1.6 and 1.7 proteins. NRT1.7 is a low-affinity nitrate transporter involved in source to sink mobilization of nitrate via the phloem (Fan et al., 2009). In a similar manner, *Pavirv00039672*, with homology to the Arabidopsis NRT1.5/NRT1.8 transporters, is also upregulated in the October harvest (**Figure 4A**). Arabidopsis NRT1.5 modulates the allocation of nitrate to the roots to mediate stress responses in concert with NRT1.8 and other proteins (Chen et al., 2012).

Ammonium is an additional source of N for plant growth. Ammonium is taken into roots by AMT1 or AMT2 family transporters (Ludewig et al., 2007). Ammonium is also generated in tissues by reduction of nitrite or breakdown of amino compounds, some of which are remobilized during leaf

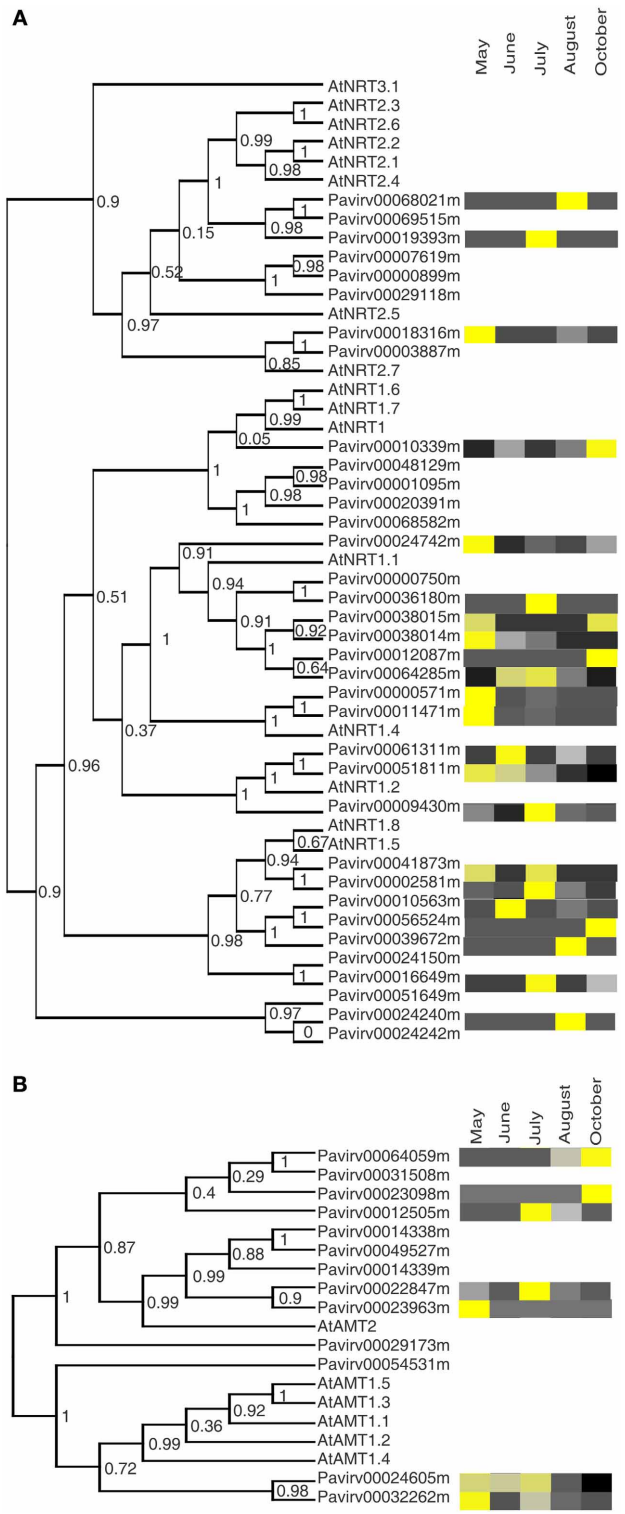

FIGURE 4 | Cladogram of the phylogentic relationships between switchgrass transporter genes to *Arabidopsis thaliana* orthologs and their expression profiles in crowns and rhizomes of field grown cv Summer plants. (A) Nitrate transporters (NRT) **(B)** Ammonium transporters (AMT). Other details are as described for **Figure 2**.

senescence, and may need to be transported into chloroplasts for reassimilation. In Arabidopsis (Ludewig et al., 2007) and poplar (Couturier et al., 2007) *AMT* genes are expressed in roots, shoots, and flower structures. Switchgrass ammonium transporters were separable into four clades based on Arabidopsis (AtAMT) protein sequences (**Figure 4B**). Of the 13 AMT sequences in the switchgrass genome, transcripts for 7 genes were identified in the crown and rhizome. Five were more abundantly expressed during the active growing phases (May-July). Two genes overexpressed in crowns and rhizomes at the October harvest, *Pavirv00064059* and *Pavirv00023098* were orthologs of AtAMT2, which is a high affinity ammonium transporter in both shoots and roots (Sohlenkamp et al., 2002).

In Arabidopsis, phosphate transporters of the PHT family are classified into 4 subfamilies (Liu et al., 2011) all of which had homologs in switchgrass. *PHT1* genes are primarily expressed in roots, where they are thought to take up phosphate from soil or mycorrhizal fungi, but are also expressed in leaves and pollen. *PHT2;1* is expressed primarily in the leaves and is thought to transport phosphate into leaves (Daram et al., 1998). PHT3 proteins are found in a variety of cellular membranes including mitochondria (Zhu et al., 2012). *PHT4* genes are mainly targeted to the plastids or Golgi (Guo et al., 2008) and are expressed in both roots and leaves. In rice (Liu et al., 2011) and Arabidopsis (Nussaume et al., 2011) *PHT* genes were expressed in numerous tissue types during the life cycle. Transcripts for ∼71% of the total *PHT* genes in the switchgrass genome were found in the crown and rhizome of field grown switchgrass. Expression of the *PHTs* predominantly tracked with active growth phases of the plant (May–July harvests), although a smaller subset was upregulated in tissues harvested near physiological maturity (August, **Figure 5A**). Many of these genes clustered with Arabidopsis protein sequences belonging to the PHT3-2, 4-1, and 4-4 genes. These phosphate transporters are thought to be involved in a number of plant processes involving the shuttling of P_i across plant compartments. In crowns and rhizomes, this could involve both the acquisition and transport of phosphate from the soil to the developing shoots during the growing season as well as potentially in the redistribution of P_i at the end of the growing season. Notably, transcripts for *Pavirv00062983* (orthologous to *AtPHT4-4*) were upregulated in tissues obtained after a killing frost (October; **Figure 5A**). AtPHT4 has been implicated in the movement of P_i between the cytosol and plastids (Guo et al., 2008).

Silicon is important for resistance to abiotic and biotic stress in grasses (Ma et al., 2011; Nabity et al., 2012). About 90% of Si taken up by roots is transported into shoots (Ma and Takahashi, 2002). Lsi1 and Lsi2 are required for uptake (Ma et al., 2006, 2007), and Lsi6 is required for Si transport throughout leaves (Yamaji et al., 2008) and through stem nodes (Yamaji and Ma, 2009). To move from roots to leaves, Si would have to pass through crown tissue. Five genes putatively code for silicon transporters in switchgrass, and transcripts were detected for all five genes in the crown and rhizome tissues (**Figure 5B**). In contrast to other transporters, silicon transporters genes were essentially downregulated as the growing season progressed. Expression patterns of these putative switchgrass Si transporters are consistent with the movement of

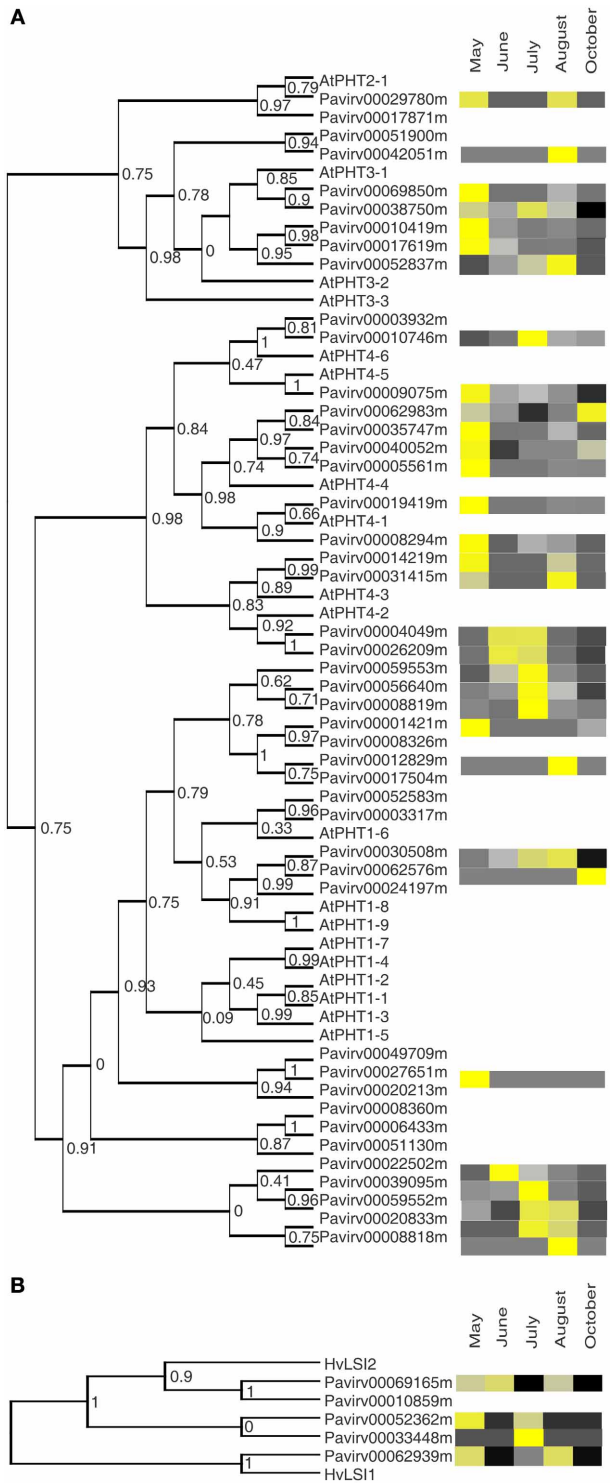

FIGURE 5 | Cladogram of the phylogentic relationships between switchgrass transporter genes to *Arabidopsis thaliana* (PHT) and barley (Hordeum vulgare; LSi) orthologs and their expression profiles in crowns and rhizomes of field grown cv Summer plants.
(A) Phosphate transporters (PHT) **(B)** Silicon transporters (LSi). Other details are as described for **Figure 2**.

Si from the soil to the shoots. The downregulation of all of these Si-transporter genes possibly resulted from death of the shoots (October harvest, **Figure 5B**).

We also analyzed the expression patterns of YSL and ZIP families of metal micronutrient transporters (**Figures 6A,B**). Yellow stripe-like (*YSL*) genes are related to the Yellow Stripe gene that encodes an Fe(III)-phytosiderophore uptake protein in maize roots (Curie et al., 2001). However, instead of functioning in uptake, YSL proteins carry out transport of nicotianamine-metal complexes within the plant, including Cu, Fe, Zn, and Mn (Waters et al., 2006; Curie et al., 2009; Ishimaru et al., 2010). Transcripts for all but two of 15 putative *YSLs* were found in the total crown and rhizome transcriptome dataset (**Figure 6A**). The expression patterns of these genes were similar to those for the *NRTs* and *PHTs*, in that certain genes were upregulated at different harvest dates, suggesting both developmental and tissue regulation. Notably, four of the *YSL* genes were at higher levels at the August harvests when minerals were being remobilized from shoots. Two Arabidopsis *YSL* genes have been implicated in remobilization of Cu, Fe, and Zn (Waters et al., 2006; Waters and Grusak, 2008). The *Pavirv00003688* transcript that is most closely related to Arabidopsis *YSL2* appeared to be strongly upregulated in the crown and rhizomes harvested in October (**Figure 6A**). Arabidopsis YSL2 probably functions in the lateral movement of Fe, Cu, and Zn in tissues (Didonato et al., 2004; Schaaf et al., 2005). Some of the switchgrass *YSL* paralogs showed a bimodal expression pattern, with higher transcript abundance in May and August harvests. The orthologous Arabidopsis genes *AtYSL4* and *YSL6* are key for iron homeostasis during plastid ontogeny, thereby modulating plant responses to iron availability (Divol et al., 2013).

Plant zinc/divalent metal transporters (ZIPs) are members of a relatively large group of related genes that participate in metal transport and homeostasis (Sinclair and Kramer, 2012), including Fe (Eide et al., 1996; Vert et al., 2009), Zn (Lin et al., 2009; Milner et al., 2013), and Mn (Milner et al., 2013), with transport across plasma or vacuolar membranes (Waters and Sankaran, 2011; Milner et al., 2013). Transcripts were detected for 19 of 27 switchgrass *ZIPs* in the crown and rhizome transcriptomes (**Figure 6B**). Two *ZIP* genes that are orthologous to Arabidopsis *ZIP2* and *ZIP7* were overexpressed in the July harvests. AtZIP2 is localized on the root plasma membrane and is thought to aid in the loading of Mn and Zn into the xylem (Milner et al., 2013). Since above ground growth in plants is active in July, it is possible that the switchgrass orthologs fill a similar role. Transcripts for nine *ZIPs* were more abundant in the early (predominately May) harvest. Among these were three genes orthologous to the Arabidopsis *ZIPs* 4, 9, and 12, which are induced by Zn deprivation (Jain et al., 2013). A related switchgrass gene (*Pavirv00015988*), however, was overexpressed in crowns and rhizomes at the end of the growing season (October; **Figure 6B**). Four switchgrass zinc transporter (*ZTP*) genes with homology to the Arabidopsis *ZTP29* were upregulated in crown and rhizome tissues later in the growing season (August and October). AtZTP29 is localized to the ER and induced in roots in response to salt stress, and is thought to play a role in the unfolded protein response (Wang et al., 2010).

The distinct temporal changes in transcript abundances in the 454 dataset were validated using qRT-PCR for arbitrarily selected genes that in the 454 datasets (See **Figures 3–6**) were at higher abundance at a specific harvest date. Six transporter genes and one reference gene were analyzed by qRT-PCR using RNA from field grown plants (**Figure 7**). In 5 out of 6 genes, transcript abundance by qRT-PCR agreed with the 454 expression datasets. Transcript abundance determined by the two methods corresponded closely for four genes (**Figures 7B,D,E,F**), where a simple regression of transcript counts at each harvest date to the relative quantities of abundance by qRT-PCR yielded R^2-values between 0.69 and 0.97. For two others (**Figures 7A,C**), the R^2-values were 0.37 (**A**) and 0.21 (**C**). In the case of the *YSL* gene (**Figure 7A**), the highest abundance for both datasets was in May, but the abundances observed in the August and November harvest dates were different, resulting in the lower correlation coefficient. The *PHT* gene *Pavirv00039095* (**Figure 7C**) had a weak correlation coefficient between the 454 dataset (highest in July) and the qRT-PCR dataset (highest in November). Taken together, these data support the findings presented in **Figures 3–6**.

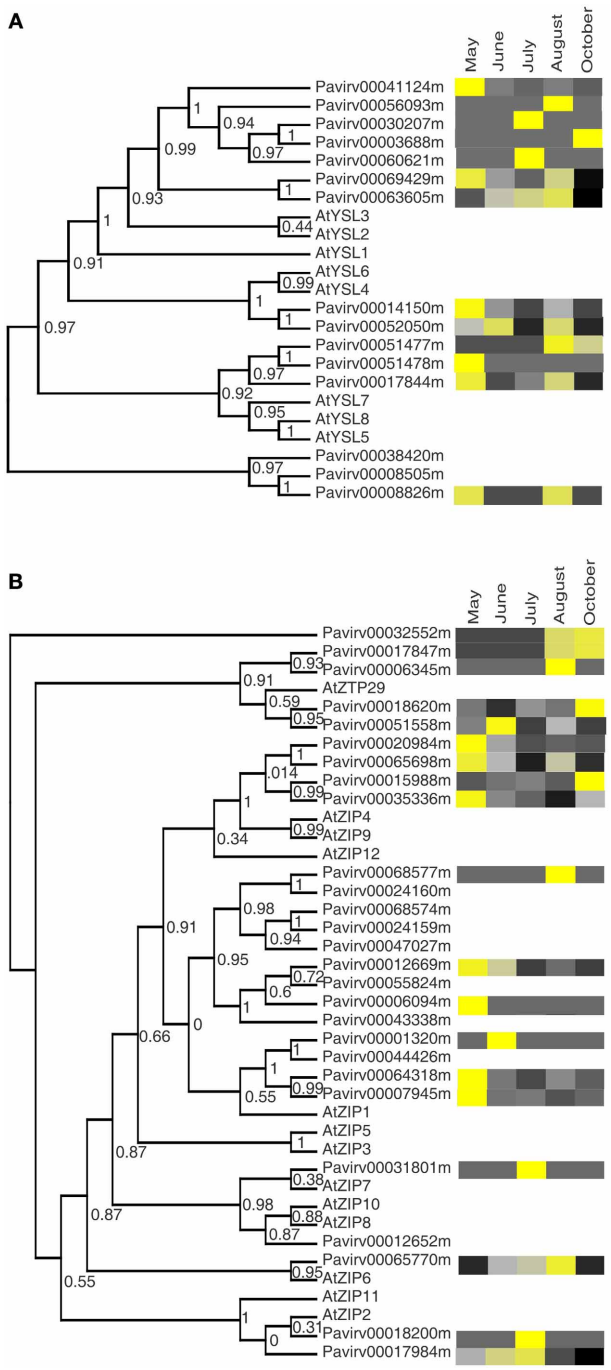

FIGURE 6 | Cladogram of the phylogentic relationships between switchgrass transporter genes to *Arabidopsis thaliana* orthologs and their expression profiles in crowns and rhizomes of field grown cv Summer plants. (A) Yellow stripe-like transporters (YSL) **(B)** Zinc transporters (ZIP). Other details are as described for **Figure 2**.

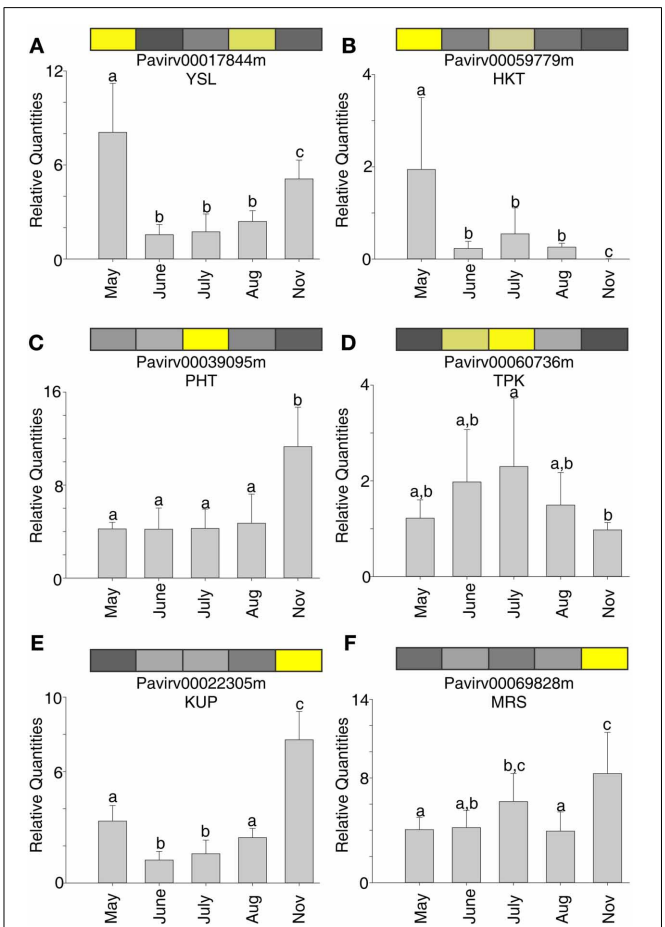

FIGURE 7 | Correspondence between expression profiles observed in NGS datasets to qRT-PCR data for six select switchgrass transporter genes. (A) YSL; **(B)** HKT; **(C)** PHT; **(D)** TPK; **(E)** KUP; **(F)** MRS. Colored bar at the top of each panel is the expression profile for each gene observed in the 454 NGS dataset (see **Figure 2**). Yellow is high expression and black is low or negligible expression. Gray bars in each panel show the relative expression ($\pm SD$) for each individual gene as determined by qRT-PCR. Different letters above each bar was significantly different expression at $P < 0.05$. See text for more details.

MINERAL DYNAMICS IN CROWN AND RHIZOME

Mineral concentrations were analyzed in crown and rhizome tissues. Of the 16 minerals analyzed, As, Fe, Na, Ni, S, and Se levels did not change significantly across harvests. The remaining 10 minerals with significant difference between any two harvest dates are shown in **Figure 8**. Excluding P and Ca, all the other minerals had lowest concentrations in crowns and rhizomes harvested in November. For K, Mg, Mn, Zn, Cu, Cd, and Co, maximal levels were detected at the August harvest, when the shoots were at an advanced stage of senescence. Highest levels of Mo were found in rhizomes harvested in June. A bimodal pattern of mineral concentrations was seen for K, Ca, P, Mg, and Mn. For K, Mg, and Mn, maximal concentrations were observed in tissues harvested in May (Mn) and June (period of active growth) and in August (shoot senescence). For Ca and P maximal amounts were observed for the June and November harvests, respectively (**Figure 8**). These fluctuating concentrations may reflect the passage of minerals through the crowns seasonally. Cu, K, and Zn are minerals known to be remobilized, and were lowest during shoot dormancy when recycled minerals would be stored in roots. Alternatively, since large fluctuations were not observed, mineral concentrations may reflect demands of the tissues themselves for growth and metabolism.

CONCLUSIONS AND FUTURE DIRECTIONS

This research represents a first step in the characterization of mineral transporter genes and associating their expression in a perennial grass. As more data for mineral dynamics becomes available, a clear picture of the genes needed for translocation to shoots in spring and to storage tissues in the fall will emerge. We have identified a number of mineral transporter genes with seasonal expression patterns that give clues to the biology of crown and rhizome tissues as a gateway between shoot growth and mineral storage and uptake. Likewise, this tissue is also a recipient of nutrients remobilized from senescing shoots. We anticipate that as additional transcriptomic and mineral datasets from other switchgrass tissues become available, these resources will be a valuable tool for plant breeders to improve production and sustainability of switchgrass.

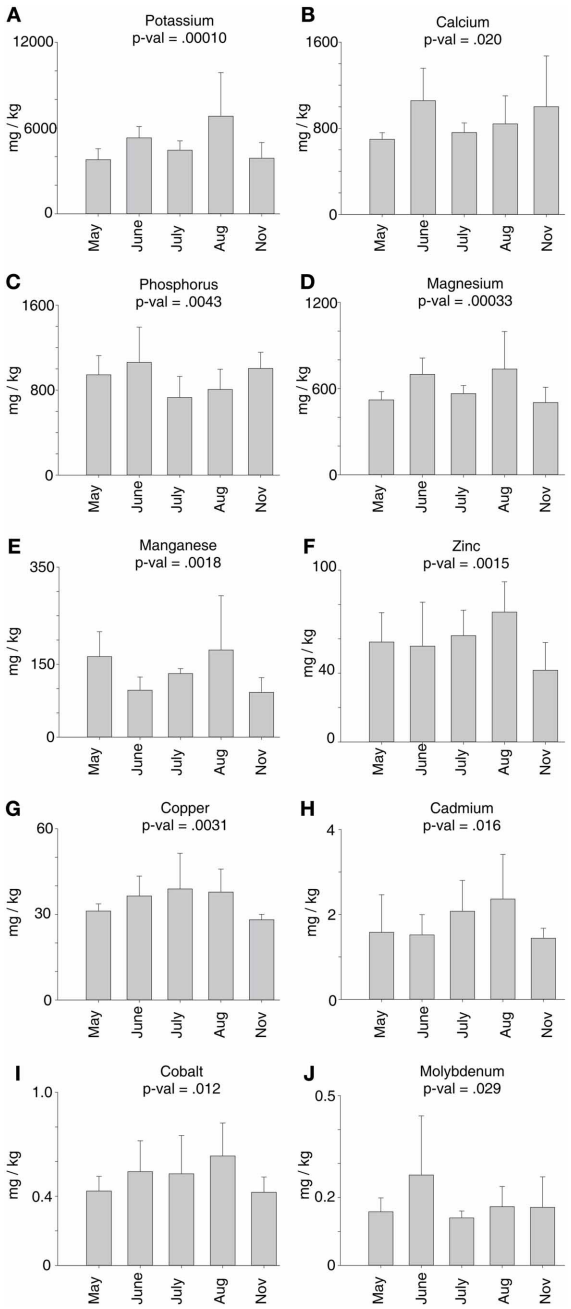

FIGURE 8 | Mineral concentrations in crowns and rhizomes of field grown cv Summer plants across harvest dates. Each panel shows the change in mineral concentration for a specific mineral. Analysis for each time point consisted of two technical replicates from each of three biological replicates ($n = 6$). Average abundances with standard deviation error bars are shown in this figure. *P*-values were calculated for each mineral using Single-Factor ANOVA. **(A)** Potassium; **(B)** Calcium; **(C)** Phosphorus; **(D)** Magnesium; **(E)** Manganese; **(F)** Zinc; **(G)** Copper; **(H)** Cadmium; **(I)** Cobalt; **(J)** Molybdenum.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00549/abstract.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Balazadeh, S., Riano-Pachon, D. M., and Mueller-Roeber, B. (2008). Transcription factors regulating leaf senescence in *Arabidopsis thaliana*. *Plant Biol. (Stuttg.)* 10(Suppl. 1), 63–75. doi: 10.1111/j.1438-8677.2008.00088.x

Bennetzen, J. L., Schmutz, J., Wang, H., Percifield, R., Hawkins, J., Pontaroli, A. C., et al. (2012). Reference genome sequence of the model plant Setaria. *Nat. Biotechnol.* 30, 555–561. doi: 10.1038/nbt.2196

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T., et al. (2003). The molecular analysis of leaf senescence–a genomics approach. *Plant Biotechnol. J.* 1, 3–22. doi: 10.1046/j.1467-7652.2003.00004.x

Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P. O., Nam, H. G., et al. (2005). Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. *Plant J.* 42, 567–585. doi: 10.1111/j.1365-313X.2005.02399.x

Chen, C. Z., Lv, X. F., Li, J. Y., Yi, H. Y., and Gong, J. M. (2012). Arabidopsis NRT1.5 is another essential component in the regulation of nitrate reallocation and stress tolerance. *Plant Physiol.* 159, 1582–1590. doi: 10.1104/pp.112.199257

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal. Biochem.* 162, 156–159. doi: 10.1016/0003-2697(87)90021-2

Couturier, J., Montanini, B., Martin, F., Brun, A., Blaudez, D., and Chalot, M. (2007). The expanded family of ammonium transporters in the perennial poplar plant. *New Phytol.* 174, 137–150. doi: 10.1111/j.1469-8137.2007.01992.x

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S. L., Briat, J. F., and Walker, E. L. (2001). Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409, 346–349. doi: 10.1038/35053080

Daram, P., Brunner, S., Persson, B. L., Amrhein, N., and Bucher, M. (1998). Functional analysis and cell-specific expression of a phosphate transporter from tomato. *Planta* 206, 225–233. doi: 10.1007/s004250050394

Dechorgnat, J., Nguyen, C. T., Armengaud, P., Jossier, M., Diatloff, E., Filleur, S., et al. (2011). From the soil to the seeds: the long journey of nitrate in plants. *J. Exp. Bot.* 62, 1349–1359. doi: 10.1093/jxb/erq409

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469. doi: 10.1093/nar/gkn180

Didonato, R. J. Jr., Roberts, L. A., Sanderson, T., Eisley, R. B., and Walker, E. L. (2004). Arabidopsis Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metal complexes. *Plant J.* 39, 403–414. doi: 10.1111/j.1365-313X.2004.02128.x

Dien, B. S., Jung, H.-J. G., Vogel, K. P., Casler, M. D., Lamb, J. F. S., Iten, L., et al. (2006). Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. *Biomass Bioenergy* 30, 880–891. doi: 10.1016/j.biombioe.2006.02.004

Divol, F., Couch, D., Conejero, G., Roschzttardtz, H., Mari, S., and Curie, C. (2013). The Arabidopsis yellow stripe LIKE4 and 6 transporters control iron release from the chloroplast. *Plant Cell* 25, 1040–1055. doi: 10.1105/tpc.112.107672

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5624–5628. doi: 10.1073/pnas.93.11.5624

El-Nashaar, H. M., Banowetz, G. M., Griffith, S. M., Casler, M. D., and Vogel, K. P. (2009). Genotypic variability in mineral composition of switchgrass. *Bioresour. Technol.* 100, 1809–1814. doi: 10.1016/j.biortech.2008.09.058

Fan, S. C., Lin, C. S., Hsu, P. K., Lin, S. H., and Tsay, Y. F. (2009). The Arabidopsis nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. *Plant Cell* 21, 2750–2761. doi: 10.1105/tpc.109.067603

Goodstein, D. M., Shu, S. Q., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., et al. (2012). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* 40, D1178–D1186. doi: 10.1093/nar/gkr944

Grabov, A. (2007). Plant KT/KUP/HAK potassium transporters: single family - multiple functions. *Ann. Bot.* 99, 1035–1041. doi: 10.1093/aob/mcm066

Guo, B., Jin, Y., Wussler, C., Blancaflor, E. B., Motes, C. M., and Versaw, W. K. (2008). Functional analysis of the Arabidopsis PHT4 family of intracellular phosphate transporters. *New Phytol.* 177, 889–898. doi: 10.1111/j.1469-8137.2007.02331.x

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19. doi: 10.1186/gb-2007-8-2-r19

Hermans, C., Vuylsteke, M., Coppens, F., Craciun, A., Inze, D., and Verbruggen, N. (2010). Early transcriptomic changes induced by magnesium deficiency in *Arabidopsis thaliana* reveal the alteration of circadian clock gene expression in roots and the triggering of abscisic acid-responsive genes. *New Phytol.* 187, 119–131. doi: 10.1111/j.1469-8137.2010.03258.x

Ishimaru, Y., Masuda, H., Bashir, K., Inoue, H., Tsukamoto, T., Takahashi, M., et al. (2010). Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. *Plant J.* 62, 379–390. doi: 10.1111/j.1365-313X.2010.04158.x

Jain, A., Sinilal, B., Dhandapani, G., Meagher, R. B., and Sahi, S. V. (2013). Effects of deficiency and excess of zinc on morphophysiological traits and spatiotemporal regulation of zinc-responsive genes reveal incidence of cross talk between micro- and macronutrients. *Environ. Sci. Technol.* 47, 5327–5335. doi: 10.1021/es400113y

Karley, A. J., and White, P. J. (2009). Moving cationic minerals to edible tissues: potassium, magnesium, calcium. *Curr. Opin. Plant Biol.* 12, 291–298. doi: 10.1016/j.pbi.2009.04.013

Kiba, T., Feria-Bourrellier, A. B., Lafouge, F., Lezhneva, L., Boutet-Mercey, S., Orsel, M., et al. (2012). The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. *Plant Cell* 24, 245–258. doi: 10.1105/tpc.111.092221

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Lemus, R., Parrish, D. J., and Wolf, D. D. (2009). Nutrient uptake by 'Alamo' switchgrass used as an energy crop. *Bioenergy Res.* 2, 37–50. doi: 10.1007/s12155-009-9032-3

Lin, Y. F., Liang, H. M., Yang, S. Y., Boch, A., Clemens, S., Chen, C. C., et al. (2009). Arabidopsis IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New Phytol.* 182, 392–404. doi: 10.1111/j.1469-8137.2009.02766.x

Liu, F., Chang, X. J., Ye, Y., Xie, W. B., Wu, P., and Lian, X. M. (2011). Comprehensive sequence and whole-life-cycle expression profile analysis of the phosphate transporter gene family in rice. *Mol. Plant* 4, 1105–1122. doi: 10.1093/mp/ssr058

Ludewig, U., Neuhauser, B., and Dynowski, M. (2007). Molecular mechanisms of ammonium transport and accumulation in plants. *FEBS Lett.* 581, 2301–2308. doi: 10.1016/j.febslet.2007.03.034

Ma, J. F., and Takahashi, E. (2002). *Soil, Fertilizer, and Plant Silicon Research in Japan*. Amsterdam; Boston: Elsevier.

Ma, J. F., Tamai, K., Yamaji, N., Mitani, N., Konishi, S., Katsuhara, M., et al. (2006). A silicon transporter in rice. *Nature* 440, 688–691. doi: 10.1038/nature04590

Ma, J. F., Yamaji, N., Mitani, N., Tamai, K., Konishi, S., Fujiwara, T., et al. (2007). An efflux transporter of silicon in rice. *Nature* 448, U209–U212. doi: 10.1038/nature05964

Ma, J. F., Yamaji, N., and Mitani-Ueno, N. (2011). Transport of silicon from roots to panicles in plants. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 87, 377–385. doi: 10.2183/pjab.87.377

Maruyama-Nakashita, A., Inoue, E., Watanabe-Takahashi, A., Yamaya, T., and Takahashi, H. (2003). Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. *Plant Physiol.* 132, 597–605. doi: 10.1104/pp.102.019802

Milner, M. J., Seamon, J., Craft, E., and Kochian, L. V. (2013). Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. *J. Exp. Bot.* 64, 369–381. doi: 10.1093/jxb/ers315

Monti, A., Bezzi, G., Pritoni, G., and Venturi, G. (2008). Long-term productivity of lowland and upland switchgrass cytotypes as affected by cutting frequency. *Bioresour. Technol.* 99, 7425–7432. doi: 10.1016/j.biortech.2008.02.034

Nabity, P. D., Orpet, R., Miresmailli, S., Berenbaum, M. R., and Delucia, E. H. (2012). Silica and nitrogen modulate physical defense against chewing insect

herbivores in bioenergy crops miscanthus × giganteus and Panicum virgatum (Poaceae). *J. Econ. Entomol.* 105, 878–883. doi: 10.1603/EC11424

Nagarajan, V. K., Jain, A., Poling, M. D., Lewis, A. J., Raghothama, K. G., and Smith, A. P. (2011). Arabidopsis Pht1;5 mobilizes phosphate between source and sink organs and influences the interaction between phosphate homeostasis and ethylene signaling. *Plant Physiol.* 156, 1149–1163. doi: 10.1104/pp.111.174805

Nour-Eldin, H. H., Andersen, T. G., Burow, M., Madsen, S. R., Jorgensen, M. E., Olsen, C. E., et al. (2012). NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature* 488, 531–534. doi: 10.1038/nature11285

Nussaume, L., Kanno, S., Javot, H., Marin, E., Pochon, N., Ayadi, A., et al. (2011). Phosphate Import in Plants: focus on the PHT1 Transporters. *Front. Plant Sci.* 2:83. doi: 10.3389/fpls.2011.00083

Palmer, N. A., Saathoff, A. J., Kim, J., Benson, A., Tobias, C. M., Twigg, P., et al. (2012). Next-generation sequencing of crown and rhizome transcriptome from an upland, tetraploid switchgrass. *Bioenergy Res.* 5, 649–661. doi: 10.1007/s12155-011-9171-1

Paterson, A. H., Bowers, J. E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., et al. (2009). The Sorghum bicolor genome and the diversification of grasses. *Nature* 457, 551–556. doi: 10.1038/nature07723

Patwardhan, P. R., Satrio, J. A., Brown, R. C., and Shanks, B. H. (2010). Influence of inorganic salts on the primary pyrolysis products of cellulose. *Bioresour. Technol.* 101, 4646–4655. doi: 10.1016/j.biortech.2010.01.112

Pilon, M., Cohu, C. M., Ravet, K., Abdel-Ghany, S. E., and Gaymard, F. (2009). Essential transition metal homeostasis in plants. *Curr. Opin. Plant Biol.* 12, 347–357. doi: 10.1016/j.pbi.2009.04.011

Ramakers, C., Ruijter, J. M., Deprez, R. H., and Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 339, 62–66. doi: 10.1016/S0304-3940(02)01423-4

Reynolds, J. H., Walker, C. L., and Kirchner, M. J. (2000). Nitrogen removal in switchgrass biomass under two harvest systems. *Biomass Bioenergy* 19, 281–286. doi: 10.1016/S0961-9534(00)00042-8

Rodriguez-Navarro, A., and Rubio, F. (2006). High-affinity potassium and sodium transport systems in plants. *J. Exp. Bot.* 57, 1149–1160. doi: 10.1093/jxb/erj068

Ruijter, J. M., Ramakers, C., Hoogaars, W. M., Karlen, Y., Bakker, O., Van Den Hoff, M. J., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37, e45. doi: 10.1093/nar/gkp045

Saathoff, A. J., Donze, T., Palmer, N. A., Bradshaw, J., Heng-Moss, T., Twigg, P., et al. (2013). Towards uncovering the roles of switchgrass peroxidases in plant processes. *Front. Plant Sci.* 4:202. doi: 10.3389/fpls.2013.00202

Sarath, G., Mitchell, R. B., Sattler, S. E., Funnell, D., Pedersen, J. F., Graybosch, R. A., et al. (2008). Opportunities and roadblocks in utilizing forages and small grains for liquid fuels. *J. Ind. Microbiol. Biotechnol.* 35, 343–354. doi: 10.1007/s10295-007-0296-3

Schaaf, G., Schikora, A., Haberle, J., Vert, G., Ludewig, U., Briat, J. F., et al. (2005). A putative function for the arabidopsis Fe-Phytosiderophore transporter homolog AtYSL2 in Fe and Zn homeostasis. *Plant Cell Physiol.* 46, 762–774. doi: 10.1093/pcp/pci081

Schwartz, C., and Amasino, R. (2013). Nitrogen recycling and flowering time in perennial bioenergy crops. *Front. Plant Sci.* 4:76. doi: 10.3389/fpls.2013.00076

Sinclair, S. A., and Kramer, U. (2012). The zinc homeostasis network of land plants. *Biochim. Biophys. Acta* 1823, 1553–1567. doi: 10.1016/j.bbamcr.2012.05.016

Sohlenkamp, C., Wood, C. C., Roeb, G. W., and Udvardi, M. K. (2002). Characterization of Arabidopsis AtAMT2, a high-affinity ammonium transporter of the plasma membrane. *Plant Physiol.* 130, 1788–1796. doi: 10.1104/pp.008599

Stacey, M. G., Patel, A., McClain, W. E., Mathieu, M., Remley, M., Rogers, E. E., et al. (2008). The Arabidopsis AtOPT3 protein functions in metal homeostasis and movement of iron to developing seeds. *Plant Physiol.* 146, 589–601. doi: 10.1104/pp.107.108183

Szczerba, M. W., Britto, D. T., and Kronzucker, H. J. (2009). K+ transport in plants: physiology and molecular biology. *J. Plant Physiol.* 166, 447–466. doi: 10.1016/j.jplph.2008.12.009

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3–new capabilities and interfaces. *Nucleic Acids Res.* 40, e115. doi: 10.1093/nar/gks596

Van Der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flugge, U. I., and Kunze, R. (2006). Transcription analysis of arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. *Plant Physiol.* 141, 776–792. doi: 10.1104/pp.106.079293

Vert, G., Barberon, M., Zelazny, E., Seguela, M., Briat, J. F., and Curie, C. (2009). Arabidopsis IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta* 229, 1171–1179. doi: 10.1007/s00425-009-0904-8

Vogel, K. P., Brejda, J. J., Walters, D. T., and Buxton, D. R. (2002). Switchgrass biomass production in the Midwest USA: harvest and nitrogen management. *Agron. J.* 94, 413–420. doi: 10.2134/agronj2002.0413

Vogel, K. P., Sarath, G., Saathoff, A. J., and Mitchell, R. B. (2011). Switchgrass. *Energy Crops* 3, 341–380. doi: 10.1039/9781849732048-00341

Wang, M., Xu, Q., Yu, J., and Yuan, M. (2010). The putative Arabidopsis zinc transporter ZTP29 is involved in the response to salt stress. *Plant Mol. Biol.* 73, 467–479. doi: 10.1007/s11103-010-9633-4

Wang, Y. Y., Hsu, P. K., and Tsay, Y. F. (2012). Uptake, allocation and signaling of nitrate. *Trends Plant Sci.* 17, 458–467. doi: 10.1016/j.tplants.2012.04.006

Waters, B. M. (2011). Moving magnesium in plant cells. *New Phytol.* 190, 510–513. doi: 10.1111/j.1469-8137.2011.03724.x

Waters, B. M., Chu, H. H., Didonato, R. J., Roberts, L. A., Eisley, R. B., Lahner, B., et al. (2006). Mutations in Arabidopsis yellow stripe-like1 and yellow stripe-like3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds. *Plant Physiol.* 141, 1446–1458. doi: 10.1104/pp.106.082586

Waters, B. M., and Grusak, M. A. (2008). Whole-plant mineral partitioning throughout the life cycle in *Arabidopsis thaliana* ecotypes Columbia, Landsberg erecta, Cape Verde Islands, and the mutant line ysl1ysl3. *New Phytol.* 177, 389–405. doi: 10.1111/j.1469-8137.2007.02288.x

Waters, B. M., McInturf, S. A., and Stein, R. J. (2012). Rosette iron deficiency transcript and microRNA profiling reveals links between copper and iron homeostasis in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 5903–5918. doi: 10.1093/jxb/ers239

Waters, B. M., and Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

Yamaji, N., and Ma, J. F. (2009). A transporter at the node responsible for intervascular transfer of silicon in rice. *Plant Cell* 21, 2878–2883. doi: 10.1105/tpc.109.069831

Yamaji, N., Mitatni, N., and Ma, J. F. (2008). A transporter regulating silicon distribution in rice shoots. *Plant Cell* 20, 1381–1389. doi: 10.1105/tpc.108.059311

Yang, J., Worley, E., Wang, M., Lahner, B., Salt, D., Saha, M., et al. (2009). Natural variation for nutrient use and remobilization efficiencies in switchgrass. *Bioenergy Res.* 2, 257–266. doi: 10.1007/s12155-009-9055-9

Yoshimoto, N., Inoue, E., Saito, K., Yamaya, T., and Takahashi, H. (2003). Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in Arabidopsis. *Plant Physiol.* 131, 1511–1517. doi: 10.1104/pp.014712

Zhu, W., Miao, Q., Sun, D., Yang, G., Wu, C., Huang, J., et al. (2012). The mitochondrial phosphate transporters modulate plant responses to salt stress via affecting ATP and gibberellin metabolism in *Arabidopsis thaliana*. *PLoS ONE* 7:e43530. doi: 10.1371/journal.pone.0043530

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Iron in seeds – loading pathways and subcellular localization

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Iron (Fe) is one of the most abundant elements on earth, but its limited bioavailability poses a major constraint for agriculture and constitutes a serious problem in human health. Due to an improved understanding of the mechanisms that control Fe homeostasis in plants, major advances toward engineering biofortified crops have been made during the past decade. Examples of successful biofortification strategies are, however, still scarce and the process of Fe loading into seeds is far from being well understood in most crop species. In particular in grains where the embryo represents the main storage compartment such as legumes, increasing the seed Fe content remains a challenging task. This review aims at placing the recently identified actors in Fe transport into the unsolved puzzle of grain filling, taking the differences of Fe distribution between various species into consideration. We summarize the current knowledge on Fe transport between symplasmic and apoplasmic compartments, and provide models for Fe trafficking and localization in different seed types that may help to develop high seed Fe germplasms.

Keywords: biofortification, grain filling, Fe transport, Fe storage, Fe in seeds

INTRODUCTION

Iron (Fe) is involved in the transport of electrons in many ubiquitous metabolic processes such as respiration and photosynthesis, and is required as a co-factor of numerous enzymes. Although highly abundant in the earth's crust, the low solubility of Fe often limits plant growth. This is largely due to the high reactivity of Fe toward oxygen: in soils, Fe tends to form highly insoluble ferric hydroxides, dramatically restricting the bioavailability of Fe. Undernourishment for Fe decreases productivity and yield, posing a major constraint for both agriculture and human health. Among the essential micronutrients, Fe is considered as the most deleterious when present in insufficient amounts (Stoltzfus, 2003; Zimmermann and Hurrell, 2007). In plant-based diets, Fe is provided as non-heme Fe that is less well absorbed than heme-bound Fe in meat, causing Fe deficiency-induced anemia (FDA) in areas where monotonous, plant-based diets are dominating (USA National Institute of Health, Office of Dietary Supplements, http://ods.od.nih.gov/factsheets/IronHealthProfessional/). Combating FDA requires strategies to increase the Fe content in crops and, as a prerequisite for the development of such strategies, a better understanding of the mechanisms that control transport of Fe to and storage of Fe in edible plant tissues, as well as the chemical forms in which Fe is present in these tissues.

Efforts toward understanding Fe uptake have been mainly focused on roots (Eide et al., 1996; Robinson et al., 1999; Curie et al., 2001; Nozoye et al., 2011). Plants are traditionally separated into two strategies by which they acquire Fe from the soil (Römheld and Marschner, 1983). Strategy I plants, which include all plants except grasses, acquire Fe after reduction of Fe^{III} chelates by a plasma membrane (PM)-bound ferric chelate reductase (FRO2 in *Arabidopsis*; Robinson et al., 1999); the resulting Fe^{II} is then taken up by a transporter of the ZIP family (Iron-Regulated Transporter1, IRT1, in *Arabidopsis*; Eide et al., 1996; Vert et al., 2002). The solubility of Fe is increased by the P-type ATPase-mediated proton excretion (AHA2 in *Arabidopsis*; Santi and Schmidt, 2009), which decreases the rhizosphere pH and increases pFe in the soil solution. Graminaceous plants take up Fe by secreting plant-borne chelators (phytosiderophores) of the mugineic acid family with high affinity to Fe^{III} via TOM1 (Nozoye et al., 2011), and the Fe^{III}-phytosiderophore complex is taken up by a YELLOW STRIPE/YELLOW STRIPE1-like (YSL) family transporter without prior reduction (Curie et al., 2001). Recent findings have faded the border between the two strategies. Iron binding compounds of the coumarin and flavin families have been identified in root exudates of *Arabidopsis* and *Medicago truncatula* (Fourcroy et al., 2013; Rodriguez-Celma et al., 2013). Similar to phytosiderophores, these compounds mobilize non-bioavailable Fe. Secretion of phenolic compounds was also observed in the strategy II plant rice (Bashir et al., 2011; Ishimaru et al., 2011). Another deviation from the initial strategy I/strategy II concept is the presence of a functional Fe^{2+} transporter (OsIRT1) in rice roots (Ishimaru et al., 2006).

The flow of Fe through the plant involves the Fe chelators nicotianamine (NA), citrate, and deoxymugineic acid (DMA), which act as chaperones to avoid precipitation and cellular damage by the formation of harmful reactive oxygen species through Fenton chemistry, as well as proteins capable of transporting either these molecules as such or their Fe chelates (Le Jean et al., 2005; Waters et al., 2006; Durrett et al., 2007; Inoue et al., 2009; Rogers et al., 2009; Yokosho et al., 2009; Nishiyama et al., 2011). Ultimately, Fe has to be transported to the places of highest demand, the photosynthetic electron transport chains in leaves,

the reproductive organs (Roschzttardtz et al., 2013), and to the seeds where Fe is stored to support embryogenesis. Despite the importance for human nutrition, the latter process is poorly understood. In this review, we are aiming at providing an update on the mechanisms that transports Fe from roots to the seed and to emphasize the current knowledge gaps in the framework of Fe transport to the seeds. We will focus on the proteins involved in this process as well as on the chemical forms of Fe they transport. Finally, we will also discuss the differences in Fe localization between seeds of various plant species and their consequences in terms of chemical speciation and nutritional properties.

IRON TRANSPORT IN THE SHOOT AND UPTAKE OF Fe INTO AERIAL PLANT PARTS

In contrast to the abundant data on root Fe uptake and its regulation (for reviews see Palmer and Guerinot, 2009; Hindt and Guerinot, 2012; Thomine and Vert, 2013), relatively little is known regarding Fe transport in shoots. Before reaching the chloroplasts and mitochondria where it is highly required, Fe has to be unloaded from the xylem, distributed to the different tissues, and transported across the PM of the sink cells. None of these mechanisms has yet been deciphered, possibly due to difficulties derived from functional redundancy of transporters involved and possible feedback loops to recalibrate Fe homeostasis in these tissues. In dicots, Fe circulates in the xylem as ferric-citrate complexes (Durrett et al., 2007; Rellan-Alvarez et al., 2010). It has been suggested that Fe uptake by shoot cells is achieved through an IRT-like transporter after reduction of Fe in Fe^{III} chelates to Fe^{II} (Jeong and Connolly, 2009; Palmer and Guerinot, 2009). A candidate for the uptake of Fe into leaf cells is the PM-localized transporter AtIRT3. The protein is highly produced in the xylem and in mesophyll cells (Lin et al., 2009), and was shown to mediate Fe uptake in *Arabidopsis* (Shanmugam et al., 2011). The Fe^{III} reduction system that is likely to co-operate with an IRT-type transporter is a ferric-chelate reductase of the FRO family (**Figure 1**). In *Arabidopsis*, the most highly expressed member of this gene family in shoots is *AtFRO6* (Wu et al., 2005; Mukherjee et al., 2006), encoding a protein located on the PM (Jeong et al., 2008). However, *fro6* knock-out lines do not display any phenotype (Jeong and Connolly, 2009), arguing against an essential function of FRO6 in leaf Fe uptake. Other members of the *FRO* family are either not expressed in shoots or localized to intracellular compartments, suggesting other, FRO-independent reduction mechanisms. Although in aerial plant parts photoreduction of Fe^{III} is likely to occur, the extent to which this process contributes to the overall reduction of Fe in leaves remains to be elucidated. Iron in ferric-citrate complexes is particularly sensitive to this process (Bienfait and Scheffers, 1992), resulting in a fast and complete reduction of Fe^{III} accompanied by degradation of citrate and a rise in pH. As Fe^{III}-citrate is the predominant form of Fe in the xylem, photoreduction may represent an important component of xylem unloading. Alternatively, Fe^{III} could also be reduced by direct reaction with molecules such as ascorbate (**Figure 1**). Ascorbic acid is present in millimolar concentrations in most tissues and in all cellular compartments including cell walls of both dicots and grasses (Rautenkranz et al., 1994; Foyer and Lelandais, 1996; Conklin et al., 2000). Ascorbic acid is able to reduce Fe^{III}-chelates *in vitro* (Grinstead, 1960; Römheld and Marschner, 1983), and its properties to facilitate Fe transport in mammalians are well described (Sayers et al., 1973; Hallberg et al., 1989; Lane and Lawen, 2008). Several studies show a strong negative correlation between Fe and ascorbic acid (AsA) concentration; therefore, Fe^{III} reduction by ascorbic acid is likely crucial for Fe transport in shoots (Zaharieva and Abadía, 2003; Urzica et al., 2012).

REMOBILIZATION AND DISTRIBUTION OF Fe

Contrary to the uptake of Fe into leaf cells, its distribution within the plants is relatively well documented. A key role in this process is played by the Fe chelator NA. Despite high Fe concentrations in leaves, the NA-deficient tomato mutant *chloronerva* develops Fe-dependent interveinal chlorosis that can be corrected by exogenous application of NA (the "normalizing factor"; Procházka and Scholz, 1984). The phenotype of *chloronerva* plants is caused by defective expression of the gene encoding NA synthase, *NAS* (Ling et al., 1999). In *Arabidopsis*, a similar phenotype was observed in the *nas4x* quadruple mutant, which harbors mutations in all four *Arabidopsis NAS* genes (Klatte et al., 2009). In *nas4x* plants, the flower, and seed Fe content were also affected, indicating a function of NA in intercellular Fe distribution (Klatte et al., 2009; Schuler et al., 2012). NA is a non-proteinogenic amino acid, structurally analogous to and a precursor for phytosiderophores of the DMA family (Noma et al., 1971). Implication of NA in Fe distribution was further demonstrated by functional characterization of the Fe-NA transporter YELLOW STRIPE1 in maize (Schaaf et al., 2004) and other members of this gene family in several species including rice and *Arabidopsis* (Le Jean et al., 2005; Waters et al., 2006; Gendre et al., 2007; Inoue et al., 2009; Ishimaru et al., 2010). In *Arabidopsis*, *AtYSL1*, and *AtYSL3* were shown to be involved in the shoot to seed translocation of Fe; loss-of-function mutants displayed a decrease of both Fe and NA in seeds (Le Jean et al., 2005; Waters et al., 2006). A similar function has been attributed to *OsYSL2* in rice (Ishimaru et al., 2010). To date, there are only two reports on Fe speciation in the phloem sap. The first study was conducted on *Ricinus communis*, revealing the presence of an Fe transport protein (RcITP; Krüger et al., 2002). A later work conducted in rice showed that DMA is the major Fe chelator in the phloem of this species, whereas NA was in fact mainly bound to zinc (Nishiyama et al., 2011).

PHLOEM LOADING AND UNLOADING OF Fe

In the current view of phloem loading, Fe is bound to NA and transported into the sieve tubes by YSL proteins (Koike et al., 2004; reviewed in Curie et al., 2009). It is, however, unclear whether the Fe transported by YSLs is localized in the cytosol or in the apoplast. The exchange of solutes between the phloem and surrounding tissues occurs either through plasmodesmata, or across the PMs of adjacent companion or vascular parenchyma cells (Lalonde et al., 2003). The later path requires Fe to be present in the cell wall. Fe-NA is unstable at the slightly acidic pH of the cell wall and Fe-citrate is the predominant form of Fe in apoplasmic environment (von Wiren et al., 1999; Rellan-Alvarez et al., 2008). In such a context, formation of the Fe-NA complex would be rate limiting for phloem loading and unloading (**Figure 1**). A major caveat to this scenario is the absence of any characterized PM-bound

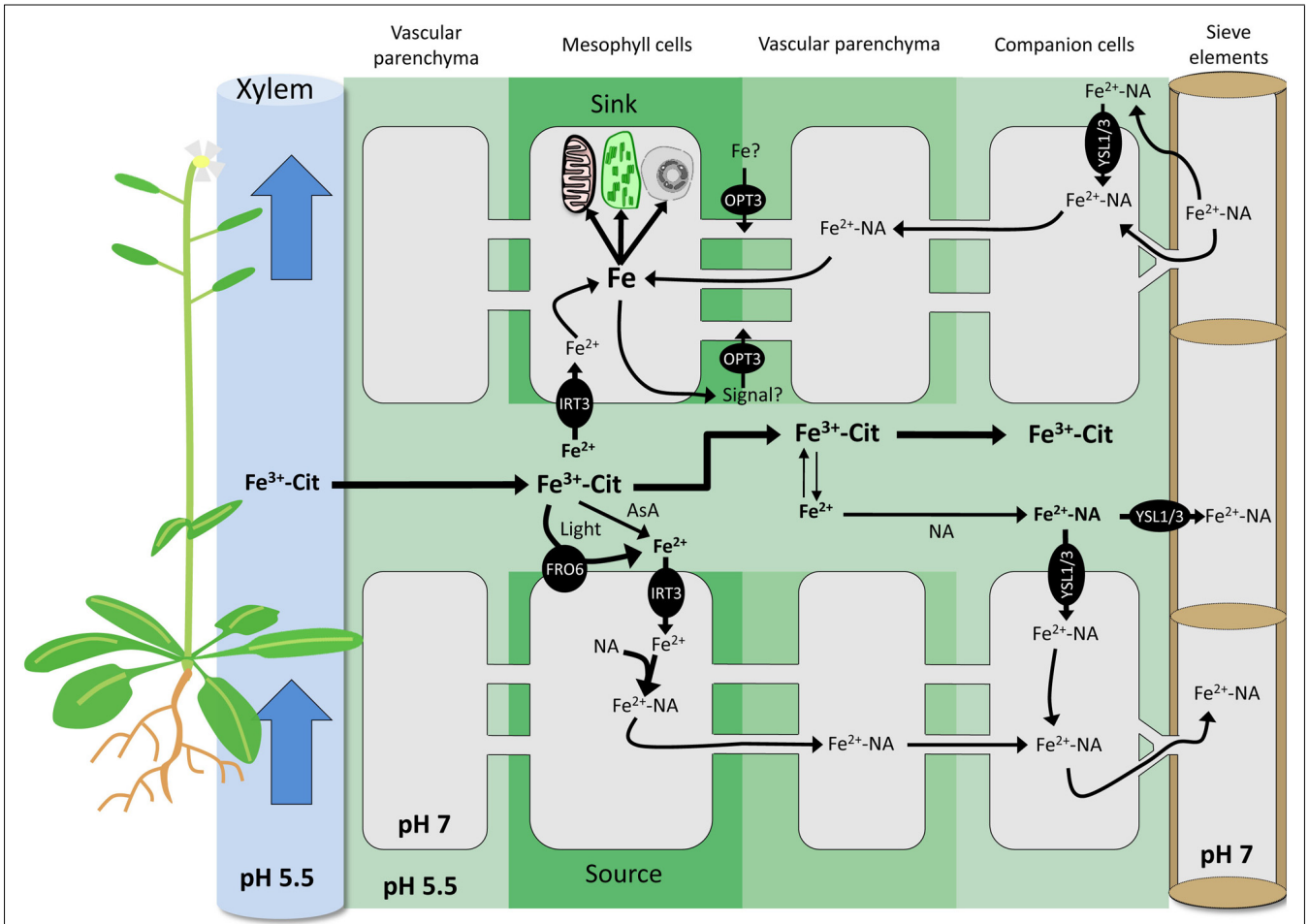

FIGURE 1 | Exchange of Fe between apoplasmic and symplasmic compartments in *Arabidopsis*. Root Fe is translocated to shoots through the xylem as Fe^{III}-citrate complexes by bulk flow. Fe^{III} can be reduced by light, extracellular AsA, and PM-bound ferric reductases of the FRO family. AtFRO6 and AtIRT3 are likely candidates to mediate Fe reduction and uptake in *Arabidopsis* shoots in both sink and source cells. In the cytoplasm, highly reactive Fe^{II} is complexed by NA. In the apoplasm, Fe^{II} is oxidized to Fe^{III} and chelated by citrate. The formation of Fe-NA complexes in the apoplasm may occur to some extent. Apoplasmic Fe-NA can be taken up by PM-localized YSL transporters. In the cytoplasm, the prevalent Fe form is Fe-NA, which can readily circulate through the symplasmic path. Fe-NA moves from cell-to-cell through plasmodesmata passively by bulk flow. In the phloem, Fe is presumably mainly present as Fe-NA. In sink organs, Fe-NA can exit the phloem via plasmodesmata, or diffuse out of sieve elements into the apoplasm. In the latter scenario, Fe is retrieved by YSLs or by AtOPT3. Iron derived both from the apoplasm and from the symplasmic pathway constitutes the intracellular Fe pool. Plants possess yet unknown sensing and signaling mechanisms to regulate the size of this pool. AtOPT3 has been also suggested to transport signaling molecules that could circulate from the shoot to the root through the phloem, thereby negatively regulating root Fe uptake. AsA, ascorbic acid; Cit, citrate; FRO, Ferric Reductase Oxidase; IRT, Iron-Regulated Transporter; NA, nicotianamine; OPT, OligoPeptide Transporter; YSL, Yellow Stripe-Like; PM, plasma membrane.

Fe efflux transporters in plants. In *Arabidopsis*, a homolog of the mammalian Fe exporter ferroportin (FPN), FPN1, was suggested to perform such a function (Morrissey et al., 2009).

Plasmodesmata function is complex and the mechanisms that determine the selectivity of the transported solutes are not clearly defined. So far, no plasmodesma-localized YSL transporter has been identified. Interestingly, such a location was demonstrated for AtOPT3 (Fernandez-Calvino et al., 2011), a member of the oligopeptide transporters (OPT) protein family with Fe transport activity in yeast (Wintz et al., 2003). Disruption of this gene severely affects cellular Fe homeostasis (Stacey et al., 2008); *opt3* knock-down lines (i.e., *opt3-2*) display constitutive overexpression of *IRT1* and *FRO2* in roots, despite of accumulation of high Fe levels in leaves. Members of the OPT family were shown to transport peptides (Lubkowitz et al., 1997; Lubkowitz et al., 1998) and AtOPT3 is thus an unlikely candidate for the transport of free Fe. Potential transport of a Fe-ligand conjugate has not been tested yet. It was hypothesized that AtOPT3 might transport signaling molecules involved in phloem loading and/or Fe sensing (Stacey et al., 2008; García et al., 2013). Iron accumulation in the distal ends of siliques, in the funiculus and in vascular tissues of the seed coat of *opt3-2* plants suggests an impairment of the unloading of Fe from the phloem rather than compromised loading. Whether AtOPT3 functions in the transport of Fe or plays other roles in Fe homeostasis, for example by transporting a signal molecule or a ligand, remains to be elucidated (**Figure 1**).

The existence of mutants with similar phenotypes in other species, such as the pea mutant *degenerative leaves* (*dgl*), which shows a constitutively activated Fe deficiency response in roots despite high Fe concentrations in leaves (Grusak and Pezeshgi, 1996), points to a conserved regulation mechanism of root Fe uptake through shoot-to-root communication. Phloem Fe is likely to be essential in this process (García et al., 2013). Thus, unraveling the loading and unloading processes would provide a comprehensive picture of plant Fe homeostasis which may reveal new targets for breeding biofortified crops.

CONTRIBUTIONS OF THE XYLEM AND THE PHLOEM TO SEED LOADING OF Fe

Iron loaded into seeds arrives either via xylem vessels or via the sieve tubes of the phloem. Both paths circulate around the seed coat. Nutrients are not directly unloaded into the endosperm (Van Dongen et al., 2003; Stadler et al., 2005), implying the need for an active and selective transport from the integument to the endosperm. The passage from the funiculus to the embryo requires at least two shifts between the symplasmic and apoplasmic path: unloading of Fe from the phloem into the endosperm and transport from the endosperm to the embryo (Patrick and Offler, 2001). Iron delivered via the xylem derives from the uptake of Fe from the rhizosphere; hence, its concentration depends directly on the expression of *IRT1* and *FRO2* (Brown and Chaney, 1971; Blair et al., 2010). This pool of Fe is readily transported as Fe^{III} chelate to the aerial parts (Durrett et al., 2007), where it is taken up by leaf cells and ultimately reaches the seed coat. Thus, xylem Fe contributes directly to both shoot Fe and seed Fe levels. Phloem Fe derives from remobilization of Fe in senescing leaves, likely present as Fe-NA complex. Therefore, the size of the phloem Fe pool is determined on one hand by remobilization mechanisms, i.e., by NA synthesis and by Fe-NA transport via YSL proteins, and on the other hand by the shoot Fe concentration, that was established by the root uptake system during the plant's life. With this in mind, it is not surprising that xylem Fe was considered as a more important contributor to seed Fe concentration, even though the ratio may greatly vary among species (Hocking and Pate, 1977). In *Arabidopsis*, it was concluded that the xylem provides 60–70% of the total seed Fe content, whereas the remaining 30–40% originates from senescing leaves, most likely via the phloem stream (Waters and Grusak, 2008). Differences between species are likely to exist, as discussed in (Stomph et al., 2009) for the case of xylem discontinuity at the base of cereal seeds. This discontinuity is absent in rice and therefore allows solutes in the xylem to flow through the seed without symplasmic unloading.

STORAGE OF Fe IN Seeds

Inside seeds, Fe is essential for embryo development (Stacey et al., 2002; Stacey et al., 2008) but might also become toxic at high concentration. Thus, Fe must be transported into embryos and stored in a stable form that can be remobilized during germination. Embryo Fe transport has rarely been addressed experimentally, although this process is highly relevant for both increasing the seed Fe content and to preserve the fitness of the embryo. Only few genes encoding proteins with functions in Fe transport within seeds have been identified. *OsYSL2* encodes a PM Fe-NA transporter and is expressed in various parts of the seed throughout its development (Koike et al., 2004). The role of OsYSL2 in the transport of Fe to the seed was further confirmed using a RNAi line (Ishimaru et al., 2010). Disruption of *AtOPT3* leads to embryo lethality and decreased expression of the gene (as observed in the *opt3-2* mutant) resulted in reduced Fe content of the embryo (Stacey et al., 2008). The substrate of AtOPT3 is unknown. The citrate efflux transporter FRD3 is expressed in the aleurone layer and in the embryo protodermis, and is known to promote Fe nutrition between symplastically disconnected tissues (Roschzttardtz et al., 2011b). For both AtOPT3 and AtFRD3 the precise function in embryo Fe uptake remains to be elucidated.

Development of elemental imaging techniques allowed accurate localization of Fe in seeds of *Arabidopsis,* rice, and pea (Kim et al., 2006; Roschzttardtz et al., 2009, 2011a; Takahashi et al., 2009; Iwai et al., 2012). The results of these studies highlighted major differences between species. In rice seeds, the highest Fe concentration was observed in aleurone layers, integument, and in the scutellum (**Figure 2A**). These tissues are discarded during processing; hence in this species, breeding focused on increasing endosperm Fe content (Goto et al., 1999; Lee et al., 2009) in order to enhance Fe bioavailability (Bashir et al., 2010). In dicots, the endosperm represents a minor portion of mature seeds, and Fe is mainly stored within the embryo. Raising the Fe content in such seeds may therefore be associated with a possible damage of the embryo by toxic Fe concentrations. To prevent such damage, plants possess two mechanisms. Iron can be stored in plastids within ferritins, which assemble as large spherical 24-mer protein complexes able to store up to 4500 Fe atoms in their internal cavities. The second mechanism consists in vacuolar sequestration.

In *Arabidopsis*, during maturation of the embryo Fe is sequestered in vacuoles of the endodermal cell layer (**Figure 2B**) through the VACUOLAR IRON TRANSPORTER1 (AtVIT1; (Kim et al., 2006), and then remobilized during germination by the NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN3 and 4 (AtNRAMP3 and AtNRAMP4; Lanquar et al., 2005; Roschzttardtz et al., 2009). Quantitatively, it has recently been shown that this particular Fe pool represents around 50% of the total Fe accumulated in *Arabidopsis* embryos (Schnell Ramos et al., 2013). The scenario is quite different in pea embryos where Fe concentration is highest in the epidermis and gradually decreases throughout the inner layers (**Figure 2C**). At the subcellular level, high Fe concentrations have been observed in nuclei and nucleoli although a quantitatively important fraction of Fe is stored in plastids, bound to ferritins (Lobréaux and Briat, 1991). This localization pattern is similar to that observed in leaves of other species (Roschzttardtz et al., 2011a) and young *Arabidopsis* embryos (Roschzttardtz et al., 2009). The diversity in Fe localization reflects different storage forms of Fe. Because of pronounced differences in bioavailability between different forms of Fe, knowledge regarding the Fe ligands is critical for an effective biofortification strategy. Iron-phytate (inositol hexakisphosphate) is an example of non-available Fe (Hallberg et al., 1989). Phytic acids bind tightly to various cations that are not readily released during digestion by mammalians. Phytates are preferentially stored in vacuolar globoids (Wada and Lott, 1997) and constitute the main Fe storage pool of *Arabidopsis* embryos (Lanquar et al., 2005). Recent research has shown that

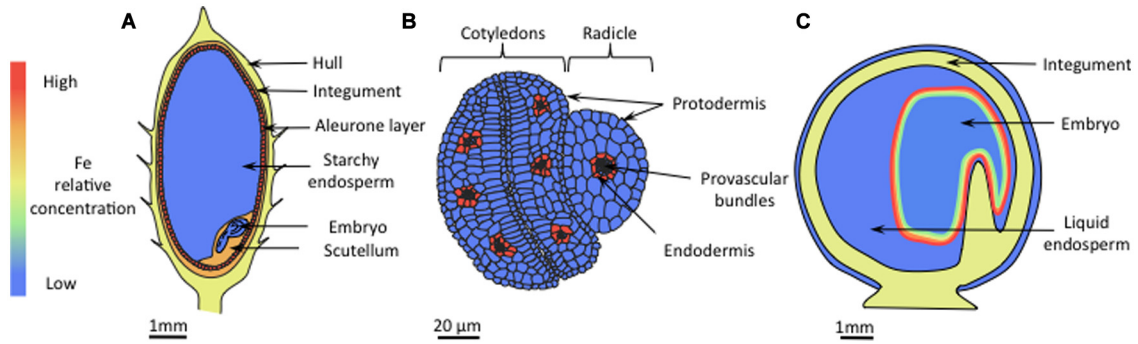

FIGURE 2 | Iron localization in seeds. (A) In rice, the concentration of Fe is highest in the aleurone layer, the integument, and the scutellum. Iron levels are also high in the hull. In the starchy endosperm the Fe content is low. **(B)** In *Arabidopsis*, Fe is mainly localized in vacuoles of endodermal cells that surround provascular tissues, whereas in other cells the Fe concentration is homogenously low. **(C)** In pea seeds, embryos constitute the main Fe storage pool, but unlike in *Arabidopsis*, Fe is localized in nuclei and plastids. The iron concentration is highest in the outer cell layers, progressively decreasing toward the inner layers.

phytate represents also the main Fe storage in the rice aleurone layer (Persson et al., 2009; Iwai et al., 2012). In legume seeds, by contrast, highly bioavailable plastidic ferritin constitutes the main storage form of Fe (Lott et al., 1984; Lobréaux and Briat, 1991; Murray-Kolb et al., 2003; Davila-Hicks et al., 2004). Ferritin was successfully used to engineer rice lines with high seed Fe (Goto et al., 1999; Wirth et al., 2009), indicating that ferritin is a good candidate for biofortification purposes. Although not considered as a natural storage form of Fe, NA was also employed to breed Fe-enriched rice varieties, and manipulating NA content represents so far the most successful attempts regarding to both content and bioavailability of Fe. The first attempt (Masuda et al., 2009) consisted in the expression of the gene encoding barley NA synthase, *HvNAS1*, in rice. Grains of lines overexpressing the NA synthase *OsNAS3* contained around 3-fold more Fe than wild-type plants and were successfully used to heal anemic mice (Lee et al., 2009). Similar results were obtained by overexpressing OsNAS2 (Lee et al., 2012) and *OsNAS1*, specifically in the endosperm (Zheng et al., 2010). In fact, constitutive expression of OsNAS genes was shown to increase the Fe content of polished grains by 2.1- to 4.2-fold (Johnson et al., 2011). Multiple transgene approaches expressing both the ferritin gene from *Phaseolus vulgaris* (*PvFERRITIN*), the *Arabidopsis* NA synthase *AtNAS1*, and the phytase from *Aspergillus fumigatus Afphytase* were successful as well, with a report of a six-fold increase in rice seed Fe content (Wirth et al., 2009). Using a different combination of transgenes (*HvNAS1*, *OsYSL2*, and *GmFERRITIN*), Masuda et al. (2012) were able to produce field-grown plants with a 4.4-fold increase in Fe content in polished seeds without yield loss. The authors concluded that for efficient biofortification introduction of multiple Fe homeostasis genes is more effective than introduction of single genes.

CONCLUSION

In the last two decades, understanding of Fe homeostasis in plants has leapt forward dramatically; molecular biology progress led to the identification and functional characterization of many genes and regulatory nodes involved in Fe transport, evolution of analytical techniques has allowed accurate determination of labile Fe species, and, more recently, elemental imaging techniques such as X-ray fluorescence provided new insights into the distribution and trafficking of Fe. Data provided by this array of techniques set the stage for producing Fe-fortified plant varieties, illustrated by the recent achievements in rice (reviewed in Sperotto et al., 2012). Critical gaps in knowledge exist regarding the mechanisms controlling Fe homeostasis in green plant parts, loading and unloading of xylem and phloem, Fe transport within seeds, and, last but not least, regarding the shoot-to-root signal adjusting root Fe uptake to the shoot demand. These questions are pending for decades, complicated by the difficulty to sample phloem sap and intracellular fluids (Kehr and Rep, 2007). Further progress in deciphering the function of several unknown Fe-responsive genes in combination with further technical progress, providing a better resolution of Fe speciation and concentration, will lead to improved strategies to generate Fe-efficient germplasms and to combat FDA.

NOTES

Since this paper was completed and accepted for publication, the role of ascorbate in iron transport by pea and *Arabidopsis* embryos was unambiguously demonstrated by Grillet et al. (2013).

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REFERENCES

Bashir, K., Ishimaru, Y., and Nishizawa, N. (2010). Iron uptake and loading into rice grains. *Rice* 3, 122–130. doi: 10.1007/s12284-010-9042-y

Bashir, K., Ishimaru, Y., Shimo, H., Kakei, Y., Senoura, T., Takahashi, R., et al. (2011). Rice phenolics efflux transporter 2 (PEZ2) plays an important role in solubilizing apoplasmic iron. *Soil Sci. Plant Nutr.* 57, 803–812. doi: 10.1080/00380768.2011.637305

Bienfait, H., and Scheffers, M. (1992). Some properties of ferric citrate relevant to the iron nutrition of plants. *Plant Soil* 143, 141–144. doi: 10.1007/BF00009139

Blair, M. W., Knewtson, S. J. B., Astudillo, C., Li, C. M., Fernandez, A. C., and Grusak, M. A. (2010). Variation and inheritance of iron reductase activity in the roots of common bean (*Phaseolus vulgaris* L.) and association with seed iron accumulation QTL. *BMC Plant Biol.* 10:215. doi: 10.1186/1471-2229-10-215

Brown, J. C., and Chaney, R. L. (1971). Effect of iron on transport of citrate into xylem of soybeans and tomatoes. *Plant Physiol.* 47, 836–840. doi: 10.1104/pp.47.6.836

Conklin, P. L., Saracco, S. A., Norris, S. R., and Last, R. L. (2000). Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. *Genetics* 154, 847–856.

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and YELLOW STRIPE 1-LIKE transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S. L., Briat, J. F., and Walker, E. L. (2001). Maize YELLOW STRIPE1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409, 346–349. doi: 10.1038/35053080

Davila-Hicks, P., Theil, E. C., and Lönnerdal, B. (2004). Iron in ferritin or in salts (ferrous sulfate) is equally bioavailable in nonanemic women. *Am. J. Clin. Nutr.* 80, 936–940.

Durrett, T. P., Gassmann, W., and Rogers, E. E. (2007). The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiol.* 144, 197–205. doi: 10.1104/pp.107.097162

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5624–5628. doi: 10.1073/pnas.93.11.5624

Fernandez-Calvino, L., Faulkner, C., Walshaw, J., Saalbach, G., Bayer, E., Benitez-Alfonso, Y., et al. (2011). *Arabidopsis* plasmodesmal proteome. *PLoS ONE* 6:e18880. doi: 10.1371/journal.pone.0018880

Fourcroy, P., Siso-Terraza, P., Sudre, D., Saviron, M., Reyt, G., Gaymard, F., et al. (2013). Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency. *New Phytol.* 201, 155–167. doi: 10.1111/nph.12471

Foyer, C. H., and Lelandais, M. (1996). A comparison of the relative rates of transport of ascorbate and glucose across the thylakoid, chloroplast and plasmalemma membranes of pea leaf mesophyll cells. *J. Plant Physiol.* 148, 391–398. doi: 10.1016/S0176-1617(96)80271-9

García, M., Romera, F., Stacey, M., Stacey, G., Villar, E., Alcántara, E., et al. (2013). Shoot to root communication is necessary to control the expression of iron-acquisition genes in Strategy I plants. *Planta* 237, 65–75. doi: 10.1007/s00425-012-1757-0

Gendre, D., Czernic, P., Conejero, G., Pianelli, K., Briat, J. F., Lebrun, M., et al. (2007). TcYSL3, a member of the YSL gene family from the hyper-accumulator *Thlaspi caerulescens*, encodes a nicotianamine-Ni/Fe transporter. *Plant J.* 49, 1–15. doi: 10.1111/j.1365-313X.2006.02937.x

Goto, F., Yoshihara, T., Shigemoto, N., Toki, S., and Takaiwa, F. (1999). Iron fortification of rice seed by the soybean ferritin gene. *Nature Biotechnol.* 17, 282–286. doi: 10.1038/7029

Grillet, L., Ouerdane, L., Hoang, M. T. T., Isaure, M. P., Lobinski, R., Curie, C., et al. (2013). Ascorbate efflux as a new strategy for iron reduction and transport in plants. *J. Biol. Chem.* doi: 10.1074/jbc.M113.514828

Grinstead, R. R. (1960). The oxidation of ascorbic acid by hydrogen peroxide - Catalysis by ethylenediaminetetraacetato-iron(III). *J. Am. Chem. Soc.* 82, 3464–3471. doi: 10.1021/ja01498a057

Grusak, M. A., and Pezeshgi, S. (1996). Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the dgl mutant of pea. *Plant Physiol.* 110, 329–334.

Hallberg, L., Brune, M., and Rossander, L. (1989). Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *Am. J. Clin. Nutr.* 49, 140–144.

Hindt, M. N., and Guerinot, M. L. (2012). Getting a sense for signals: regulation of the plant iron deficiency response. *Biochim. Biophys. Acta* 1823, 1521–1530. doi: 10.1016/j.bbamcr.2012.03.010

Hocking, P. J., and Pate, J. S. (1977). Mobilization of minerals to developing seeds of legumes. *Ann. Bot.* 41, 1259–1278.

Inoue, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., et al. (2009). Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J. Biol. Chem.* 284, 3470–3479. doi: 10.1074/jbc.M806042200

Ishimaru, Y., Kakei, Y., Shimo, H., Bashir, K., Sato, Y., Sato, Y., et al. (2011). A rice phenolic efflux transporter is essential for solubilizing precipitated apoplasmic iron in the plant stele. *J. Biol. Chem.* 286, 24649–24655. doi: 10.1074/jbc.M111.221168

Ishimaru, Y., Masuda, H., Bashir, K., Inoue, H., Tsukamoto, T., Takahashi, M., et al. (2010). Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. *Plant J.* 62, 379–390. doi: 10.1111/j.1365-313X.2010.04158.x

Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., et al. (2006). Rice plants take up iron as an Fe3+-phytosiderophore and as Fe2+. *Plant J.* 45, 335–346. doi: 10.1111/j.1365-313X.2005.02624.x

Iwai, T., Takahashi, M., Oda, K., Terada, Y., and Yoshida, K. T. (2012). Dynamic changes in the distribution of minerals in relation to phytic acid accumulation during rice seed development. *Plant Physiol.* 160, 2007–2014. doi: 10.1104/pp.112.206573

Jeong, J., Cohu, C., Kerkeb, L., Pilon, M., Connolly, E. L., and Guerinot, M. L. (2008). Chloroplast Fe(III) chelate reductase activity is essential for seedling viability under iron limiting conditions. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10619–10624. doi: 10.1073/pnas.0708367105

Jeong, J., and Connolly, E. L. (2009). Iron uptake mechanisms in plants: functions of the FRO family of ferric reductases. *Plant Sci.* 176, 709–714. doi: 10.1016/j.plantsci.2009.02.011

Johnson, A. a. T., Kyriacou, B., Callahan, D. L., Carruthers, L., Stangoulis, J., Lombi, E., et al. (2011). Constitutive overexpression of the OsNAS gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS ONE* 6:e24476. doi:10.1371/journal.pone.0024476

Kehr, J., and Rep, M. (2007). Protein extraction from xylem and phloem sap. *Methods Mol. Biol.* 355, 27–35.

Kim, S. A., Punshon, T., Lanzirotti, A., Li, L. T., Alonso, J. M., Ecker, J. R., et al. (2006). Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science* 314, 1295–1298. doi: 10.1126/science.1132563

Klatte, M., Schuler, M., Wirtz, M., Fink-Straube, C., Hell, R., and Bauer, P. (2009). The analysis of *Arabidopsis* nicotianamine synthase mutants reveals functions for nicotianamine in seed iron loading and iron deficiency responses. *Plant Physiol.* 150, 257–271. doi: 10.1104/pp.109.136374

Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori, S., et al. (2004). OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J.* 39, 415–424. doi: 10.1111/j.1365-313X.2004.02146.x

Krüger, C., Berkowitz, O., Stephan, U. W., and Hell, R. (2002). A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of *Ricinus communis* L. *J. Biol. Chem.* 277, 25062–25069. doi: 10.1074/jbc.M201896200

Lalonde, S., Tegeder, M., Throne-Holst, M., Frommer, W. B., and Patrick, J. W. (2003). Phloem loading and unloading of sugars and amino acids. *Plant Cell Environ.* 26, 37–56. doi: 10.1046/j.1365-3040.2003.00847.x

Lane, D. J. R., and Lawen, A. (2008). Non-transferrin iron reduction and uptake are regulated by transmembrane ascorbate cycling in K562 cells. *J. Biol. Chem.* 283, 12701–12708. doi: 10.1074/jbc.M800713200

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., et al. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24, 4041–4051. doi: 10.1038/sj.emboj.7600864

Le Jean, M., Schikora, A., Mari, S., Briat, J. F., and Curie, C. (2005). A loss-of-function mutation in AtYSL1 reveals its role in iron and nicotianamine seed loading. *Plant J.* 44, 769–782. doi: 10.1111/j.1365-313X.2005.02569.x

Lee, S., Jeon, U. S., Lee, S. J., Kim, Y. K., Persson, D. P., Husted, S., et al. (2009). Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22014–22019. doi: 10.1073/pnas.0910950106

Lee, S., Kim, Y. -S., Jeon, U., Kim, Y.-K., Schjoerring, J., and An, G. (2012). Activation of rice nicotianamine synthase 2 (OsNAS2) enhances iron availability for biofortification. *Mol. Cells* 33, 269–275. doi: 10.1007/s10059-012-2231-3

Lin, Y. F., Liang, H. M., Yang, S. Y., Boch, A., Clemens, S., Chen, C. C., et al. (2009). *Arabidopsis* IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New Phytol.* 182, 392–404. doi: 10.1111/j.1469-8137.2009.02766.x

Ling, H. Q., Koch, G., Baumlein, H., and Ganal, M. W. (1999). Map-based cloning of chloronerva, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7098–7103. doi: 10.1073/pnas.96.12.7098

Lobréaux, S., and Briat, J. F. (1991). Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development. *Biochem. J.* 274, 601–606.

Lott, J., Goodchild, D., and Craig, S. (1984). Studies of mineral reserves in pea (*Pisum sativum*) cotyledons using low-water-content procedures. *Funct. Plant Biol.* 11, 459–469.

Lubkowitz, M. A., Hauser, L., Breslav, M., Naider, F., and Becker, J. M. (1997). An oligopeptide transport gene from *Candida albicans*. *Microbiology* 143, 387–396. doi: 10.1099/00221287-143-2-387

Lubkowitz, M. A., Barnes, D., Breslav, M., Burchfield, A., Naider, F., and Becker, J. M. (1998). *Schizosaccharomyces pombe* isp4 encodes a transporter representing a novel family of oligopeptide transporters. *Mol. Microbiol.* 28, 729–741. doi: 10.1046/j.1365-2958.1998.00827.x

Masuda, H., Usuda, K., Kobayashi, T., Ishimaru, Y., Kakei, Y., Takahashi, M., et al. (2009). Overexpression of the barley nicotianamine synthase gene HvNAS1 increases iron and zinc concentrations in rice grains. *Rice* 2, 155–166. doi: 10.1007/s12284-009-9031-1

Masuda, H., Ishimaru, Y., Sann Aung, M., Kobayashi, T., Kakei, Y., Takahashi, M., et al. (2012). Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci. Rep.* 2:543. doi: 10.1038/srep 00543

Morrissey, J., Baxter, I. R., Lee, J., Li, L. T., Lahner, B., Grotz, N., et al. (2009). The ferroportin metal efflux proteins function in iron and cobalt homeostasis in *Arabidopsis*. *Plant Cell* 21, 3326–3338. doi: 10.1105/tpc.109. 069401

Mukherjee, I., Campbell, N. H., Ash, J. S., and Connolly, E. L. (2006). Expression profiling of the *Arabidopsis* ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper. *Planta* 223, 1178–1190. doi: 10.1007/s00425-005-0165-0

Murray-Kolb, L. E., Welch, R., Theil, E. C., and Beard, J. L. (2003). Women with low iron stores absorb iron from soybeans. *Am. J. Clin. Nutr.* 77, 180–184.

Nishiyama, R., Kato, M., Nagata, S., Yanagisawa, S., and Yoneyama, T. (2011). Identification of Zn-nicotianamine and Fe-2'-deoxymugineic acid in the phloem sap from rice plants (*Oryza sativa* L.). *Plant Cell Physiol.* 53, 381–390. doi: 10.1093/pcp/pcr188

Noma, M., Noguchi, M., and Tamaki, E. (1971). New amino acid, nicotianamine, from tobacco leaves. *Tetrahedron Lett. 12,* 2017–2020. doi: 10.1016/S0040-4039(01)96769-3

Nozoye, T., Nagasaka, S., Kobayashi, T., Takahashi, M., Sato, Y., Uozumi, N., et al. (2011). Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. *J. Biol. Chem.* 286, 5446–5454. doi: 10.1074/jbc.M110.180026

Palmer, C. M., and Guerinot, M. L. (2009). Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nat. Chem. Biol.* 5, 333–340. doi: 10.1038/nchembio.166

Patrick, J. W., and Offler, C. E. (2001). Compartmentation of transport and transfer events in developing seeds. *J. Exp. Bot.* 52, 551–564. doi: 10.1093/jexbot/52.356.551

Persson, D. P., Hansen, T. H., Laursen, K. H., Schjoerring, J. K., and Husted, S. (2009). Simultaneous iron, zinc, sulfur and phosphorus speciation analysis of barley grain tissues using SEC-ICP-MS and IP-ICP-MS. *Metallomics* 1, 418–426. doi: 10.1039/b905688b

Procházka, Ž., and Scholz, G. (1984). Nicotianamine, the 'normalizing factor' for the auxotroph tomato mutant Chloronerva; a representative of a new class of plant effectors. *Experientia* 40, 794–801. doi: 10.1007/BF01951961

Rautenkranz, A. A. F., Li, L. J., Machler, F., Martinoia, E., and Oertli, J. J. (1994). Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (*Hordeum vulgare* cv Gerbel) leaves. *Plant Physiol.* 106, 187–193.

Rellan-Alvarez, R., Abadia, J., and Alvarez-Fernandez, A. (2008). Formation of metal-nicotianamine complexes as affected by pH, ligand exchange with citrate and metal exchange. a study by electrospray ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom* 22, 1553–1562. doi: 10.1002/rcm.3523

Rellan-Alvarez, R., Giner-Martinez-Sierra, J., Orduna, J., Orera, I., Rodriguez-Castrillon, J. A., Garcia-Alonso, J. I., et al. (2010). Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol.* 51, 91–102. doi: 10.1093/pcp/pcp170

Robinson, N. J., Procter, C. M., Connolly, E. L., and Guerinot, M. L. (1999). A ferric-chelate reductase for iron uptake from soils. *Nature* 397, 694–697. doi: 10.1038/17800

Rodriguez-Celma, J., Lin, W. D., Fu, G. M., Abadia, J., Lopez-Millan, A. F., and Schmidt, W. (2013). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by *Arabidopsis* and *Medicago truncatula*. *Plant Physiol.* 162, 1473–1485. doi: 10.1104/pp.113. 220426

Rogers, E. E., Wu, X., Stacey, G., and Nguyen, H. T. (2009). Two MATE proteins play a role in iron efficiency in soybean. *J. Plant Physiol.* 166, 1453–1459. doi: 10.1016/j.jplph.2009.02.009

Römheld, V., and Marschner, H. (1983). Mechanism of iron uptake by peanut plants - I. Fe(III) reduction, chelate splitting, and release of phenolics. *Plant Physiol.* 71, 949–954.

Roschzttardtz, H., Conéjéro, G., Curie, C., and Mari, S. (2009). Identification of the endodermal vacuole as the iron storage compartment in the *Arabidopsis* embryo. *Plant Physiol.* 151, 1–10. doi: 10.1104/pp.109.144444

Roschzttardtz, H., Conèjèro, G., Divol, F., Alcon, C., Verdeil, J. -L., Curie, C., et al. (2013). New insights into Fe localization in plant tissues. *Front. Plant Sci.* 4, 350. doi: 10.3389/fpls.2013.00350

Roschzttardtz, H., Grillet, L., Isaure, M. P., Conejero, G., Ortega, R., Curie, C., et al. (2011a). Plant cell nucleolus as a hot spot for iron. *J. Biol. Chem.* 286, 27863–27866. doi: 10.1074/jbc.C111.269720

Roschzttardtz, H., Séguéla-Arnaud, M., Briat, J. -F., Vert, G., and Curie, C. (2011b). The FRD3 citrate effluxer promotes iron nutrition between symplastically disconnected tissues throughout *Arabidopsis* development. *Plant Cell* 23, 2725–2737. doi: 10.1105/tpc.111.088088

Santi, S., and Schmidt, W. (2009). Dissecting iron deficiency-induced proton extrusion in *Arabidopsis* roots. *New Phytol.* 183, 1072–1084. doi: 10.1111/j.1469-8137.2009.02908.x

Sayers, M. H., Lynch, S. R., Jacobs, P., Charlton, R. W., Bothwell, T. H., Walker, R. B., et al. (1973). The Effects of ascorbic acid supplementation on the absorption of iron in maize, wheat and soya. *Br. J. Haematol.* 24, 209–218. doi: 10.1111/j.1365-2141.1973.tb05741.x

Schaaf, G., Ludewig, U., Erenoglu, B. E., Mori, S., Kitahara, T., and Von Wiren, N. (2004). ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *J. Biol. Chem.* 279, 9091–9096. doi: 10.1074/jbc.M311799200

Schnell Ramos, M., Khodja, H., Mary, V., and Thomine, S. (2013). Using μPIXE for quantitative mapping of metal concentration in *Arabidopsis thaliana* seeds. *Front. Plant Sci.* 4:168. doi: 10.3389/fpls.2013.00168

Schuler, M., Rellàn-Alvarez, R., Fink-Straube, C., Abadia, J., and Bauer, P. (2012). Nicotianamine functions in the phloem-based transport of iron to sink organs, in pollen development and pollen tube growth in *Arabidopsis*. *Plant Cell* 24, 2380–2400. doi: 10.1105/tpc.112.099077

Shanmugam, V., Lo, J. C., Wu, C. L., Wang, S. L., Lai, C. C., Connolly, E. L., et al. (2011). Differential expression and regulation of iron-regulated metal transporters in *Arabidopsis halleri* and *Arabidopsis thaliana* - The role in zinc tolerance. *New Phytol.* 190, 125–137. doi: 10.1111/j.1469-8137.2010.03606.x

Sperotto, R. A., Ricachenevsky, F. K., Waldow, V. D. A., and Fett, J. P. (2012). Iron biofortification in rice: it's a long way to the top. *Plant Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Stacey, M. G., Koh, S., Becker, J., and Stacey, G. (2002). AtOPT3, a member of the oligopeptide transporter family, is essential for embryo development in *Arabidopsis*. *Plant Cell* 14, 2799–2811. doi: 10.1105/tpc.005629

Stacey, M. G., Patel, A., Mcclain, W. E., Mathieu, M., Remley, M., Rogers, E. E., et al. (2008). The *Arabidopsis* AtOPT3 protein functions in metal homeostasis and movement of iron to developing seeds. *Plant Physiol.* 146, 589–601. doi: 10.1104/pp.107.108183

Stadler, R., Lauterbach, C., and Sauer, N. (2005). Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in *Arabidopsis* seeds and embryos. *Plant Physiol.* 139, 701–712. doi: 10.1104/pp.105.065607

Stoltzfus, R. J. (2003). Iron deficiency: global prevalence and consequences. *Food Nutr. Bull.* 24, 99–103.

Stomph, J. T., Jiang, W., and Struik, P. C. (2009). Zinc biofortification of cereals: rice differs from wheat and barley. *Trends Plant Sci.* 14, 123–124. doi: 10.1016/j.tplants.2009.01.001

Takahashi, M., Nozoye, T., Kitajima, N., Fukuda, N., Hokura, A., Terada, Y., et al. (2009). In vivo analysis of metal distribution and expression of metal transporters in rice seed during germination process by microarray and X-ray fluorescence

imaging of Fe, Zn, Mn, and Cu. *Plant Soil* 325, 39–51. doi: 10.1007/s11104-009-0045-7

Thomine, S., and Vert, G. (2013). Iron transport in plants: better be safe than sorry. *Curr. Opin. Plant Biol.* 16, 322–327. doi: 10.1016/j.pbi.2013.01.003

Urzica, E. I., Casero, D., Yamasaki, H., Hsieh, S. I., Adler, L. N., Karpowicz, S. J., et al. (2012). Systems and trans-system level analysis identifies conserved iron deficiency responses in the plant lineage. *Plant Cell* 24, 3921–3948. doi: 10.1105/tpc.112.102491

Van Dongen, J. T., Ammerlaan, A. M. H., Wouterlood, M., Van Aelst, A. C., and Borstlap, A. C. (2003). Structure of the developing pea seed coat and the post-phloem transport pathway of nutrients. *Ann. Bot.* 91, 729–737. doi: 10.1093/aob/mcg066

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., et al. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

von Wiren, N., Klair, S., Bansal, S., Briat, J. F., Khodr, H., Shioiri, T., et al. (1999). Nicotianamine chelates both Fe-III and Fe-II. Implications for metal transport in plants. *Plant Physiol.* 119, 1107–1114. doi: 10.1104/pp.119.3.1107

Wada, T., and Lott, J. N. A. (1997). Light and electron microscopic and energy dispersive X-ray microanalysis studies of globoids in protein bodies of embryo tissues and the aleurone layer of rice (*Oryza sativa* L.) grains. *Can. J. Bot.* 75, 1137–1147. doi: 10.1139/b97-125

Waters, B. M., Chu, H. H., Didonato, R. J., Roberts, L. A., Eisley, R. B., Lahner, B., et al. (2006). Mutations in *Arabidopsis* YELLOW STRIPE-LIKE1 and YELLOW STRIPE-LIKE3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds. *Plant Physiol.* 141, 1446–1458. doi: 10.1104/pp.106.082586

Waters, B. M., and Grusak, M. A. (2008). Whole-plant mineral partitioning throughout the life cycle in *Arabidopsis thaliana* ecotypes Columbia, Landsberg erecta, Cape Verde Islands, and the mutant line ysl1ysl3. *New Phytol.* 177, 389–405.

Wintz, H., Fox, T., Wu, Y. Y., Feng, V., Chen, W. Q., Chang, H. S., et al. (2003). Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *J. Biol. Chem.* 278, 47644–47653. doi: 10.1074/jbc.M309338200

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., et al. (2009). Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnol. J.* 7, 631–644. doi: 10.1111/j.1467-7652.2009.00430.x

Wu, H. L., Li, L. H., Du, J., Yuan, Y. X., Cheng, X. D., and Ling, H. Q. (2005). Molecular and biochemical characterization of the Fe(III) chelate reductase gene family in *Arabidopsis thaliana*. *Plant Cell Physiol.* 46, 1505–1514. doi: 10.1093/pcp/pci163

Yokosho, K., Yamaji, N., Ueno, D., Mitani, N., and Ma, J. F. (2009). OsFRDL1 is a citrate transporter required for efficient translocation of iron in rice. *Plant Physiol.* 149, 297–305. doi: 10.1104/pp.108.128132

Zaharieva, T. B., and Abadía, J. (2003). Iron deficiency enhances the levels of ascorbate, glutathione, and related enzymes in sugar beet roots. *Protoplasma* 221, 269–275.

Zheng, L. Q., Cheng, Z. Q., Ai, C. X., Jiang, X. H., Bei, X. S., Zheng, Y., et al. (2010). Nicotianamine, a novel enhancer of rice iron bioavailability to humans. *PLoS ONE* 5:e10190. doi:10.1371/journal.pone.0010190

Zimmermann, M. B., and Hurrell, R. F. (2007). Nutritional iron deficiency. *Lancet* 370, 511–520. doi: 10.1016/S0140-6736(07)61235-5

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Autophagy as a possible mechanism for micronutrient remobilization from leaves to seeds

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Seed formation is an important step of plant development which depends on nutrient allocation. Uptake from soil is an obvious source of nutrients which mainly occurs during vegetative stage. Because seed filling and leaf senescence are synchronized, subsequent mobilization of nutrients from vegetative organs also play an essential role in nutrient use efficiency, providing source-sink relationships. However, nutrient accumulation during the formation of seeds may be limited by their availability in source tissues. While several mechanisms contributing to make leaf macronutrients available were already described, little is known regarding micronutrients such as metals. Autophagy, which is involved in nutrient recycling, was already shown to play a critical role in nitrogen remobilization to seeds during leaf senescence. Because it is a non-specific mechanism, it could also control remobilization of metals. This article reviews actors and processes involved in metal remobilization with emphasis on autophagy and methodology to study metal fluxes inside the plant. A better understanding of metal remobilization is needed to improve metal use efficiency in the context of biofortification.

Keywords: transition metal, isotopic labeling, nutrient use efficiency, leaf senescence, nutrient fluxes, *atg*, Fe, Zn

INTRODUCTION

Micronutrients, such as metals, are essentials for cell functions. Zinc (Zn), which exists only as divalent cation, plays an important role in protein structure and function thank to its Lewis acids properties. Transition metals such as iron (Fe), copper (Cu), or manganese (Mn), which have unpaired electrons that promote their involvement in oxido-reduction reactions, are used in a wealth of biological processes (Pierre and Fontecave, 1999). A third of the proteins characterized at the structural level are metalloproteins, highlighting the need of metals for cell functions (Finney and O'Halloran, 2003).

In plants, transition metal functions are mainly associated to energy production mechanisms, thereby about 80% of Fe in mesophyll cell is localized in chloroplasts (Nouet et al., 2011). Fe is essential for chlorophyll synthesis, nitrogen fixation, DNA replication, reactive oxygen species (ROS) detoxification, and electron transport chain in both mitochondria and chloroplasts (Nouet et al., 2011; Yruela, 2013). Mn plays a central role in the photosystem II (PS II) where it catalyzes water oxidation (Tommos et al., 1998). This element is also involved in sugar metabolism, Mn-superoxide dismutase (SOD), and chloroplastic enzymes such as decarboxylases and dehydrogenases (Luk and Culotta, 2001; Horsburgh et al., 2002; Aggarwal et al., 2012). Cu is integrated into plastocyanines involved in electron transfer of chloroplasts (Yruela, 2013). It plays also an essential role in the cytochrome oxidase of mitochondria (Bleackley and Macgillivray, 2011). Zn is required for carbon fixation through the carbonic anhydrase (Badger and Price, 1994). It is also needed for the Cu/Zn-SOD, transcriptional regulation by zinc-finger DNA binding proteins and for the turnover of PSII in chloroplasts (Kurepa et al., 1997; Bleackley and Macgillivray, 2011; Lu et al., 2011). Therefore, plants need metals to achieve vital functions in all their organs.

Among all plant organs, seed is a special one because it has to store metals required for germination and during the first days of seedling development. Hence in annual plants, seed formation is a crucial step in which plant sacrifices itself to store nutrients for its offspring. Seed filling depends on nutrient originating from *de novo* uptake by roots or remobilization from senescent organs.

Here, we review genes and processes involved in metal remobilization during seed filling. We will discuss methodologies that can be used to study metal fluxes in plants and thereby determine the relative contribution of uptake and remobilization pathways. Autophagy is a ubiquitous process involved in cellular nutrient recycling. Because it was recently shown to play a critical role in nitrogen remobilization (Htwe et al., 2011; Guiboileau et al., 2012), this review focuses on autophagy as a potential mechanism to make metal available for subsequent remobilization during senescence.

ORIGIN OF SEED METALS: UPTAKE FROM SOIL VS REMOBILIZATION FROM SENESCENT TISSUES

CIRCULATION OF METALS INTO THE PLANT AND MICRONUTRIENT USE EFFICIENCY

Understanding metal seed filling requires knowledge on the general micronutrient pathways which was already summarized in several recent reviews (Pittman, 2005; Palmgren et al., 2008; Morrissey and Guerinot, 2009; Puig and Peñarrubia, 2009; Yruela,

2009; Pilon, 2011; Waters and Sankaran, 2011; Thomine and Vert, 2013).

On the whole, both uptake from soil and remobilization from senescent organs may participate in metal loading in seeds (**Figure 1**). To date, little is known about the contribution of metal remobilization from senescent organs to seed filling. In contrast, this topic is well documented regarding nitrogen. It was shown that uptake and fixation of nitrogen dramatically decrease at the onset of reproductive stage in cereals, oilseed rape and legumes (Salon et al., 2011). Accordingly, 50 to 90% of nitrogen grain of rice, wheat, or maize originate from leaf remobilization (Masclaux et al., 2001). This highlights that the importance of nitrogen remobilization for seed filling is conserved in most plants. However, some species, such as oilseed rape, have a low nitrogen remobilization capacity resulting in low nitrogen use efficiency (Schjoerring et al., 1995; Etienne et al., 2007).

As for nitrogen, it is necessary to better understand metal remobilization from senescent organs during seed filling with the aim to increase micronutrient use efficiency in the context of intensive agriculture, fertilization limitations, and biofortification. This is especially important as metal availability may become limiting under certain environmental conditions (drought, low temperature) and soil characteristics (low metal content, high salt content, ionic unbalance, low pH, high bicarbonate concentration; Chen and Barak, 1982; Karamanos et al., 1986; Graham, 1988; Alloway, 2009).

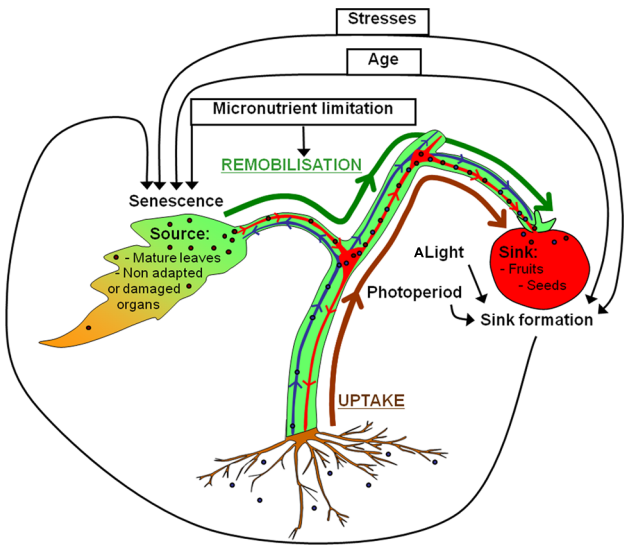

FIGURE 1 | Uptake and remobilization pathways involved in seed filling with emphasis on source-sink relationships. Micronutrients from the rhizosphere (brown arrow) are taken up into roots and transported to the xylem vessels (shown in blue). After xylem loading, micronutrients are translocated into shoots for subsequent unloading. Micronutrients located in the xylem can also be unloaded into the xylem parenchyma of nodes to be transferred to phloem vessels (shown in red) by specific transporters (Sondergaard et al., 2004; Tanaka et al., 2008; Yamaji and Ma, 2009). This is essential for seed filling which is only achieved by the phloem sap (Patrick and Offler, 2001). Phloem micronutrients are unloaded to fill seeds. Because seed filling is also achieved by nutrient remobilized from senescent tissues (green arrow), seed formation requires close synchronization between sink formation and source organ senescence. Age, biotic and abiotic stresses contribute to orchestrate nutrient mobilization during leaf senescence with the formation of reproductive organs and seed filling (black arrows). Light and photoperiod act indirectly on leaf senescence by stimulating the development of the reproductive organs.

METHODOLOGIES TO DETERMINE NUTRIENT FLUX

The most common way to study nutrient fluxes within the plant is to determine the "apparent remobilization" which consists in the measurement of the total amount of element of interest present in different plant organs at different times (Masclaux-Daubresse et al., 2010). However, this approach does not provide sufficient resolution and does not allow distinguishing nutrients coming from different pathways, such as nutrient uptake from soil and nutrient remobilization from senescent leaves.

The most appropriate approach to study short-term accumulation, uptake from soil and fluxes between tissues is the use of isotopes as tracers. Isotopic labeling can be implemented with different protocols (Grusak, 1994; Wu et al., 2010; Erenoglu et al., 2011; Hegelund et al., 2012).

Metal fluxes may be monitored by pulse-chase labeling using radioactive or stable isotopes. The ^{59}Fe, ^{65}Zn, and ^{68}Zn radioisotopes have been used for pulse labeling on specific organs followed by a chase period to facilitate the identification of source organs contributing to seed filling in peas, wheat and rice (Grusak, 1994; Wu et al., 2010; Erenoglu et al., 2011; Zheng et al., 2012). Following this approach, it was demonstrated that nutrient supply can affect Zn remobilization in wheat (Erenoglu et al., 2011). In rice, differences in Zn remobilization efficiency between genotypes were observed using isotopic pulse-chase on specific organs (Wu et al., 2010).

Recently, pulse labeling using very short life ß$^+$ radioisotope like ^{52}Fe, ^{52}Mn, and ^{62}Zn has been used to image metal fluxes within a plant *via* a real-time and non-destructive technique called Positron-Emitting Tracer Imaging System (Kume et al., 1997; Tsukamoto et al., 2006; Tsukamoto et al., 2009).

Non-radioactive isotope is also used for pulse labeling on specific organs. Application of ^{65}Cu to one individual leaf of rice allowed to study Cu redistribution between the different leaves during vegetative stage (Zheng et al., 2012). Non-radioactive isotopes can be also added in the nutrient solution for labeling plants early during development in order to monitor nutrient movement during vegetative stages or later at reproductive stage to study remobilization and seed filling. Using Zn isotopes, this pulse-chase approach has been used to quantify the effect of nutrient limitation on Zn fluxes between organs in rice and wheat (Wu et al., 2010; Erenoglu et al., 2011). Moreover, ^{70}Zn pulse-chase labeling combined with laser ablation-inductively coupled plasma-mass spectrometry has provided a spatial distribution of Zn within wheat seeds revealing zinc transport barriers during grain filling in wheat (Wang et al., 2010).

Long term labeling in nutrient solution may be performed to address the contribution of uptake from soil to organs during a specific developmental stage, with respect to the contribution of endogenous remobilization. Continuous application of ^{68}Zn provided evidence that Zn uptake before anthesis contributes to

more than 50% to the total Zn grain content in rice (Wu et al., 2010). Shorter continuous labeling can also be used to determine the uptake capacity by measuring isotope accumulation in roots (Hegelund et al., 2012) or isotope depletion in the nutritive solution (Erenoglu et al., 2011).

Isotopic labeling is an essential tool to study metal fluxes within the plant but require the availability of enriched isotopes and adequate analytical tools. Initially, isotopic labeling was mainly performed using radioactive isotope despite the risk for humans. Nowadays, enriched stable isotopes are more and more accessible at least for Fe, Ni, Cu, Zn, and Mo. They represent a healthier and less restrictive alternative but their analysis requires the use of mass spectrometry, such as inductively coupled plasma-mass spectrometry.

THE COUPLING BETWEEN SENESCENCE AND MICRONUTRIENT REMOBILIZATION

CONTROL OF SENESCENCE AND REMOBILIZATION AT THE WHOLE PLANT LEVEL

Senescence is an active process controlled by age whereby sink tissues performing photosynthesis and anabolism become source tissues undergoing catabolism (**Figure 2**). Senescence makes nutrients available for further plant organs (Hörtensteiner and Feller, 2002), contributing to nutrient use efficiency. Optimal remobilization requires close synchronization between sink formation and source organ senescence (**Figure 1**). It was observed that the removal of sink tissues delays senescence in oilseed rape, soybean and wheat and decrease nitrogen remobilization in oilseed rape and soybean (Patterson and Brun, 1980; Crafts-Brandner and Egli, 1987; Noquet et al., 2004; Htwe et al., 2011). However, senescence and remobilization are also controlled by other parameters such as nutrient availability (**Figure 1**). In *Arabidopsis*, nitrogen limitation triggers leaf senescence (Lemaître et al., 2008). In wheat, remobilization of Fe and Zn from flag leaves to seeds is increased under nutrient-limiting conditions (Waters et al., 2009; Wu et al., 2010; Sperotto et al., 2012b). Conversely continuous nutrient uptake during seed formation may account for low nutrient remobilization in some species (Masclaux-Daubresse and Chardon, 2011; Waters and Sankaran, 2011). However, an opposite behavior was observed in barley plants for which remobilization increased upon high Zn supply. This illustrates the diversity of Zn management at the whole plant level (Hegelund et al., 2012). Moreover, other abiotic and biotic stresses such as pathogen attack, high salinity, drought, low temperature, modifications of light intensity, and quality can also cause premature senescence and remobilization (Nooden et al., 1996; Buchanan-Wollaston, 1997; Gan and Amasino, 1997). Because they are sessile, plants developed high plasticity to respond to environment conditions, triggering cell death and remobilization in order to save nutrients and produce more adapted organs and tissues.

CONTROL OF SENESCENCE AND REMOBILIZATION AT THE MOLECULAR LEVEL

Transcript analysis, comparing green and senescing leaves, led to the identification of senescence-associated genes (SAG) in different species (Hensel et al., 1993; Buchanan-Wollaston, 1994; Smart et al., 1995; Guo et al., 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Breeze et al., 2011). Irrevocably, the expression of genes encoding cysteine proteases is strongly induced in senescent leaves (Hensel et al., 1993; Smart et al., 1995; Bhalerao et al., 2003; Andersson et al., 2004; Guo

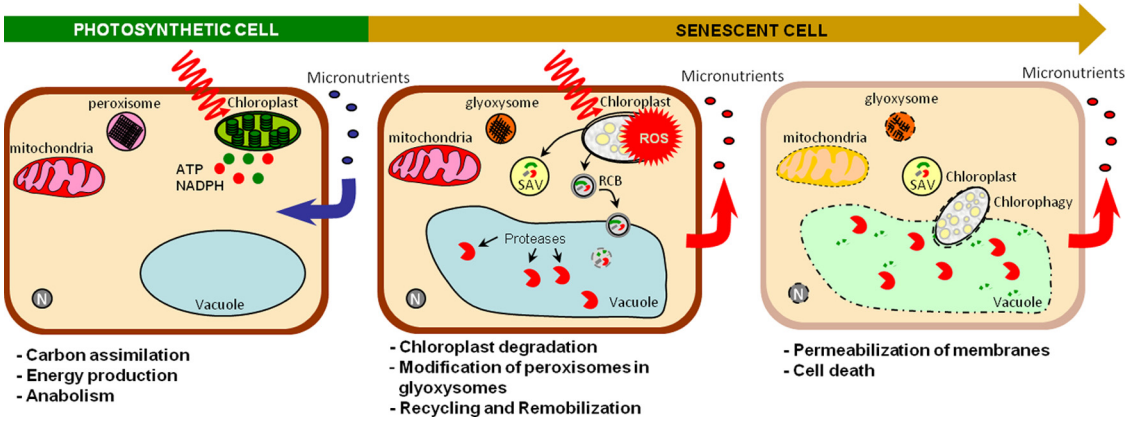

FIGURE 2 | Sink/source transition at the cellular level. Active photosynthetic cells perform carbon fixation, energy production and anabolism and require micronutrients for these functions. Senescence modifies these sink cells into a source cells undergoing catabolism. Intense catabolism activities and nutrient recycling occurs in chloroplasts, cytosol, and vacuole allowing nutrient remobilization. Chloroplasts, which concentrate a large part of metals, are first affected (Zavaleta-Mancera et al., 1999). Pigment degradation directly takes place in chloroplasts (Hörtensteiner et al., 1995; Park et al., 2007). However, stromal proteins are degradated into the central vacuole through rubisco containing body (RCB: autophagosome) or into senescence associated vacuoles (SAV) through an ATG-independent route which is not well understand yet (Hörtensteiner and Feller, 2002; Ishida et al., 2008; Ishida et al., 2013). These dismantling mechanisms decrease chloroplast seize enabling whole chloroplast degradation via chlorophagy (Ishida et al., 2013). Peroxisomes are modified to glyoxysomes, which produce energy and soluble sugars from lipid catabolism (Buchanan-Wollaston, 1997; del Rio et al., 1998). Mitochondria that remain intact until late after senescence onset, are in turn degraded when the energy demand decreases (Yoshida, 2003). Finally, membrane permeabilization causes loss of cytoplasm that finally leads to death. ROS, reactive oxygen species; SAV, senescence-associated vacuoles; RCB, rubisco containing body; N, nucleus.

et al., 2004; Breeze et al., 2011). As expected, these analyses confirmed induction of genes involved in hormonal pathways (Andersson et al., 2004; van der Graaff et al., 2006; Breeze et al., 2011). Indeed, senescence is regulated by the balance between senescence promoting hormones, namely jasmonic acid, abscisic acid, salicylic acid, and ethylene, and senescence repressing hormones such as cytokinins, auxins, and gibberellins (van der Graaff et al., 2006). As hormones, sugars are known to act as signaling molecules and several lines of evidence indicate that they also contribute to senescence regulation. Sugar concentrations rise in senescent leaves. Moreover, overexpression of hexokinase, a sugar sensor, accelerates senescence whereas antisense expression delays senescence in *Arabidopsis* (Nooden et al., 1997; Masclaux et al., 2000; Xiao et al., 2000; Watanabe et al., 2013).

Genes coding metal ion binding proteins such as metallothioneins, ferritins, zinc-finger proteins, metalloproteases (Ftsh) and metal transporters were also frequently found to be upregulated in senescent leaves (Buchanan-Wollaston, 1994; Bhalerao et al., 2003; Andersson et al., 2004; Guo et al., 2004; Zelisko et al., 2005). This may illustrate the involvement of metals in degradation mechanisms and/or the importance of their remobilization (Breeze et al., 2011). Furthermore, these transcriptomic analyses highlighted the significant induction of autophagy related genes (*ATG* genes) and genes encoding NAC and WRKY transcription factors (Andersson et al., 2004; Guo et al., 2004; van der Graaff et al., 2006; Breeze et al., 2011). Whereas NAC have already been demonstrated to be involved in micronutrient remobilization during senescence (Olmos et al., 2003; Guo and Gan, 2006; Uauy et al., 2006; Sperotto et al., 2009, 2010; Waters et al., 2009), nothing is known about the implication of *ATG* genes in this process.

ROLE OF AUTOPHAGY IN NUTRIENT RECYCLING AND REMOBILIZATION

INVOLVEMENT OF AUTOPHAGY IN NUTRIENT RECYCLING

Autophagy catabolizes cytoplasmic components that are no longer useful. It eliminates aberrant proteins and damaged organelles for the maintenance of essential cellular function by vacuole internalization mediated by double membrane vesicles called autophagosomes (Yoshimoto, 2012). Genes involved in autophagy (*ATG*) were first defined by a genetic screen in yeast (Matsuura et al., 1997), thereby molecular mechanisms have been well described on this organism (for reviews see Thompson and Vierstra, 2005; Bassham, 2007; Li and Vierstra, 2012; Yoshimoto, 2012). Most of these genes turned out to have conserved functions in all eukaryotic cells. They encode proteins involved in the induction of autophagy, membrane delivery for autophagosome formation, nucleation, expansion, and enclosure of autophagosomes (Thompson and Vierstra, 2005).

Autophagy can be triggered upon nutrient starvation and stress leading to intracellular remodeling, which allows plants to respond to environmental constraints (Yoshimoto, 2012). Accordingly, mutants impaired in *ATG* genes exhibit decreased growth associated with premature senescence when they develop under carbon or nitrogen starvation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Phillips et al., 2008; Chung et al., 2010; Suttangkakul et al., 2011). Plants defective in autophagy are thus unable to cope with nutrient starvation suggesting that autophagy is an important mechanism for nutrient use efficiency and cellular homeostasis.

AUTOPHAGY CONTROLS NUTRIENT REMOBILIZATION DURING SENESCENCE

During senescence, cytoplasmic components such as organelles are gradually dismantled and degraded. Autophagy is an essential degradation process for nutrient recycling and remobilization. Accordingly, up-regulation of *ATG* genes is observed during leaf senescence in *Arabidopsis* (Doelling et al., 2002; van der Graaff et al., 2006; Chung et al., 2010; Breeze et al., 2011) and the decrease of chloroplast number and chloroplast size during senescence is affected in *Arabidopsis atg4a4b-1* mutant (Wada et al., 2009).

Because of its key role in the degradation of cellular components during nutrient recycling and its up-regulation and involvement during senescence, it was hypothesized that autophagy could play a role in nutrient remobilization. During senescence, autophagy was shown to be involved in the degradation of chloroplasts and specifically of RuBisCO which is the most abundant leaf protein containing about 80% of the cellular nitrogen (**Figure 2**; Chiba et al., 2003; Ishida et al., 2008; Wada et al., 2009; Guiboileau et al., 2012; Ishida et al., 2013). In addition, pulse-chase experiments in which ^{15}N labeling was applied in nutrient solution during vegetative stage revealed a significant decrease of nitrogen remobilization from vegetative tissues to seeds in *atg* mutants. These results demonstrated that autophagy is required for nitrogen remobilization and seed filling (Guiboileau et al., 2012).

Chloroplast is the organelle where metals are most intensively used. Thereby about 80% of the cellular Fe is localized in chloroplasts (Nouet et al., 2011). Because autophagy is involved in the degradation of organelles, including chloroplasts, the role of autophagy in metal recycling in source tissues for remobilization to the seeds has to be considered. In plants, autophagy leads to the degradation of autophagosome cargo within the vacuole. Hence, tonoplastic metal efflux transporters are needed to retrieve metals from the vacuole. Interestingly, transcriptomic analyses that highlight autophagy induction during senescence in *Arabidopsis* leaf also show specific up-regulation of *NRAMP3*, a gene encoding a transporter involved in metal mobilization from vacuoles (Thomine et al., 2003; Lanquar et al., 2005, 2010; Breeze et al., 2011). Availability of metals in source tissues may therefore also be dependent on autophagy and subsequent mobilization from vacuole during senescence.

REMOBILIZATION AND AUTOPHAGY IN THE CONTEXT OF BIOFORTIFICATION

BIOFORTIFICATION TO IMPROVE HUMAN DIET

Key micronutrients are often not sufficiently available in human diet (Kennedy et al., 2003). Over 60% of the world population are Fe deficient and over 30% are Zn deficient (White and Broadley, 2009). Staple food crops such as cereal grains are poor sources of some mineral nutrients, including Fe and Zn. Thus, the importance of cereals in human diet accounts

in large part for micronutrients deficiencies (Gomez-Galera et al., 2010).

Biofortification aims at increasing the availability of key micronutrients such as Fe and Zn in crops (White and Broadley, 2009). For this purpose, conventional breeding and genetic engineering are performed in rice, which is the major staple crop in most countries affected by Fe-deficiency (Juliano, 1993; WHO, 2002; Sperotto et al., 2012a). Single or multiple metal homeostasis genes were already introduced in rice through genetic engineering to improve grain Fe content (Sperotto et al., 2012a). By pyramiding transgenes conferring strong sink strength in seeds, high metal translocation and enhancing phloem unloading during seed maturation, it was possible to increase Fe concentration by 4.4 in rice seeds (Masuda et al., 2012).

ENGINEERING AUTOPHAGY AS A NEW WAY FOR BIOFORTIFICATION

Another option to increase seed micronutrient content could be to improve their availability in source tissues for remobilization during seed formation. Himelblau and Amasino (2001) showed that senescence of *Arabidopsis* leaves only leads to a decrease by 40% of leaf concentrations of metals such as Mo, Fe, Cu, and Zn. Thus, about 60% of these micronutrients are not remobilized and can therefore not participate to seed filling. Up-regulating autophagy in source tissues specifically during seed formation could improve intracellular nutrient recycling and thereby increase the nutrient pool available for reallocation. However, because autophagy is not specific, this approach may increase seed yield without increasing Zn or Fe concentrations. To improve seed quality, up-regulation of autophagy should be combined with a strategy that specifically targets a metal, such as the expression of ferritin under the control of a seed endosperm promoter in the case of Fe (Sperotto et al., 2012a).

More than thirty genes are involved in autophagy (Yoshimoto, 2012). It might therefore not be straightforward to increase autophagy by overexpressing autophagy related genes during seed formation. However, autophagy is regulated at the post-transcriptional level by the target of rapamycin (TOR) kinase complex (Noda and Ohsumi, 1998; Kamada et al., 2000). Because TOR is a negative regulator of autophagy, its specific inhibition in vegetative tissues during seed formation may be the best approach to stimulate autophagy and nutrient recycling. On the other hand, TOR kinase complex is not a specific regulator of autophagy. It controls many others aspect of metabolism (Diaz-Troya et al., 2008). Besides, autophagy itself is not only involved in nutrient recycling. It also controls the hypersensitive response (Yoshimoto et al., 2009). Therefore, further investigations are necessary to determine if TOR inactivation during senescence is efficient for biofortification and to identify more specific regulators.

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REFERENCES

Aggarwal, A., Sharma, I., Tripathi, B. N., Munjal, A. K., Baunthiyal, M., and Sharma, V. (2012). "Metal toxicity and photosynthesis," in *Photosynthesis: Overviews on Recent Progress and Future Perspectives*, eds S. Itoh, P. Mohanty, and K. N. Guruprasad (New Delhi: IK International Publishing House (Pvt) Limited).

Alloway, B. J. (2009). Soil factors associated with zinc deficiency in crops and humans. *Environ. Geochem. Health* 31, 537–548. doi: 10.1007/s10653-009-9255-4

Andersson, A., Keskitalo, J., Sjodin, A., Bhalerao, R., Sterky, F., Wissel, K., et al. (2004). A transcriptional timetable of autumn senescence. *Genome Biol.* 5, R24. doi: 10.1186/gb-2004-5-4-r24

Badger, M. R., and Price, G. D. (1994). The role of carbonic anhydrase in photosynthesis. *Annu. Rev. Plant Biol.* 45, 369–392. doi: 10.1146/annurev.pp.45.060194.002101

Bassham, D. C. (2007). Plant autophagy-more than a starvation response. *Curr. Opin. Plant Biol.* 10, 587–593. doi: 10.1016/j.pbi.2007.06.006

Bhalerao, R., Keskitalo, J., Sterky, F., Erlandsson, R., Björkbacka, H., Birve, S. J., et al. (2003). Gene expression in autumn leaves. *Plant Physiol.* 131, 430–442. doi: 10.1104/pp.012732

Bleackley, M. R., and Macgillivray, R. T. A. (2011). Transition metal homeostasis: from yeast to human disease. *Biometals* 24, 785–809. doi: 10.1007/s10534-011-9451-4

Breeze, E., Harrison, E., Mchattie, S., Hughes, L., Hickman, R., Hill, C., et al. (2011). High-resolution temporal profiling of transcripts during *Arabidopsis* leaf senescence reveals a distinct chronology of processes and regulation. *Plant Cell* 23, 873–894. doi: 10.1105/tpc.111.083345

Buchanan-Wollaston, V. (1994). Isolation of cDNA clones for genes that are expressed during leaf senescence in *Brassica napus* (identification of a gene encoding a senescence-specific metallothionein-like protein). *Plant Physiol.* 105, 839–846. doi: 10.1104/pp.105.3.839

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *J. Exp. Bot.* 48, 181–199. doi: 10.1093/jxb/48.2.181

Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P. O., Nam, H. G., et al. (2005). Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. *Plant J.* 42, 567–585. doi: 10.1111/j.1365-313X.2005.02399.x

Chen, Y., and Barak, P. (1982). Iron nutrition of plants in calcareous soils. *Adv. Agron.* 35, 217–240. doi: 10.1016/S0065-2113(08)60326-0

Chiba, A., Ishida, H., Nishizawa, N. K., Makino, A., and Mae, T. (2003). Exclusion of ribulose-1, 5-bisphosphate carboxylase/oxygenase from chloroplasts by specific bodies in naturally senescing leaves of wheat. *Plant Cell Physiol.* 44, 914–921. doi: 10.1093/pcp/pcg118

Chung, T., Phillips, A. R., and Vierstra, R. D. (2010). ATG8 lipidation and ATG8-mediated autophagy in *Arabidopsis* require ATG12 expressed from the differentially controlled ATG12A AND ATG12B loci. *Plant J.* 62, 483–493. doi: 10.1111/j.1365-313X.2010.04166.x

Crafts-Brandner, S. J., and Egli, D. B. (1987). Sink removal and leaf senescence in soybean: cultivar effects. *Plant Physiol.* 85, 662–666. doi: 10.1104/pp.85.3.662

del Rìo, L. A., Pastori, G. M., Palma, J. M., Sandalio, L. M., Sevilla, F., Corpas, F. J., et al. (1998). The activated oxygen role of peroxisomes in senescence. *Plant Physiol.* 116, 1195–1200. doi: 10.1104/pp.116.4.1195

Diaz-Troya, S., Pérez-Pérez, M. E., Florencio, F. J., and Crespo, J. L. (2008). The role of TOR in autophagy regulation from yeast to plants and mammals. *Autophagy* 4, 851–865.

Doelling, J. H., Walker, J. M., Friedman, E. M., Thompson, A. R., and Vierstra, R. D. (2002). The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in *Arabidopsis thaliana*. *J. Biol. Chem.* 277, 33105–33114. doi: 10.1074/jbc.M204630200

Erenoglu, E. B., Kutman, U. B., Ceylan, Y., Yildiz, B., and Cakmak, I. (2011). Improved nitrogen nutrition enhances root uptake, root-to-shoot translocation and remobilization of zinc (65Zn) in wheat. *New Phytol.* 189, 438–448. doi: 10.1111/j.1469-8137.2010.03488.x

Etienne, P., Desclos, M., Le Gou, L., Gombert, J., Bonnefoy, J., Maurel, K., et al. (2007). N-protein mobilisation associated with the leaf senescence process in oilseed rape is concomitant with the disappearance of trypsin inhibitor activity. *Funct. Plant Biol.* 34, 895–906. doi: 10.1071/FP07088

Finney, L. A., and O'Halloran, T. V. (2003). Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Sci. Signal.* 300, 931. doi: 10.1126/science.1085049

Gan, S., and Amasino, R. M. (1997). Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). *Plant Physiol.* 113, 313.

Gomez-Galera, S., Rojas, E., Sudhakar, D., Zhu, C., Pelacho, A. M., Capell, T., et al. (2010). Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Res.* 19, 165–180. doi: 10.1007/s11248-009-019311-y

Graham R. D. (1988). "Genotypic diferences in tolerance to manganese deficiency," in *Manganese in Soils and Plants*, eds R. D. Graham, R. J. Hannam, and N. C. Uren (Dordrecht: Kluwer Academic Publishers), 261–276.

Grusak, M. A. (1994). Iron transport to developing ovules of *Pisum sativum* (I. Seed import characteristics and phloem iron-loading capacity of source regions*). Plant Physiol.* 104, 649–655. doi: 10.1104/pp.104.2.649

Guiboileau, A., Yoshimoto, K., Soulay, F., Bataillé, M.-P., Avice, J.-C., et al. (2012). Autophagy machinery controls nitrogen remobilization at the whole-plant level under both limiting and ample nitrate conditions in *Arabidopsis. New Phytol.* 194, 732–740. doi: 10.1111/j.1469-8137.2012.04084.x

Guo, Y., Cai, Z., and Gan, S. (2004). Transcriptome of *Arabidopsis* leaf senescence. *Plant Cell Environ.* 27, 521–549. doi: 10.1111/j.1365-3040.2003.01158.x

Guo, Y., and Gan, S. (2006). AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *Plant J.* 46, 601–612. doi: 10.1111/j.1365-313X.2006.02723.x

Hanaoka, H., Noda, T., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., et al. (2002). Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an *Arabidopsis* autophagy gene. *Plant Physiol.* 129, 1181–1193. doi: 10.1104/pp.011024

Hegelund, J. N., Pedas, P., Husted, S., Schiller, M., and Schjoerring, J. K. (2012). Zinc fluxes into developing barley grains: use of stable Zn isotopes to separate root uptake from remobilization in plants with contrasting Zn status. *Plant Soil* 361, 241–250. doi: 10.1007/s11104-012-1272-x

Hensel, L. L., Grbic, V., Baumgarten, D. A., and Bleecker, A. B. (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis. Plant Cell* 5, 553–564. doi: 10.1105/tpc.5.5.553

Himelblau, E., and Amasino, R. M. (2001). Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. *J. Plant Physiol.* 158, 1317–1323. doi: 10.1078/0176-1617-00608

Horsburgh, M. J., Wharton, S. J., Karavolos, M., and Foster, S. J. (2002). Manganese: elemental defence for a life with oxygen. *Trends Microbiol.* 10, 496–501. doi: 10.1016/S0966-842X(02)02462-9

Hörtensteiner, S., and Feller, U. (2002). Nitrogen metabolism and remobilization during senescence. *J. Exp. Bot.* 53, 927–937. doi: 10.1093/jexbot/53.370.927

Hörtensteiner, S., Vicentini, F., and Matile, P. (1995). Chlorophyll breakdown in senescent cotyledons of rape, *Brassica napus* L: enzymatic cleavage of pheophorbide a in vitro. *New Phytol.* 129, 237–246. doi: 10.1111/j.1469-8137.1995.tb04293.x

Htwe, N. M. P. S., Yuasa, T., Ishibashi, Y., Tanigawa, H., Okuda, M., Zheng, S.-H., et al. (2011). Leaf senescence of soybean at reproductive stage is associated with induction of autophagy-related genes, GmATG8c, GmATG8i and GmATG4. *Plant Prod. Sci.* 14, 141–147. doi: 10.1626/pps.14.141

Ishida, H., Izumi, M., Wada, S., and Makino, A. (2013). Roles of autophagy in chloroplast recycling. *Biochim. Biophys. Acta* doi: 10.1016/j.bbabio.2013.11.009 [Epub ahead of print].

Ishida, H., Yoshimoto, K., Izumi, M., Reisen, D., Yano, Y., Makino, A., et al. (2008). Mobilization of rubisco and stroma-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process. *Plant Physiol.* 148, 142–155. doi: 10.1104/pp.108.122770

Juliano, B. O. (1993). *Rice in Human Nutrition.* Rome: International Rice Research Institute & FAO.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* 150, 1507–1513. doi: 10.1083/jcb.150.6.1507

Karamanos, R. E., Kruger, G. A., and Stewart, J. W. B. (1986). Copper deficiency in cereal and oilseed crops in northern Canadian prairie soils. *Agron. J.* 78, 317–323. doi: 10.2134/agronj1986.00021962007800020021x

Kennedy, G., Nantel, G., and Shetty, P. (2003). The scourge of "hidden hunger": global dimensions of micronutrient deficiencies. *Food Nutr. Agric.* 8–16.

Kume, T., Matsuhashi, S., Shimazu, M., Ito, H., Fujimura, T., Adachi, K., et al. (1997). Uptake and transport of positron-emitting tracer (18 F) in plants. *Appl. Radiat. Isot.* 48, 1035–1043. doi: 10.1016/S0969-8043(97)00117-6

Kurepa, J., Hérouart, D., Van Montagu, M., and Inzé, D. (1997). Differential expression of CuZn-and Fe-superoxide dismutase genes of tobacco during development, oxidative stress, and hormonal treatments. *Plant Cell Physiol.* 38, 463–470. doi: 10.1093/oxfordjournals.pcp.a029190

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., et al. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24, 4041–4051. doi: 10.1038/sj.emboj.7600864

Lanquar, V., Ramos, M. S., Lelièvre, F., Barbier-Brygoo, H., Krieger-Liszkay, A., Kramer, U., et al. (2010). Export of vacuolar manganese by AtNRAMP3 and AtNRAMP4 is required for optimal photosynthesis and growth under manganese deficiency. *Plant Physiol.* 152, 1986–1999. doi: 10.1104/pp.109.150946

Lemaître, T., Gaufichon, L., Boutet-Mercey, S., Christ, A., and Masclaux-Daubresse, C. (2008). Enzymatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana* Wassileskija accession. *Plant Cell Physiol.* 49, 1056–1065. doi: 10.1093/pcp/pcn081

Li, F., and Vierstra, R. D. (2012). Autophagy: a multifaceted intracellular system for bulk and selective recycling. *Trends Plant Sci.* 17, 526–537. doi: 10.1016/j.tplants.2012.05.006

Lu, Y., Hall, D. A., and Last, R. L. (2011). A small zinc finger thylakoid protein plays a role in maintenance of photosystem II in *Arabidopsis thaliana. Plant Cell* 23, 1861–1875. doi: 10.1105/tpc.111.085456

Luk, E. E. C., and Culotta, V. C. (2001). Manganese superoxide dismutase in *Saccharomyces cerevisiae* acquires its metal co-factor through a pathway involving the Nramp metal transporter, Smf2p. *J. Biol. Chem.* 276, 47556–47562. doi: 10.1074/jbc.M108923200

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., and Suzuki, A. (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* 105, 1141–1157. doi: 10.1093/aob/mcq028

Masclaux-Daubresse, C. L., and Chardon, F. (2011). Exploring nitrogen remobilization for seed filling using natural variation in *Arabidopsis thaliana. J. Exp. Bot.* 62, 2131–2142. doi: 10.1093/jxb/erq405

Masclaux, C., Quilleré, I., Gallais, A., and Hirel, B. (2001). The challenge of remobilisation in plant nitrogen economy. A survey of physio-agronomic and molecular approaches. *Ann. Appl. Biol.* 138, 69–81. doi: 10.1111/j.1744-7348.2001.tb00086.x

Masclaux, C., Valadier, M.-H., Brugière, N., Morot-Gaudry, J.-F., and Hirel, B. (2000). Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta* 211, 510–518. doi: 10.1007/s004250000310

Masuda, H., Ishimaru, Y., Aung, M. S., Kobayashi, T., Kakei, Y., Takahashi, M., et al. (2012). Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci. Rep.* 2, 543. doi:10.1038/srep00543

Matsuura, A., Tsukada, M., Wada, Y., and Ohsumi, Y. (1997). Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae. Gene* 192, 245–250. doi: 10.1016/S0378-1119(97)00084-X

Morrissey, J., and Guerinot, M. L. (2009). Iron uptake and transport in plants: the good, the bad, and the ionome. *Chem. Rev.* 109, 4553–4567. doiI: 10.1021/cr900112r

Noda, T., and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* 273, 3963–3966. doi: 10.1074/jbc.273.7.3963

Nooden, L. D., Guiamet, J. J., and John, I. (1997). Senescence mechanisms. *Physiol. Plant.* 101, 746–753. doi: 10.1111/j.1399-3054.1997.tb01059.x

Nooden, L. D., Hillsberg, J. W., and Schneider, M. J. (1996). Induction of leaf senescence in *Arabidopsis thaliana* by long days through a light-dosage effect. *Physiol. Plant.* 96, 491-495. doi: 10.1111/j.1399-3054.1996.tb00463.x

Noquet, C., Avice, J.-C., Rossato, L., Beauclair, P., Henry, M.-P., and Ourry, A. (2004). Effects of altered source-sink relationships on N allocation and vegetative storage protein accumulation in *Brassica napus* L. *Plant Sci.* 166, 1007–1018. doi: 10.1016/j.plantsci.2003.12.014

Nouet, C., Motte, P., and Hanikenne, M. (2011). Chloroplastic and mitochondrial metal homeostasis. *Trends Plant Sci.* 16, 395–404. doi: 10.1016/j.tplants.2011.03.005

Olmos, S., Distelfeld, A., Chicaiza, O., Schlatter, A. R., Fahima, T., Echenique, V., et al. (2003). Precise mapping of a locus affecting grain protein content in

durum wheat. *Theor. Appl. Genet.* 107, 1243–1251. doi: 10.1007/s00122-003-1377-y

Palmgren, M. G., Clemens, S., Williams, L. E., Kramer, U., Borg, S., Schjorring, J. K., et al. (2008). Zinc biofortification of cereals: problems and solutions. *Trends Plant Sci.* 13, 464–473. doi: 10.1016/j.tplants.2008.06.005

Park, S.-Y., Yu, J.-W., Park, J.-S., Li, J., Yoo, S.-C., Lee, N.-Y., et al. (2007). The senescence-induced staygreen protein regulates chlorophyll degradation. *Plant Cell* 19, 1649–1664. doi: 10.1105/tpc.106.044891

Patrick, J. W., and Offler, C. E. (2001). Compartmentation of transport and transfer events in developing seeds. *J. Exp. Bot.* 52, 551–564. doi: 10.1093/jexbot/52.356.551

Patterson, T. G., and Brun, W. A. (1980). Influence of sink removal in the senescence pattern of wheat. *Crop Sci.* 20, 19–23. doi: 10.2135/cropsci1980.0011183X002000010006x

Phillips, A. R., Suttangkakul, A., and Vierstra, R. D. (2008). The ATG12-conjugating enzyme ATG10 is essential for autophagic vesicle formation in *Arabidopsis thaliana. Genetics* 178, 1339–1353. doi: 10.1534/genetics.107.086199

Pierre, J. L., and Fontecave, M. (1999). Iron and activated oxygen species in biology: the basic chemistry. *Biometals* 12, 195–199. doi: 10.1023/A:1009252919854

Pilon, M. (2011). Moving copper in plants. *New Phytol.* 192, 305–307. doi: 10.1111/j.1469-8137.2011.03869.x

Pittman, J. K. (2005). Managing the manganese: molecular mechanisms of manganese transport and homeostasis. *New Phytol.* 167, 733–742. doi: 10.1111/j.1469-8137.2005.01453.x

Puig, S., and Peñarrubia, L. (2009). Placing metal micronutrients in context: transport and distribution in plants. *Curr. Opin. Plant Biol* 12, 299–306. doi: 10.1016/j.pbi.2009.04.008

Salon, C., Avice, J. C., Alain, O., Prudent, M., and Voisin, A.-S. (2011). "Plant N fluxes and modulation by nitrogen, heat, and water stresses: a review based on comparison of legumes and non legume plants," in *Abiotic Stress in Plants – Mechanisms and Adaptations*, eds A. Shanker and B. Venkateswarlu (Rijeka: InTech), 78–118.

Schjoerring, J. K., Bock, J. G. H., Gammelvind, L., Jensen, C. R., and Mogensen, V. O. (1995). Nitrogen incorporation and remobilization in different shoot components of field-grown winter oilseed rape (*Brassica napus* L.) as affected by rate of nitrogen application and irrigation. *Plant Soil* 177, 255–264. doi: 10.1007/BF00010132

Smart, C. M., Hosken, S. E., Thomas, H., Greaves, J. A., Blair, B. G., and Schuch, W. (1995). The timing of maize leaf senescence and characterisation of senescence-related cDNAs. *Physiol. Plant.* 93, 673–682. doi: 10.1111/j.1399-3054.1995.tb05116.x

Sondergaard, T. E., Schulz, A., and Palmgren, M. G. (2004). Energization of transport processes in plants. Roles of the plasma membrane H+-ATPase. *Plant Physiol.* 136, 2475–2482. doi: 10.1104/pp.104.048231

Sperotto, R. A., Boff, T., Duarte, G. L., Santos, L. S., Grusak, M. A., and Fett, J. P. (2010). Identification of putative target genes to manipulate Fe and Zn concentrations in rice grains. *J. Plant Physiol.* 167, 1500–1506. doi: 10.1016/j.jplph.2010.05.003

Sperotto, R. A., Ricachenevsky, F. K., Duarte, G. L., Boff, T., Lopes, K. L., Sperb, E. R., et al. (2009). Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. *Planta* 230, 985–1002. doi: 10.1007/s00425-009-1000-9

Sperotto, R. A., Ricachenevsky, F. K., Waldow, V. D. A., and Fett, J. P. (2012a). Iron biofortification in rice: it's a long way to the top. *Plant Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Sperotto, R. A., Vasconcelos, M. W., Grusak, M. A., and Fett, J. P. (2012b). Effects of different Fe supplies on mineral partitioning and remobilization during the reproductive development of rice (*Oryza sativa* L.). *Rice* 5, 1–11. doi: 10.1186/1939-8433-5-27

Suttangkakul, A., Li, F., Chung, T., and Vierstra, R. D. (2011). The ATG1/ATG13 protein kinase complex is both a regulator and a target of autophagic recycling in *Arabidopsis. Plant Cell* 23, 3761–3779. doi: 10.1105/tpc.111.090993

Tanaka, M., Wallace, I. S., Takano, J., Roberts, D. M., and Fujiwara, T. (2008). NIP6; 1 is a boric acid channel for preferential transport of boron to growing shoot tissues in *Arabidopsis. Plant Cell* 20, 2860–2875. doi: 10.1105/tpc.108.058628

Thomine, S., Lelièvre, F., Debarbieux, E., Schroeder, J. I., and Barbier-Brygoo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant J.* 34, 685–695. doi: 10.1046/j.1365-313X.2003.01760.x

Thomine, S., and Vert, G. (2013). Iron transport in plants: better be safe than sorry. *Curr. Opin. Plant Biol* 16, 322–327. doi: 10.1016/j.pbi.2013.01.003

Thompson, A. R., and Vierstra, R. D. (2005). Autophagic recycling: lessons from yeast help define the process in plants. *Curr. Opin. Plant Biol* 8, 165–173. doi: 10.1016/j.pbi.2005.01.013

Tommos, C., Hoganson, C. W., Di Valentin, M., Lydakis-Simantiris, N., Dorlet, P., Westphal, K., et al. (1998). Manganese and tyrosyl radical function in photosynthetic oxygen evolution. *Curr. Opin. Chem. Biol* 2, 244–252. doi: 10.1016/S1367-5931(98)80066-5

Tsukamoto, T., Nakanishi, H., Kiyomiya, S., Watanabe, S., Matsuhashi, S., Nishizawa, N. K., et al. (2006). 52Mn translocation in barley monitored using a positron-emitting tracer imaging system. *Soil Sci. Plant Nutr.* 52, 717–725. doi:10.1111/j.1747-0765.2006.00096.x

Tsukamoto, T., Nakanishi, H., Uchida, H., Watanabe, S., Matsuhashi, S., Mori, S., et al. (2009). 52Fe Translocation in barley as monitored by a positron-emitting tracer imaging system (PETIS): evidence for the direct translocation of Fe from roots to young leaves via phloem. *Plant Cell Physiol.* 50, 48–57. doi: 10.1093/pcp/pcn192

Uauy, C., Distelfeld, A., Fahima, T., Blechl, A., and Dubcovsky, J. (2006). A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* 314, 1298–1301. doi: 10.1126/science.1133649

van der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flugge, U.-I., and Kunze, R. (2006). Transcription analysis of *Arabidopsis* membrane transporters and hormone pathways during developmental and induced leaf senescence. *Plant Physiol.* 141, 776–792. doi: 10.1104/pp.106.079293

Wada, S., Ishida, H., Izumi, M., Yoshimoto, K., Ohsumi, Y., Mae, T., et al. (2009). Autophagy plays a role in chloroplast degradation during senescence in individually darkened leaves. *Plant Physiol.* 149, 885–893. doi: 10.1104/pp.108.130013

Wang, Y. X., Specht, A., and Horst, W. J. (2010). Stable isotope labelling and zinc distribution in grains studied by laser ablation ICP-MS in an ear culture system reveals zinc transport barriers during grain filling in wheat. *New Phytol.* 189, 428–437. doi: 10.1111/j.1469-8137.2010.03489.x

Watanabe, M., Balazadeh, S., Tohge, T., Erban, A., Giavalisco, P., Kopka, J., et al. (2013). Comprehensive dissection of spatio-temporal metabolic shifts in primary, secondary and lipid metabolism during developmental senescence in *Arabidopsis thaliana. Plant Physiol.* 162, 1290–1310. doi:10.1104/pp.113.217380

Waters, B. M., and Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

Waters, B. M., Uauy, C., Dubcovsky, J., and Grusak, M. A. (2009). Wheat (*Triticum aestivum*) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain. *J. Exp. Bot.* 60, 4263–4274. doi: 10.1093/jxb/erp257

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

WHO. (2002). *World Health Report 2002: World Health Report: Reducing Risks to Health Noncommunicable Diseases.* Geneva: World Health Organization.

Wu, C.-Y., Lu, L.-L., Yang, X.-E., Feng, Y., Wei, Y.-Y., Hao, H.-L., et al. (2010). Uptake, translocation, and remobilization of zinc absorbed at different growth stages by rice genotypes of different Zn densities. *J. Agric. Food Chem.* 58, 6767–6773. doi: 10.1021/jf100017e

Xiao, W., Sheen, J., and Jang, J.-C. (2000). The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Mol. Biol.* 44, 451–461. doi: 10.1023/A:1026501430422

Yamaji, N., and Ma, J. F. (2009). A transporter at the node responsible for intervascular transfer of silicon in rice. *Plant Cell* 21, 2878–2883. doi: 10.1105/tpc.109.069831

Yoshida, S. (2003). Molecular regulation of leaf senescence. *Curr. Opin. Plant Biol* 6, 79–84. doi: 10.1016/S1369526602000092

Yoshimoto, K. (2012). Beginning to understand autophagy, an intracellular self-degradation system in plants. *Plant Cell Physiol.* 53, 1355–1365. doi: 10.1093/pcp/pcs099

Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T., et al. (2004). Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. *Plant Cell* 16, 2967–2983. doi: 10.1105/tpc.104.025395

Yoshimoto, K., Jikumaru, Y., Kamiya, Y., Kusano, M., Consonni, C., Panstruga, R., et al. (2009). Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in *Arabidopsis*. *Plant Cell* 21, 2914–2927. doi: 10.1105/tpc.109.068635

Yruela, I. (2009). Copper in plants: acquisition, transport and interactions. *Funct. Plant Biol.* 36, 409–430. doi: 10.1071/FP08288

Yruela, I. (2013). Transition metals in plant photosynthesis. *Metallomics* 5, 1090–1109. doi: 10.1039/C3MT00086A

Zavaleta-Mancera, H., Franklin, K., Ougham, H., Thomas, H., and Scott, I. (1999). Regreening of senescent *Nicotiana* leaves. II. Redifferenciation of plastids. *J. Exp. Biol.* 50, 1683-1689. doi: 10.1093/jxb/50.340.1683

Zelisko, A., Garcia-Lorenzo, M., Jackowski, G., Jansson, S., and Funk, C. (2005). AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13699–13704. doi: 10.1073/pnas.0503472102

Zheng, L., Yamaji, N., Yokosho, K., and Ma, J. F. (2012). YSL16 is a phloem-localized transporter of the copper-nicotianamine complex that is responsible for copper distribution in rice. *Plant Cell* 24, 3767–3782. doi: 10.1105/tpc.112.103820

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Zinc allocation and re-allocation in rice

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Aims: Agronomy and breeding actively search for options to enhance cereal grain Zn density. Quantifying internal (re-)allocation of Zn as affected by soil and crop management or genotype is crucial. We present experiments supporting the development of a conceptual model of whole plant Zn allocation and re-allocation in rice.

Methods: Two solution culture experiments using ^{70}Zn applications at different times during crop development and an experiment on within-grain distribution of Zn are reported. In addition, results from two earlier published experiments are re-analyzed and re-interpreted.

Results: A budget analysis showed that plant zinc accumulation during grain filling was larger than zinc allocation to the grains. Isotope data showed that zinc taken up during grain filling was only partly transported directly to the grains and partly allocated to the leaves. Zinc taken up during grain filling and allocated to the leaves replaced zinc re-allocated from leaves to grains. Within the grains, no major transport barrier was observed between vascular tissue and endosperm. At low tissue Zn concentrations, rice plants maintained concentrations of about 20 mg Zn kg^{-1} dry matter in leaf blades and reproductive tissues, but let Zn concentrations in stems, sheath, and roots drop below this level. When plant zinc concentrations increased, Zn levels in leaf blades and reproductive tissues only showed a moderate increase while Zn levels in stems, roots, and sheaths increased much more and in that order.

Conclusions: In rice, the major barrier to enhanced zinc allocation towards grains is between stem and reproductive tissues. Enhancing root to shoot transfer will not contribute proportionally to grain zinc enhancement.

Keywords: ^{70}Zn, zinc allocation, rice, *Oryza sativa*, stable isotope, re-allocation

INTRODUCTION

Until recently, crop research on micronutrients largely focussed on alleviating limitations to crop production under deficiency or toxicity conditions (Marschner, 1995; Welch, 1995; Rengel, 1999; Fageria et al., 2002). To this zinc (Zn) was no exception. The interest in plant research on micronutrients changed when it was realized that human mineral deficiencies might be alleviated—at least partly—through improved mineral concentrations in edible parts of major food crops (Graham and Welch, 1996; Welch and Graham, 1999, 2004). This shift from a purely plant production focus to a food chain (Slingerland et al., 2006, 2009) or food systems (Graham et al., 2007) focus also shifted the attention in cereal crops from mainly uptake during early crop development, when Zn deficiency problems tend to be severest, to research on Zn husbandry during all stages of the crop's life cycle.

Such research on Zn husbandry included an analysis of Zn allocation to the grains and the relative role of Zn taken up at different crop development stages. Early research had established there is a strong potential for re-allocation of Zn from vegetative tissues to filling grains in wheat (*Triticum aestivum* L.) when post-flowering uptake was absent (Pearson and Rengel, 1994), despite a partial sequestration of Zn in vegetative organs. Later studies on wheat showed that when post-flowering uptake was made possible also substantial proportions of grain Zn could be accounted for by post-flowering uptake (Garnett and Graham, 2005; Kutman et al., 2011). In nutrient solution experiments with rice (*Oryza sativa* L.), post-flowering Zn uptake equaled or surpassed grain Zn content at maturity over a wide range of applied Zn levels (Jiang et al., 2008a) and over a range of genotypes (Jiang et al., 2008b). Also Mabesa et al. (2013) observed that post-flowering uptake was larger than total grain zinc content over a range of tested rice genotypes.

In the above-mentioned studies, the potential contribution of post-flowering uptake to grain Zn was assessed on the basis of budget analyses. Such Zn budget analyses, however, do not provide full insight into the relative role of re-allocation vs. the role of direct allocation, or into the role of different plant tissues in providing Zn to the grain through re-allocation. Theoretically all Zn taken up after flowering could be allocated to the major transpiring organs (i.e., the leaves and glumes) replacing there the earlier accumulated Zn which could in turn be re-allocated to the grains. This putative role of transpiration in directing allocation of xylem transported Zn has been postulated before (Pearson et al., 1995; Wolswinkel, 1999), but to the best of our knowledge

direct evidence is still lacking in literature. Zn is mobile, both in phloem and xylem (Marschner, 1995; Wolswinkel, 1999; Rengel, 2001). Further insight into whether direct allocation implies xylem to phloem transfer in the stem, rachis or grains, or rather in leaves and glumes, and into whether newly acquired Zn in fact substitutes re-allocated zinc will help in identifying most rate-limiting, underlying physiological processes and thus processes to target in breeding for improved Zn content in harvestable parts.

The aim of the current paper is to contribute to our insight into the most rate-limiting steps at organ level throughout crop development. In a next step it would then be possible to couple the insights in and models of the respective membrane transfer, xylem or phloem transport and cell level processes (Grusak et al., 1999; Palmgren et al., 2008; Waters and Sankaran, 2010) to the tissues where and developmental phases when rate limitations seem most important. In addition, Zn re-allocation implies that once allocated to and incorporated into a tissue some Zn can be released again. However, dead leaf tissue contains some Zn, even under severe plant Zn deficiency (Reuter et al., 1997); therefore, part of the Zn, once allocated to an organ, can be considered as sequestered, while additional Zn that is taken up above this minimum level can still be re-allocated. A quantification of Zn sequestration is essential to understand allocation and re-allocation dynamics throughout crop development.

Targeted studies on (re-)allocation that go beyond budget analyses, have been carried out in rice using labeled zinc, either radio-active (Jiang et al., 2007) or stable (Wu et al., 2010, 2011) isotope forms. Jiang et al. (2007) found that most of the Zn taken up by roots after flowering accumulated in grains while only a small portion of leaf-applied Zn was re-allocated from leaves to grains. Recent work of Mabesa et al. (2013) seems to hint into the same direction although there might be genetic variation worth pursuing. These data do not provide information on the contribution of re-allocation from leaves of Zn that had been taken up through the roots at different stages. The study by Wu et al. (2010) focussed on re-allocation under Zn deprivation during a 3-week period directly following a 30-day application of stable isotope Zn providing valuable insights in the way Zn recently taken up is allocated and subsequently re-allocated at different growth stages. However, these data do not provide insight into the long-term re-allocation of Zn taken up during the vegetative stage or during panicle formation to the filling grains after flowering.

In the current paper we report on two experiments with ^{70}Zn to study long-term re-allocation of Zn and to distinguish allocation and re-allocation during grain filling of Zn applied during different growth stages. We also report on an experiment carried out to assess whether the observed bottleneck for endosperm loading of Zn in wheat (Stomph et al., 2011) is also present in rice. Moreover, we re-analyse and re-interpret earlier published data on Zn allocation with the aim to identify possible bottlenecks in Zn transfer between tissues.

MATERIALS AND METHODS

EXPERIMENT 1

A greenhouse experiment was carried out in the UNIFARM facilities of Wageningen University, Wageningen, the Netherlands, in 2009. Seeds of rice (*Oryza sativa* L.) cv. Qinai-3-hun were surface sterilized with 1% sodium hypochlorite for 5 min followed by 70% ethanol for 1 min, after which they were rinsed with deionized water. Sterilized seeds were sown in quartz sand with standing water. Eight days after sowing visibly viable seedlings were rinsed with deionized water to wash off sand and fixed in polyethylene foam discs that fitted in lids of 20 L containers. Per container 20 plants were grown in a 7×7 cm grid. The containers were filled with a half strength Hoagland solution without Zn and Fe. This nutrient solution contained 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2.4H_2O$, 1 mM $MgSO_4.7H_2O$, 0.5 mM KH_2PO_4, 46.3 μM H_3BO_3, 9.1 μM $MnCl_2.4H_2O$, 0.6 μM $CuSO_4.5H_2O$, 0.03 μM $(NH_4)_6Mo_7O_{24}.4H_2O$. To this 2.5 ml Fe-EDDHMA per L nutrient solution was added. This chelate is a stable Fe source between pH 3.5 and pH 12. The stable isotope zinc was obtained as ZnO (99.5% ^{70}Zn), this was acidified with H_2SO_4 (0.01 M ZnO + 0.0195 M H_2SO_4) to form $^{70}ZnSO_4$. Per 20-L container 1.54 ml $ZnSO_4$ 0.01 M (0.05 mg Zn L^{-1}) or 1.43 ml $^{70}ZnSO_4$ 0.01 M (0.05 mg Zn L^{-1}) was added to account for differences in molecular weight of isotopes. The nutrient solution was replaced only at intermediate harvests, i.e., 7 days after panicle initiation (36 days after transplanting) and at flowering (54 days after transplanting). At both moments plant roots were rinsed twice with deionized water and then placed in deionized water for 2 h after which they were placed in the new nutrient solution. This procedure limited carry-over of nutrients. The temperatures in the climate controlled greenhouse compartment were set at 26°C during the 12 h daylight period and 23°C during the 12 h dark period. Nutrient solutions were aerated continuously.

There were three treatments. While growing conditions and nutrient solutions were identical during the full experimental period the plants received stable isotope ^{70}Zn during either the period between transplanting and 7 days after panicle initiation (Treatment 1), or the 18-day period prior to flowering (Treatment 2), or the period between flowering and full maturity (Treatment 3). Plants of Treatment 1 were harvested 7 days after panicle initiation, at flowering and at maturity, plants of Treatment 2 were harvested at flowering and maturity, and plants of Treatment 3 were harvested at maturity only. The experiment was carried out in duplicate in a randomized block design. Per replicate of each harvest by treatment combination one container was available with 20 plants arranged as 4 by 5 plants of which only the central 2 by 3 plants were used for observations on biomass and Zn distribution. The organ Zn concentrations for plants of treatments that were not harvested a week after panicle initiation (Treatments 2 and 3) or at flowering (Treatment 3) were assumed to be identical to those of the plants that received stable isotope Zn, while the abundance of ^{70}Zn in those plants were assumed to be the natural abundance (0.6%).

Panicle initiation was monitored on some additional plants grown on a separate container. The containers received deionized water three times per week as needed to keep water levels constant. The harvest a week after panicle initiation (36 days after transplanting) was scheduled when panicle initiation was observed on two consecutive harvested plants, the harvest at flowering was scheduled when three of the six central plants on all containers had at least one flowering panicle (54 days after

transplanting), while the harvest at maturity was scheduled 96 days after transplanting.

Roots of harvested plants were rinsed with deionized water and then blotted with tissues to remove adhering water. Plants were then dissected into roots, leaf sheaths, leaf blades and, when present, further into stems, rachis and glumes, and grains. At maturity some newly formed tillers were observed and these were harvested separately. Dissected material was dried at 70°C for 48 h before weighing. Hereafter samples were ground in a stainless steel hammer mill and then analyzed for total Zn and percentage ^{70}Zn at the Chemical Biological Laboratory of Wageningen UR-Soil Centre. Samples were microwave destructed using HNO_3-HF-H_2O_2 and subsequently samples were analyzed through Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

EXPERIMENT 2

Using the same cultivation method in 20-L containers as reported above, plants of two accessions, Qinai-3-Hun and 90B290, both tested earlier for grain Zn levels under field conditions in China (Jiang et al., 2008b), were grown in growth cabinets of the UNIFARM facilities of Wageningen University, Wageningen, the Netherlands. The basic plant nutrition, day length and temperature settings were as in Experiment 1, but in this experiment two seedlings were placed per foam. In all containers nutrient solutions were only replaced 28 days after transplanting, at panicle initiation of 90B290 (50 days after transplanting) and again at flowering (64 and 79 days for Qinai-3-hun and 90B290, respectively); the experiment was conducted in two replications. The two cultivars were raised at one of three Zn treatments: (1) a control in which plants were grown at 0.05 mg Zn L^{-1}, (2) a high Zn treatment in which plants were grown at 3.00 mg Zn L^{-1} during the first 28 days after transplanting, then placed at 1.00 mg Zn L^{-1} until harvest, and (3) a high Zn treatment in which plants were grown at 3.00 mg Zn L^{-1} during the first 28 days after transplanting, then placed at 2.00 mg Zn L^{-1} until harvest. In all three treatments plants received 99.5% ^{70}Zn from flowering onwards. Upon harvesting grains were hulled and then polished using a Pearlest® grain polisher (Kett Electric Laboratory, Tokyo, Japan). The polished rice was analyzed at the Waite Analytical Services laboratory in Adelaide, SA, Australia (Wheal et al., 2011).

EXPERIMENT 3

Plants of cultivar Qinai-3-Hun were grown at two Zn treatments on 165 L containers of 1.48 × 0.86 × 0.13 m. The nutrient solution (same as in Experiment 1) was continuously circulated to avoid local nutrient depletion and to provide aeration. The nutrient solution was replaced 15 days after transplanting, at panicle initiation (34 days after transplanting), and at flowering (54 days after transplanting). In the lids of the containers a total of 250 foams could be placed in a 7 × 7 cm grid, and in each foam 2 plants were placed to create a high plant density from the start avoiding profuse tillering and synchronizing flowering. A selection of plants in each container was tagged at flowering with their individual flowering date for later harvesting for this experiment, remaining plants were used for another experiment. A plant was considered to flower when the anthers from at least 5 florets were protruding. Based on the flowering dates individual panicles were harvested at 7, 14, 21, or 28 days after their flowering and at full maturity (35 days after flowering). For each date four individual panicles were selected among the tagged plants. In total six containers were available. Four containers received a standard Zn level (Treatment 1) and two containers a high Zn level (Treatment 2). The four plants were taken one from each standard Zn container providing four true experimental repeats and two plants were taken from each high Zn container providing four replicated plants, but only two true experimental repeats.

Containers for Treatment 1 received 0.05 mg Zn L^{-1} at the start and when the nutrient solution was replaced. Containers for Treatment 2 received 3.00 mg Zn L^{-1} at the start and at the first nutrient solution replacement, at panicle initiation the nutrient solution was replaced by one with 2.00 mg Zn L^{-1}; at flowering the nutrient solution was again brought to 2.00 mg Zn L^{-1}.

Upon harvesting the fresh grains were dissected under a binocular microscope (×16) into glumes, pericarp, seed coat, vascular bundle, endosperm, and embryo. At 7 days after flowering 20 grains were thus dissected and tissue samples bulked, at 14 days after flowering and later 10 grains were dissected and tissue samples bulked as grain tissue weights had increased. The separation between embryo, seed coat, and endosperm was rather difficult 7 days after flowering and these tissues were later bulked. At grain maturity dissection failed and only whole grains were analyzed. After dissection tissues were dried at 70°C for 24 h and weighed. Thereafter the samples were sent to the Waite Analytical Services laboratory in Adelaide, SA, Australia for chemical analyses (Wheal et al., 2011).

RE-ANALYSIS OF DATA FROM THE LITERATURE

Data from Impa et al. (2013) were taken from the supplementary material presented on the journal website (last consulted 30 October 2013). Data from Wu et al. (2010) were taken from their tables presented in the paper. In both case only averages were used. The data from Jiang et al. (2008a) are our own so were used as raw data and further referred to as Experiment 4. Here we will mainly use data not published in the mentioned paper so we provide the essentials on the methods for a quick insight, full details can be found in the original paper (Jiang et al., 2008a). The experiment was carried out in 2005 in the greenhouses of the Chinese Academy of Agricultural Sciences, Beijing. Fifteen-day old seedlings of aerobic rice cultivars Handao502 and Baxiludao were transplanted into foam disks fitted in the lids of 70-liter containers with 55 seedlings per container. Containers were filled with half strength Hoagland solution (pH 5.6 ± 0.1) without Zn. Following the method used by Hoffland et al. (2000) for P, Zn (as $ZnSO_4$) was added every 3 days to the solution on the basis of expected dry matter increase, and seven target plant Zn concentrations for total plant dry matter (10, 15, 25, 50, 100, 150, and 200 mg kg^{-1}). Total Zn applied between start and end of the experiment for the seven target levels was: 142, 166, 251, 350, 558, 768, 979 μg $plant^{-1}$. Temperatures were set to 28°C/21°C (day/night) and 1000 μmol m^{-2} s^{-1} light was supplemented when it was cloudy.

Plant samples were dried at 75°C for 48 h and ground with a stainless-steel blade blender to a particle size of 0.25 mm. Dried ground plant samples (0.50 g) were digested in a bi-acid mixture (HNO_3: $HClO_4$ = 4:1) and Zn was determined by atomic

absorption spectroscopy (AAS SPECTRAA-55; Varian Australia, Mulgrave, VIC, Australia) at wavelength 213.9 nm. Zinc analyses were checked with certified Zn values in standard samples obtained from the Wageningen Evaluating Programme for Analytical Laboratories (WEPAL, Wageningen University, the Netherlands).

STATISTICAL ANALYSIS

Provided statistics are based on GENSTAT analyses. In Experiment 2 contrast analyses were used for the three Zn treatments to make single degree of freedom comparisons first between the low and the two high Zn treatments and then between the two high Zn treatments.

RESULTS

Per plant brown rice dry matter differed among experiments. Values were 3.0, 2.2, and 1.3 g plant^{-1} in Experiments 1–3, respectively. As plant density was twice as high in Experiments 2 and 3 (408 plants m^{-2}) as in Experiment 1 (204 plants m^{-2}), differences in brown rice dry matter per unit area were smaller: 612, 880, and 510 g m^{-2} in Experiments 1–3, respectively. For comparison brown rice dry matter in Experiment 4 was 1.4 g plant^{-1} and Impa et al. (2013) report brown rice dry weights of 0.2 to 4.5 g plant^{-1} depending on genotype. In Experiments 1–3 the pH of the nutrient solution increased from the original 5.6 to about 7.5 when solutions were replaced.

EXPERIMENT 1

The Zn level applied in the nutrient solution led to plants with a rather low plant zinc concentration towards maturity (<15 mg kg^{-1}, **Figure 1**). This low overall plant Zn concentration combined reasonable concentrations in leaves during early growth and in grains with very low stem Zn concentrations.

One week after panicle initiation the ^{70}Zn accumulated since transplanting was allocated to all three distinguished organs, but more than half was observed to be present in the leaf blades (**Figure 2A**).

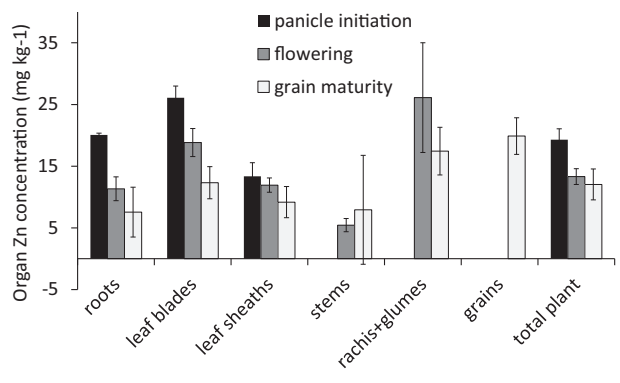

FIGURE 1 | Zinc concentrations (mg kg^{-1}) in the different organs at three harvests averaged across the different ^{70}Zn application treatments (Experiment 1). Error bars indicate standard errors where $n = 2$, 4, and 6 at panicle initiation, flowering, and grain maturity, respectively.

At flowering, plants that had received ^{70}Zn in the preceding 18 days had allocated roughly half of the accumulated ^{70}Zn to their leaf blades, while the remaining organs all received some of the other half, with small differences among organs (**Figure 2B**, grey bars). Plants that had received ^{70}Zn prior to panicle initiation had re-allocated a substantial part of that Zn at flowering. Main net source of ^{70}Zn had been the leaf blades (cf. **Figures 2A,B**), but also roots and leaf sheaths contributed some ^{70}Zn to the newly formed stems, rachis, and glumes, whereas the latter two received most of the re-allocated ^{70}Zn. Overall, most of the newly acquired Zn went to the leaf blades while these same contributed most to newly formed organs through re-allocation of earlier accumulated Zn.

The ^{70}Zn distribution at grain maturity indicated that roughly half of the Zn that had been taken up between flowering and maturity had accumulated in the grains (brown rice), while a further 18% had been accumulated in rachis and glumes, and the remaining was allocated to the other organs, including newly formed tillers (**Figure 2C**). Of the Zn that had been taken up between panicle initiation and flowering, 45% had been re-allocated to the grains. This re-allocation was mainly at the expense of the leaf blades, but also leaf sheaths contributed (compare grey bars in **Figures 2B,C**). The ^{70}Zn accumulated prior to panicle initiation and that had not been re-allocated to reproductive tissues (rachis and glumes) by the time of flowering (**Figure 2B,** dark bars) did not contribute during grain filling through re-allocation to rachis, glumes, or grains. Some re-allocation between leaf sheaths and blades seems to have occurred, while roots re-allocated also some of their Zn. The largest change was the re-allocation of Zn from rachis and glumes to grains (compare dark bars in **Figures 2B,C**).

The observed allocation and re-allocation could also be used to assess how much Zn accumulated at each stage could have minimally contributed to total grain Zn (**Figure 2D**). For Zn taken up during grain filling this is a direct estimate. For Zn accumulated earlier the estimate is lower than the actual value as the observed contribution of ^{70}Zn to total grain Zn does not include the re-allocation of non ^{70}Zn that had been accumulated prior to transplanting and that had been allocated to ^{70}Zn providing tissues simultaneously with the ^{70}Zn. The total reported in **Figure 2D** only accounts for 80% of grain Zn. Possible reasons for this low percentage are discussed below.

EXPERIMENT 2

The rather high Zn levels in Treatments 2 and 3 did not compromise the white rice production per plant (**Table 1**). In fact Qinai-3-hun produced slightly better at the higher Zn nutrition levels, while there were no effects on the other cultivar as indicated by single degree of freedom contrast analysis. The Zn concentrations in the polished grains (white rice) were roughly tripled at the higher Zn nutrition level compared to the standard Zn nutrition across the two cultivars. The ^{70}Zn enrichment also differed between the high and standard level Zn treatments. With ^{70}Zn accounting for about 30% of the grain Zn at the lower Zn level and between 42 and 55% for the high Zn level, the role of re-allocation from vegetative tissues seems to be slightly suppressed when Zn supply levels and plant Zn status are high.

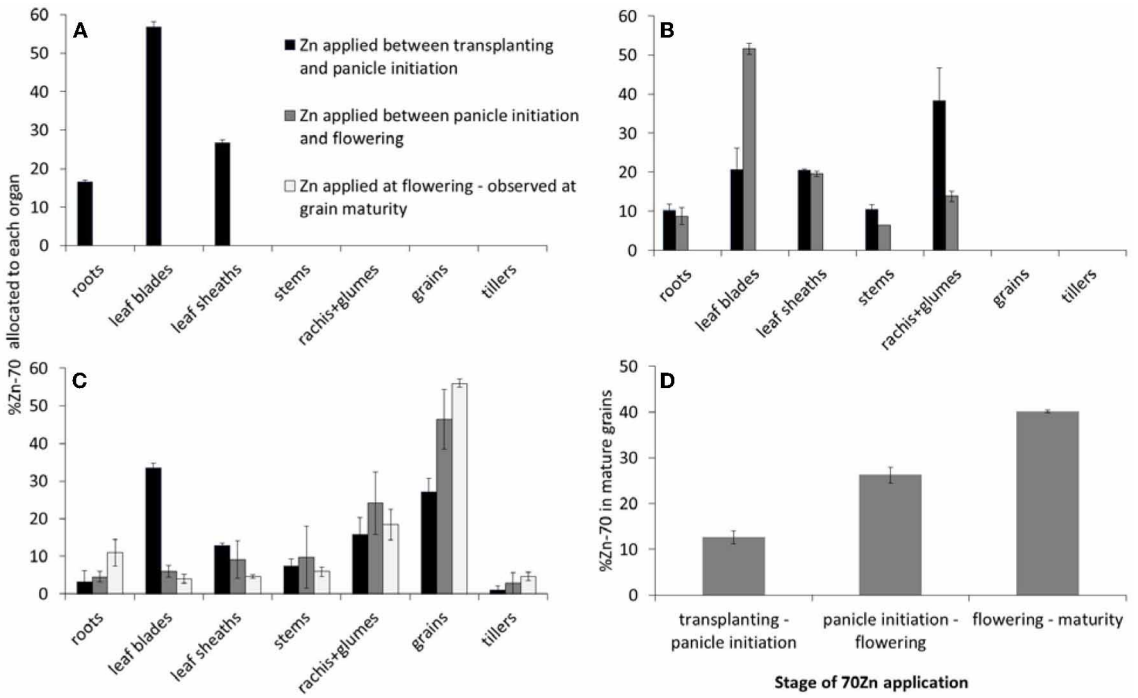

FIGURE 2 | Allocation of ^{70}Zn as observed by destructive sampling at 1 week after panicle initiation (A), at flowering (B), and at grain maturity (C), and as influenced by the period during which the plants received the ^{70}Zn (D) (Experiment 2). Panel **(D)** shows the calculated percentage of total grain ^{70}Zn at grain maturity that can be traced back to ^{70}Zn that was applied during the three distinguished development stages. Error bars indicate standard errors ($n = 2$).

EXPERIMENT 3

Total grain Zn concentration dropped between 7 and 14 days after flowering and remained more or less constant for the remaining time of the grain filling period (**Figure 3A**). The trends were independent of Zn supply levels, while concentrations were approximately 40 mg kg^{-1} higher at the higher Zn supply level.

The concentrations in the endosperm showed differences between Zn application levels comparable to those for total grain concentrations (**Figures 3A,C**). Embryo concentrations were an order of magnitude above those of the endosperm and tended to converge for the two Zn application levels (**Figure 3D**). Vascular bundle concentrations were intermediate between embryo and endosperm and a difference between the two supply levels of about 100 mg kg^{-1} was maintained (**Figure 3B**). Both embryo and vascular bundle concentrations rather seemed to decrease with time while those in the endosperm slightly increased with time.

RE-ANALYSIS OF DATA FROM THE LITERATURE

Root to shoot transfer

Data from Experiment 4 indicate that the concentrations in roots tended to be higher than those in leaf sheath (when no stems were present) or in stems at the lower range of tissue Zn concentrations for both tested cultivars (**Figures 4A,B**). Combining these data at the lower plant tissue concentration range with those from Impa et al. (2013) (**Figures 5A–C**) shows possible genotypic differences in the root to shoot transfer. While at the early vegetative phase some genotypes showed a much higher concentration in the shoots than in the roots, at later stages the opposite trend was observed as the higher concentrations were only observed in roots rather than in the shoots. In other words root to shoot transfer efficiency seems to differ both among genotypes and between developmental stages.

Within-shoot allocation

The allocation between leaf blades and leaf sheaths during the vegetative stage (**Figures 4C,D**) shows that the two cultivars tested maintained a largely comparable allocation pattern at increasing plant Zn concentrations. The same pattern was observed during reproductive stages between leaf blades and stems. While sheath concentrations in the experiment were not lower than 40–60 ppm during the vegetative stage they dropped well below 20 ppm during the reproductive stages. At these stages plants maintained lower tissue Zn concentrations in the leaf blades than in the leaf sheaths when sheath concentrations were above 20–30 ppm, but maintained leaf blade concentrations consistently above 20 ppm while allowing sheath concentrations to drop as low as 10–15 ppm.

A comparison of leaf sheath and stem concentrations during these reproductive stages shows differences between the two cultivars: Baxiludao maintained higher sheath Zn concentrations than Handao 502 at increasing stem Zn concentrations. Both cultivars, though, had higher stem than sheath Zn concentrations when

Table 1 | Grain weight per plant (brown rice), total Zn concentrations in the polished (white) rice and ^{70}Zn (% of total Zn) observed for three treatments imposed on two cultivars (cv) (Experiment 2).

Cultivar	Zn treatments		Grain weight (g (plant^{-1})	Zinc in polished rice (mg kg^{-1})	^{70}Zn in polished rice (%)
Qinai-3-hun	1[1]		1.48	14.9	30.1
	2[2]		2.10	51.3	42.0
	3[3]		2.29	52.9	44.8
90B290	1[1]		1.35	14.8	29.6
	2[2]		1.19	38.7	50.3
	3[3]		1.63	48.7	55.2
P	Cv		0.005	ns	0.03
	Zn	1 vs. 2 and 3	0.02	<0.001	<0.001
		2 vs. 3	ns	ns	ns
	Int	Cv × 1 vs. 2 and 3	0.048	ns	ns
		Cv × 2 vs. 3	ns	ns	ns
SED	Cv		–	3.04	2.11
	Zn		–	3.73	2.58
	Int		0.41	–	–

[1] *0.05 mg Zn L^{-1} nutrient solution;*

[2] *3.00 mg Zn L^{-1} during first 28 days after transplanting followed by 1.00 mg L^{-1} until harvest;*

[3] *3.00 mg Zn L^{-1} during first 28 days after transplanting followed by 2.00 mg L^{-1} until harvest.*

stem concentrations were above 40–50 ppm, but lower when stem concentrations decreased to 20 ppm.

As Impa et al. (2013) only reported Zn concentrations of leaf blades on the one hand and the combination of sheaths and stem on the other, these have been combined with the same data for Experiment 4 (**Figures 5D,E**). The data from Impa et al. essentially only report the tissue Zn concentrations where leaf blade and stems plus sheath concentrations were approximately equal with a tendency for leaf blade Zn concentrations to be slightly lower than stem plus sheath concentrations when leaf blade concentrations were above 20 ppm. The data from Experiment 4 overlap with those from Impa et al. (2013) at the lower levels of tissue concentrations and add information for conditions when more Zn would be accumulated.

Once the stem is elongating following panicle initiation the stem plays a central role in the transport between vegetative and reproductive tissues. The different concentrations of organs are therefore plotted against the stem concentrations with the exception of the early leaf blades which are plotted against either the sheath concentration (during vegetative growth) or the stem concentration (during reproductive growth).

Generative tissues

As for leaf blades the panicle tissues other than grains (rachis and glumes) had higher concentrations than the stem when the latter had concentrations below 40 ppm, while at higher stem concentrations rachis and glumes maintained concentrations over a much smaller concentration span (**Figures 6A,B**). The data from Impa et al. (2013) show a different picture at flowering when rachis and glume tissues tended to be higher than stems and sheath concentrations reaching up to 75 ppm at stem concentrations of around 40 ppm while in Experiment 4 such levels were only reached at stem concentrations around 180 ppm (**Figure 5G**). At grain maturity, panicle tissue Zn concentrations had dropped below that in the stem and sheath generally at stem and sheath concentrations even below 20 ppm (**Figure 5H**). The data from Experiment 4 seem to be at the lower end of panicle tissue concentrations of the cultivars tested by Impa et al. (2013) especially at flowering (**Figure 5G**).

During and at the end of grain filling in Experiment 4 (**Figures 5C,D**) the concentrations in brown rice were always lower than those observed in panicle tissues, while both remained above 20 ppm during these experiments. In the experiments by Impa et al. (2013) lower grain and panicle tissue concentrations were obtained but over the full range of observed concentrations brown rice concentrations were usually higher than panicle tissue concentrations (**Figure 5F**).

DISCUSSION

In Experiments 1–3 effects of Zn nutrition were studied over prolonged periods of time using stable isotopes. In contrast to more standard practices the nutrient solution was only replaced a few times in order to avoid regular replacement of the nutrient solution, which would have meant discarding very expensive stable isotopes. The attained grain weights of 510–880 g m^{-2} are reasonable to good rice yields. The un-orthodox nutritional strategy seemed to have resulted in decent plant growth providing results that are comparable to those of other studies. As a consequence of the prolonged plant growth on the same solution, the pH gradually increased from the original 5.6 to about 7.5 by the time the solution was replaced. In soils such high pH values could have led to low availability of Zn to plant uptake. Within this pH range of nutrient solutions, however, Zn availability was hardly reduced. We surmise that Zn availability was not a limiting factor.

The data from Experiment 1 on allocation and re-allocation of Zn in rice suggest that about 40% of seed Zn at grain maturity originated from uptake after flowering, against 26% originating from uptake between panicle initiation and flowering and 12% originating from uptake between transplanting and panicle initiation. The remaining 20% could not be accounted for. This 20% loss is at least partly caused by the root washing method. Root washing might have caused some loss of adhering Zn during transfer from the natural abundance Zn solution to the ^{70}Zn containing solution, and vice versa. This effect would have been larger the higher the total root mass was at transfer. So the reported contributions of Zn taken up during grain filling and during the 18 days preceding flowering might have been relatively underestimated compared to the contribution of Zn applied prior to flowering and the Zn applied during the vegetative growth. Despite this imprecision we surmise that, for the accession tested, both allocation of pre- and post-flowering acquired Zn contributed approximately equally to grain Zn. This conclusion could also be drawn from Experiment 2 (**Table 1**), but this experiment

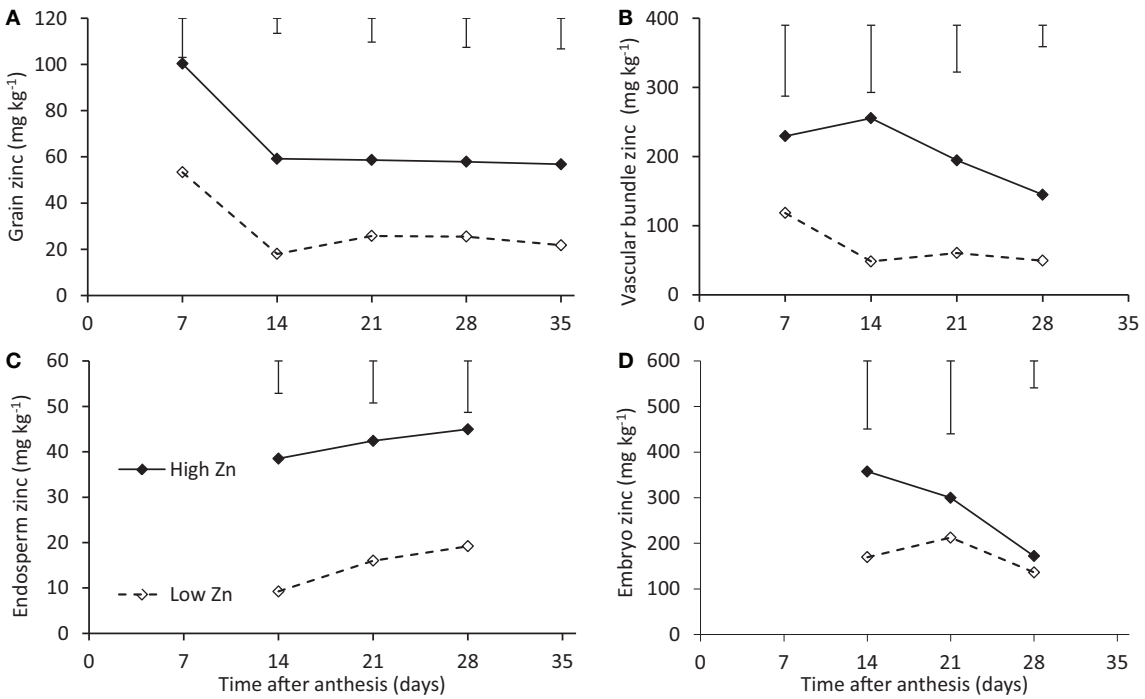

FIGURE 3 | Zinc concentrations of rice seeds of cv. Qinai-3-hun during grain filling when cultivated on a nutrient solution with either a normal (open symbols) or an extremely high (closed symbols) Zn level (Experiment 3). Total grain concentrations **(A)** and concentrations of the vascular bundle **(B)**, endosperm **(C)**, and embryo **(D)** are shown, at 7 days after anthesis no good distinction could be made between embryo and endosperm so tissues were lumped. Note that the scales along the y-axes differ.

further hints at possible genotypic differences in the relative roles of direct allocation and of re-allocation during grain filling, and at a possible effect of the amounts of Zn that are taken up during grain filling.

These findings contribute to the data from other authors that together provide evidence for genotypic differences in the relative roles of Zn from different sources to grain Zn. Many data at the level of budget analysis indicate that total uptake after flowering at least equals grain allocation (Jiang et al., 2008a,b; Impa et al., 2013; Mabesa et al., 2013). This could be interpreted as an indication that re-allocation would play a very limited role in grain Zn allocation. Yet the data presented here indicate that the dynamics of allocation and re-allocation within the plant are more complex than can be inferred on the basis of budget analyses alone. We have provided arguments that newly acquired Zn may partly replaces earlier acquired Zn in vegetative tissues while the earlier acquired Zn is re-allocated to the seeds. The relevance of such dynamics lies in the possibility to use this information for a more targeted breeding approach. While Jiang et al. (2007) and Wu et al. (2010) found that foliar applied Zn hardly ended up in the grains, recent studies (Phattarakul et al., 2012; Mabesa et al., 2013) suggest that enhancing plant Zn by foliar application does lead to higher grain Zn concentrations. One possible explanation for this apparent discrepancy among studies is that genotypes react differently; an equally plausible explanation based on results presented here is that as leaves are enriched by foliar-applied Zn more Zn from direct uptake is directed towards the grains.

The reported important role for Zn re-allocation from rice stems rather than leaves (Jiang et al., 2008a; Wu et al., 2010) seems to contrast with the results from Experiment 1. Taking a closer look at those data from Jiang et al. (2008a), though, provides evidence that the role of the stem in re-allocation of Zn is a direct function of its role in sequestering Zn that is taken up during pre-grain filling stages in excess of what is needed for optimal functioning of the physiologically most active tissues (e.g., leaf blades). The plant tissue levels in Experiment 1 were such that no extra Zn could be stored in the stem and thus it could not play the same role in providing Zn to the grains that it seemed to have played in the studies by Wu et al. (2010) and Jiang et al. (2008a). The concentration of Zn in the stems observed (10 mg kg^{-1}) is of the order indicated by Reuter et al. (1997) to be critical for normal tissue functioning. Most likely the remaining Zn is not available for re-allocation. By combining these results, it can be postulated that plants preferentially re-translocate Zn from the stem unless stem Zn concentration reaches a minimum level below which the remaining Zn is locked in.

The ability to re-allocate Zn from vegetative tissues during grain filling and translocate this Zn to grains will be an important trait especially under field conditions where additional Zn uptake during grain filling might not always be easy. However, an important aspect in breeding for this trait will be to avoid that re-allocation leads to too low levels of Zn in the vegetative tissues that would compromise productivity, especially in leaves. The re-allocation that would make use of senescence enhancing

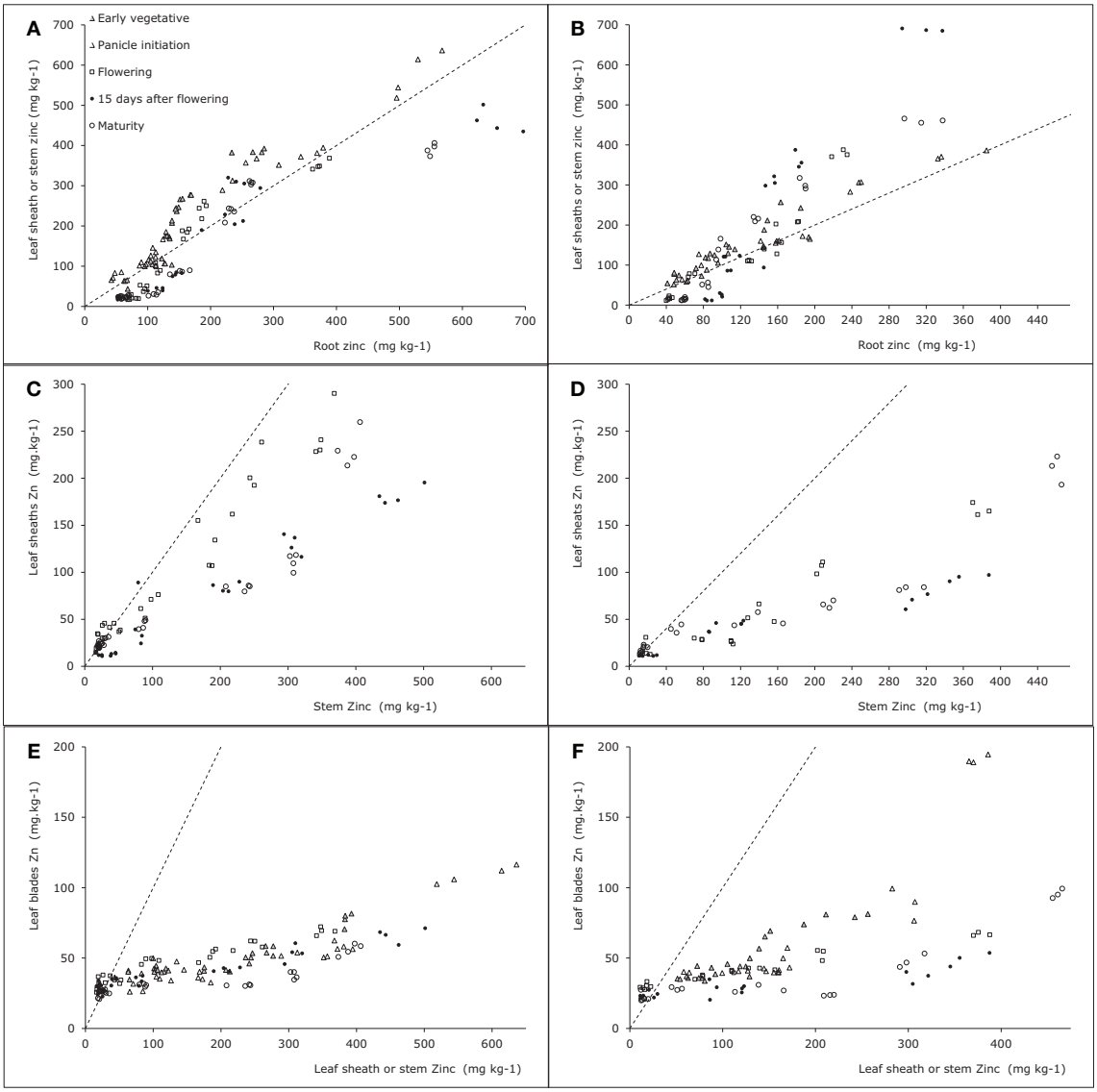

FIGURE 4 | Zinc concentrations in vegetative plant tissues as indicated along the axes for two rice genotypes tested in Experiment 4 [see also Jiang et al. (2008a)]. Panels **(A)**, **(C)**, and **(E)** are for Baxiludao, Panels **(B)**, **(D)**, and **(F)** for Handao 502. Data represent individual replications. The filled and open triangles, open squares, black dots, and open circles represent plants during early vegetative growth, panicle initiation, flowering, mid grain filling, and grain maturity respectively, the broken lines indicate the 1:1 line in each panel.

genes to improve grain nutritional quality (e.g., Uauy et al., 2006) seems much less appropriate in environments with a high potential for enhanced production by a prolonged grain-filling phase. Combining the ability to maintain photosynthesis and to accumulate grain Zn would then seem most profitable. In other words a combination of the abilities (1) to accumulate more Zn and maintain higher Zn concentrations during the vegetative stage than is necessary for optimal plant functioning, (2) to re-allocate such Zn to generative tissue while keeping production-relevant tissues at levels that allow optimal functioning, (3) to maintain Zn uptake during grain filling, and (4) to maintain full functioning at relatively low Zn levels in tissues, i.e., a high Zn use efficiency (*in sensu* Hacisalihoglu and Kochian, 2003).

The cultivar studied in Experiment 1 seemed rather effective in re-allocating Zn from its leaves to other plant parts, with a large portion presumably being re-allocated to generative tissues including the grain. This contrasts with observations on re-allocation of foliar-applied labeled Zn in rice. Jiang et al. (2007) found that about 50% of radioactive ^{65}Zn applied to the flag leaf was re-allocated, but only around 2% was re-allocated to grains. Wu et al. (2010), working among others with a high Zn density cultivar from IRRI, observed that 18–29% of flag leaf-applied ^{68}Zn was re-allocated to other plant tissues and only 4.1–8.9% of flag leaf applied Zn was found in grains. Re-allocation of endogenous Zn might be more effective than re-allocation of leaf-applied Zn in rice. This seems to contrast with what has been observed

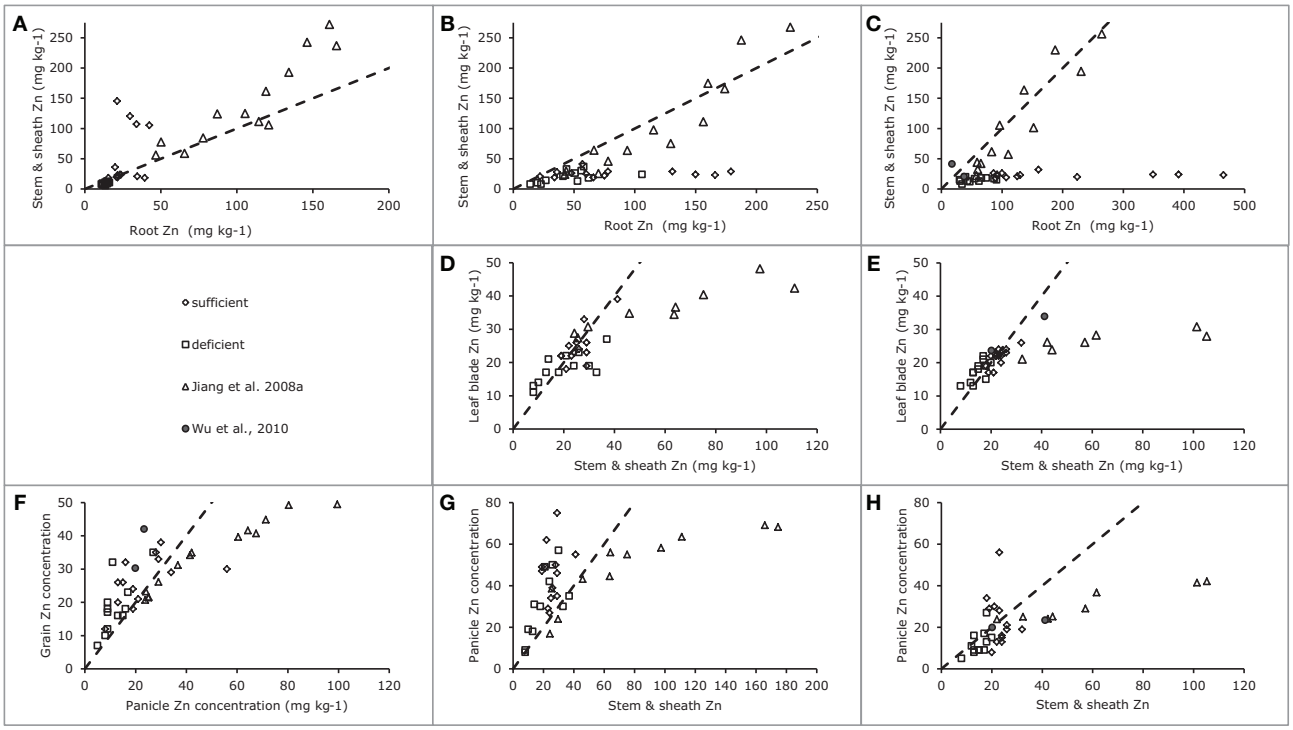

FIGURE 5 | Zinc concentrations in generative plant tissues as indicated along the axes for data from Impa et al. (2013), Wu et al. (2010) and Experiment 4 [see also Jiang et al. (2008a)]. Panel **(A)** for plants harvested during the early vegetative stage, Panels **(B)**, **(D)**, and **(G)** for plants harvested at flowering and Panels **(C)**, **(E)**, **(F)**, and **(H)** for plants harvested at grain maturity. Data points from Impa et al. (2013) represent averages per tested genotype grown at either sufficient (diamonds) of deficient (squares) Zn nutrition, data points from Wu et al. (2010) (filled circles) represent averages of two genotypes grown at sufficient Zn nutrition and data points from Jiang et al. (2008a). (Experiment 4) (triangles) represent averages for two genotypes grown at a range of Zn nutrition levels. The broken lines indicate the 1:1 line in each panel.

for foliar-applied labeled Zn in wheat (Erenoglu et al., 2011) and could be an important aspect in the explanation why rice was less responsive to leaf Zn applications than has been reported for wheat (cf. Yilmaz et al., 1997; Phattarakul et al., 2012).

Wu et al. (2011) also showed patterns of accumulation of labeled Zn (^{68}Zn) in the rice grain providing further insight into dynamics of direct allocation and re-allocation. Until 15 days after flowering the concentration of directly allocated ^{68}Zn increased steeply and also the relative contribution of ^{68}Zn increased; thereafter the relative contribution of ^{68}Zn remained more or less stable. Direct allocation seems to be of increasing importance at the start of grain filling while later on the relative roles of direct allocation and re-allocation flows remained the same. The role of transpiration in maintaining a xylem influx could be important (cf. Pearson et al., 1996) as transpiration of glumes is bound to decline during late grain filling.

Rice seems to function differently from wheat in the grain transport of Zn during grain filling. For wheat an increase in vascular tissue Zn concentrations was observed towards later stages of grain filling while endosperm concentrations slightly dropped (Stomph et al., 2011). We observed for rice (Experiment 3) rather a decreasing vascular tissue concentration and a slightly increasing trend in the endosperm. This further emphasizes that Zn transport processes differ between plant species even for grasses as closely related as these two cereals (cf. Palmgren et al., 2008; Stomph et al., 2009). The reasons for these differences still need to be elucidated but the role of anatomical differences, like those between rice and wheat grains, should definitely be included in further studies.

Combining the data presented here and in the literature the overall picture that emerges is as follows (**Figure 7**). During the vegetative growth allocation between roots and shoots is directed at maintaining approximately the same concentrations in both tissues, with a slightly higher root concentration. There is some evidence for genotypic differences that may allow breeding for more shoot allocation (**Figure 5A**). The extent to which there may be some physiological barrier to root-to-shoot transfer varies among genotypes as is evident from the work by Wu et al. (2010) who showed that of the Zn applied during the vegetative stage very different proportions ended up in the shoot (their **Table 1**). For later stages of plant development the data from Experiment 4 suggest that the relative concentrations maintained in roots and leaf sheath (or sheath and stems when the latter are present) are not very susceptible to the total Zn that is taken up, both increasing proportionally. The data from Impa et al. (2013), though, indicate that there might be genotypes with much greater root-to-shoot transfer barriers as plants develop (**Figures 5B,C**). For all leaf blades and reproductive tissues there is evidence from

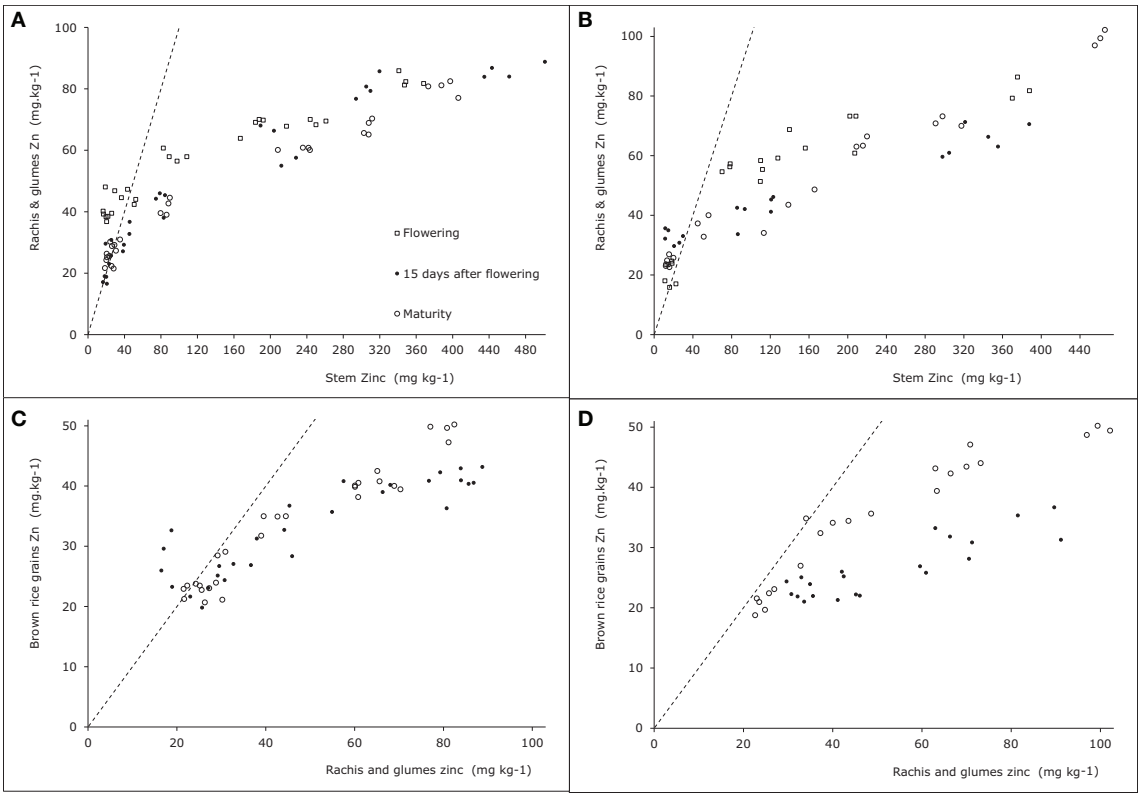

FIGURE 6 | Zinc concentrations in generative plant tissues as indicated along the y-axes for two rice genotypes tested in Experiment 4 [see also Jiang et al. (2008a)]. Panels **(A)** and **(C)** are for Baxiludao, Panels **(B)** and **(D)** for Handao 502. Data represent individual replications. The open squares, black dots, and open circles represent plants during flowering, mid grain filling, and grain maturity respectively, the broken lines indicate the 1:1 line in each panel.

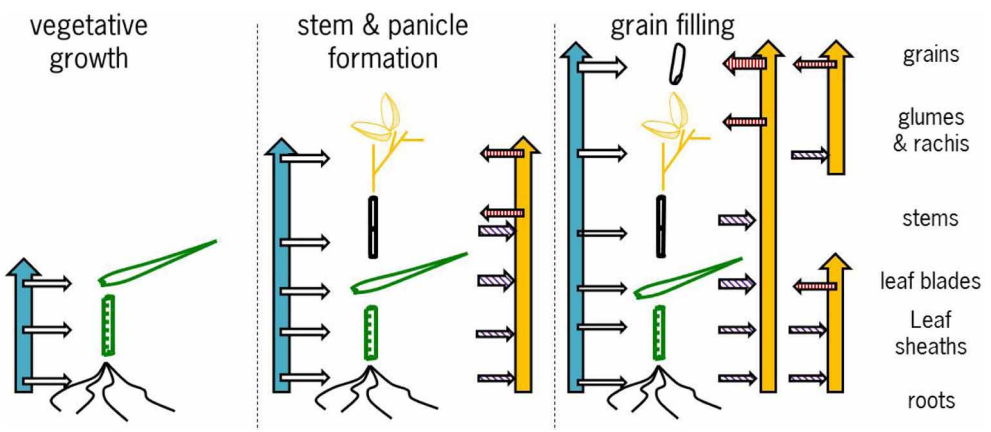

FIGURE 7 | Graphical representation of the proposed conceptual model of allocation and re-translocation of zinc during the subsequent stages of cereal development. The blue vertical and the horizontal arrows to the left of each panel indicate uptake and direct allocation to individual tissues, while the yellow vertical arrows to the right of each panel indicate re-allocation flows. The horizontal arrows with purple hatching indicate when tissues are a source of Zn for re-translocation and the horizontal arrows with a red hatching indicate when tissues are sink for Zn from re-translocation. For further explanation see the text.

Experiment 4 that as overall plant Zn concentrations increase above 40 mg kg^{-1} the Zn concentrations of these tissues increase proportionally much less than Zn concentrations in roots, sheaths and stem (**Figures 4–6**).

Zinc that is taken up between panicle initiation and flowering (**Figure 7**, middle panel) is allocated to all developing tissues (data Experiment 1) while also re-allocation takes place from vegetative to reproductive tissues. Depending on the actual total plant

Zn uptake this re-allocation between tissues is hidden in a budget analysis as all tissues may increase in both or either Zn content and concentration. The stable isotope studies (Experiment 1; Wu et al., 2010, 2011) clearly show much larger dynamics of allocation and re-allocation of Zn, drivers for which are still not fully understood. For breeding for high grain Zn the exact routes of Zn allocation and re-allocation are not very important, a major point is to enhance the ability to accumulate enough Zn at this stage to allow later re-allocation to the grains.

Comparable dynamics of allocation and re-allocation are also seen during the grain filling stages (**Figure 7**, right panel). Both direct allocation of newly acquired Zn and re-shuffling of earlier acquired Zn occurred in Experiment 1. The relative Zn concentrations in non-grain panicle tissues and rice stem plus sheaths showed large differences among genotypes (data from Impa et al., 2013, **Figures 5G,H**) with in general higher concentrations in panicle structures than in sheaths and stem at flowering but equal or lower concentrations in panicle structures at grain maturity. The general decrease in panicle structure Zn concentration between flowering and grain maturity at comparable stem and sheath concentrations hints at a relative transfer barrier between the vegetative and reproductive tissues. For the same set of genotypes grain Zn concentrations were higher than panicle structure concentrations (**Figure 5F**). Taken together this implies that there is much less barrier for an effective loading of the grain from the panicle structures than for an effective loading of the panicle from the stem and sheath. This loading becomes increasingly less effective when stem and sheath levels are higher as was observed in Experiment 4.

Within the grains, concentrations in the vascular tissues were clearly higher than in the endosperm (**Figure 3**). When the concentration in the endosperm was corrected for the presence of chemically inactive starch assuming roughly two thirds starch (Stomph et al., 2009), the drop in concentration between vascular tissue and endosperm became negligible and thus does not seem to imply a transport barrier.

In support of breeding the question to answer remains which barriers to tackle in the route from soil to seed. This paper has not addressed the soil to root barriers, which are highly relevant as well. The study by Wissuwa et al. (2008) showed how genotypes that differed substantially at high Zn soil (between 28.2 and 37.7 mg Zn kg^{-1} grain) averaged around 7.8 mg Zn kg^{-1} grain on severely Zn-deficient soils with limited differences between genotypes. Enhancing plant Zn uptake on these Zn deficient soils would at first have increased productivity and thereafter enhanced grain Zn levels. Within the plant there seem to be genotypic and development stage differences in the ease in transfer from root to shoots that could be further studied and linked to quantitative trait loci or markers for marker-assisted breeding. The data presented here clearly indicate that a larger shoot allocation of root Zn will mainly lead to an enhanced Zn concentration in stems and sheath. Priority should therefore arguably be given to the barrier between stem (or stem and sheaths) and panicle tissues. As the transfer between these and the grain seems relatively easy, enhancing stem to panicle transfer is probably going to have a much larger impact on grain Zn concentration. The data from the experiments at the International Rice Research Institute (Impa et al., 2013) suggest some but not a very large genotypic variation among the currently used high Zn lines towards grain maturity but a larger variation at flowering. Further characterization of these dynamics during grain filling could provide insight into possible interesting lines.

CONCLUSIONS

- Both pre- and post-flowering Zn uptake contributes substantially to grain Zn loading when Zn uptake continues after flowering.
- Post-flowering Zn uptake also serves to replace Zn that is remobilized from other organs to the grains, thus maintaining Zn concentrations in tissues like leaf blades.
- The Zn that was taken up prior to panicle initiation was in the stable isotope study remobilized to a lower extent than Zn that was taken up between panicle initiation and flowering.
- In all tissues only part of earlier allocated Zn was remobilized; there seems to be both a pool of Zn that can be re-allocated and Zn that is not available for re-allocation, but is sequestered in vegetative or structural tissues.
- There are transfer bottlenecks between root and shoot and between stem and panicle, alleviating the latter is most effectively going to contribute to grain Zn enhancement.
- The within grain Zn allocation from vascular tissue to endosperm in rice differs substantially from that in wheat.

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REFERENCES

Erenoglu, E. B., Kutman, U. B., Ceylan, Y., Yildiz, B., and Cakmak, I. (2011). Improved nitrogen nutrition enhances root uptake, root-to-shoot translocation and remobilization of zinc (^{65}Zn) in wheat. *New Phytol.* 189, 438–448. doi: 10.1111/j.1469-8137.2010.03488.x

Fageria, N. K., Baligar, V. C., and Clark, R. B. (2002). Micronutrients in crop production. *Adv. Agron.* 77, 185–268. doi: 10.1016/S0065-2113(02)77015-6

Garnett, T. P., and Graham, R. D. (2005). Distribution and remobilization of iron and copper in wheat. *Ann. Bot.* 95, 817–826. doi: 10.1093/aob/mci085

Graham, R. D., and Welch, R. M. (1996). "Breeding for staple food crops with high micronutrient density," in *Working Papers on Agricultural Strategies for Micronutrients No.3* (Washington, DC: International food policy research institute (IFPRI)), 72.

Graham, R. D., Welch, R. M., Saunders, D. A., Ortiz Monasterio, I., Bouis, H. E., Bonierbale, M., et al. (2007). Nutritious subsistence food systems. *Adv. Agron.* 92, 1–74. doi: 10.1016/S0065-2113(04)92001-9

Grusak, M. A., Pearson, J. N., and Marentes, E. (1999). The physiology of micronutrient homeostasis in field crops. *Field Crops Res.* 60, 41–56. doi: 10.1016/S0378-4290(98)00132-4

Hacisalihoglu, G., and Kochian, V. L. (2003). How do plants tolerate low levels of zinc? Mechanisms of zinc efficiency in crop plants. *New Phytol.* 159, 341–350. doi: 10.1046/j.1469-8137.2003.00826.x

Hoffland, E., Jeger, M. J., and van Beusichem, M. L. (2000). Effect of nitrogen supply rate on disease resistance in tomato depends on the pathogen. *Plant Soil* 218, 239–247. doi: 10.1023/A:1014960507981

Impa, S. M., Morete, M. J., Ismail, A. M., Schulin, R., and Johnson-Beebout, S. E. (2013). Zn uptake, translocation and grain Zn loading in rice (*Oryza sativa* L.) genotypes selected for Zn deficiency tolerance and high grain Zn. *J. Exp. Bot.* 64, 2739–2751. doi: 10.1093/jxb/ert118

Jiang, W., Struik, P. C., Lingna, J., van Keulen, H., Zhao, M., and Stomph, T. J. (2007). Uptake and distribution of root-applied or foliar-applied ^{65}Zn after flowering in aerobic rice. *Ann. Appl. Biol.* 150, 383–391. doi: 10.1111/j.1744-7348.2007.00138.x

Jiang, W., Struik, P. C., van Keulen, H., Zhao, M., Jin, L. N., and Stomph, T. J. (2008a). Does increased zinc uptake enhance grain zinc mass concentration in rice? *Ann. Appl. Biol.* 153, 135–147. doi: 10.1111/j.1744-7348.2008.00243.x

Jiang, W., Struik, P. C., Zhao, M., van Keulen, H., Fan, T. Q., and Stomph, T. J. (2008b). Indices to screen for grain yield and grain zinc mass concentrations in aerobic rice at different soil-Zn levels. *NJAS Wagen. J. Life Sci.* 55, 181–197. doi: 10.1016/S1573-5214(08)80036-X

Kutman, U. B., Yildiz, B., and Cakmak, I. (2011). Effect of nitrogen on uptake, remobilization and partitioning of zinc and iron throughout the development of durum wheat. *Plant Soil* 342, 149–164. doi: 10.1007/s11104-010-0679-5

Mabesa, R. L., Impa, S. M., Grewal, S., and Johnson-Beebout, S. E. (2013). Contrasting grain-Zn response of biofortification rice (*Oryza sativa* L.) breeding lines to foliar Zn application. *Field Crops Res* 149, 223–233. doi: 10.1016/j.fcr.2013.05.012

Marschner, H. (1995). *Mineral Nutrition of Higher Plants, 2nd Edn.* London: Academic Press.

Palmgren, M. G., Clemens, S., Williams, L. E., Kraemer, U., Borg, S., Schjoerring, J. K., et al. (2008). Zinc biofortification of cereals: problems and solutions. *Trends Plant Sci.* 13, 464–473. doi: 10.1016/j.tplants.2008.06.005

Pearson, J. N., and Rengel, Z. (1994). Distribution and remobilization of Zn and Mn during grain development in wheat. *J. Exp. Bot.* 45, 1829–1835. doi: 10.1093/jxb/45.12.1829

Pearson, J. N., Rengel, Z., Jenner, C. F., and Graham, R. D. (1995). Transport of zinc and manganese to developing wheat grains. *Physiol. Plant.* 95, 449–455. doi: 10.1111/j.1399-3054.1995.tb00862.x

Pearson, J. N., Rengel, Z., Jenner, C. F., and Graham, R. D. (1996). Manipulation of xylem transport affects Zn and Mn transport into developing wheat grains of cultured ears. *Physiol. Plant.* 98, 229–234. doi: 10.1034/j.1399-3054.1996.980202.x

Phattarakul, N., Rerkasem, B., Li, L. J., Wu, L. H., Zou, C. Q., Ram, H., et al. (2012). Biofortification of rice grain with zinc through zinc fertilization in different countries. *Plant Soil* 361, 131–141. doi: 10.1007/s11104-012-1211-x

Rengel, Z. (ed.). (1999). *Mineral Nutrition of Crops: Fundamental Mechanisms and Implications*. New York, London, Oxford: The Haworth Press.

Rengel, Z. (2001). "Xylem and phloem transport of micronutrients," in *Plant Nutrition – Food Security and Sustainability of Agro-Ecosystems,* ed W. J. Horst (Dordrecht: Kluwer Academic Publishers), 628–629.

Reuter, D. J., Robinson, J. B., and Dutkiewicz, C. (1997). *Plant Analysis: an Interpretation Manual, 2nd Edn.* Collingwood, VIC: CSIRO Publishing.

Slingerland, M. A., Traore, K., Kayodé, A. P. P., and Mitchikpe, C. E. S. (2006). Fighting Fe deficiency malnutrition in West Africa: an interdisciplinary programme on a food chain approach. *NJAS Wagen. J. Life Sci.* 53, 253–279. doi: 10.1016/S1573-5214(06)80009-6

Slingerland, M. A., Zhang, F., Stomph, T. J., Gao, X., Liang, J., and Jiang, W. (2009). "Biofortification in a food chain approach for rice in China," in *Development and Uses of Biofortified Agricultural Products,* eds G. S. Banuelos and Z. Q. Lin (Boca Raton, London, New York: CRC Press), 181–203.

Stomph, T. J., Choi, E. Y., and Stangoulis, J. C. R. (2011). Temporal dynamics in wheat grain zinc distribution: is sink limitation the key? *Ann. Bot.* 107, 927–937. doi: 10.1093/aob/mcr040

Stomph, T. J., Jiang, W., and Struik, P. C. (2009). Zinc biofortification of cereals: rice differs from wheat and barley. *Trends Plant Sci.* 14, 23–24. doi: 10.1016/j.tplants.2009.01.001

Uauy, C., Distelfeld, A., Fahima, T., Blechi, A., and Dubcovsky, J. (2006). A NAC gene regulating senescence improves grain protein, zinc and iron content in wheat. *Science* 314, 1298–1301. doi: 10.1126/science.1133649

Waters, B. M., and Sankaran, R. P. (2010). Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

Welch, R. M. (1995). Micronutrient nutrition of plants. *Crit. Rev. Plant Sci.* 14, 49–82. doi: 10.1080/07352689509701922

Welch, R. M., and Graham, R. D. (1999). A new paradigm for world agriculture: meeting human needs - productive, sustainable, nutritious. *Field Crops Res.* 60, 1–10. doi: 10.1016/S0378-4290(98)00129-4

Welch, R. M., and Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* 33, 353–364. doi: 10.1093/jxb/erh064

Wheal, M. S., Fowles, T. O., and Palmer, L. T. (2011). A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of plant essential elements. *Anal. Methods* 3, 2854–2863. doi: 10.1039/c1ay05430a

Wissuwa, M., Ismail, A. M., and Graham, R. D. (2008). Rice grain zinc concentrations as affected by genotype, native soil-zinc availability, and zinc fertilization. *Plant Soil* 306, 37–48. doi: 10.1007/s11104-007-9368-4

Wolswinkel, P. (1999). "Long-distance nutrient transport in plants and movement into developing grains," in *Mineral Nutrition of Crops: Fundamental Mechanisms and Implications,* ed Z. Rengel (New York, London, Oxford: The Haworth press), 91–113.

Wu, C., Feng, Y., Shohag, J. I., Lu, L., Wei, Y., Gao, C., et al. (2011). Characterization of Zn uptake, translocation and accumulation into developing grains and young leaves of high Zn-density rice genotype. *J. Zhejiang Univ. Sci. B* 12, 408–418. doi: 10.1631/jzus.B1000291

Wu, C., Lu, L., Yang, X., Feng, Y., Wei, Y., Hao, H., et al. (2010) Uptake, translocation, and remobilization of Zn absorbed at different growth stages by rice genotypes of different Zn densities. *J. Agric. Food Chem.* 58, 6767–6773. doi: 10.1021/jf100017e

Yilmaz, A., Ekiz, H., Torun, B., Gultekin, I., Karanlik, S., Babci, S. A., et al. (1997). Effect of different zinc application methods on grain yield and zinc concentration in wheat grown on zinc-deficient calcareous soils in Central Anatolia. *J. Plant Nutr.* 20, 461–471. doi: 10.1080/01904169709365267

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Vacuolar sequestration capacity and long-distance metal transport in plants

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The vacuole is a pivotal organelle functioning in storage of metabolites, mineral nutrients, and toxicants in higher plants. Accumulating evidence indicates that in addition to its storage role, the vacuole contributes essentially to long-distance transport of metals, through the modulation of Vacuolar sequestration capacity (VSC) which is shown to be primarily controlled by cytosolic metal chelators and tonoplast-localized transporters, or the interaction between them. Plants adapt to their environments by dynamic regulation of VSC for specific metals and hence targeting metals to specific tissues. Study of VSC provides not only a new angle to understand the long-distance root-to-shoot transport of minerals in plants, but also an efficient way to biofortify essential mineral nutrients or to phytoremediate non-essential metal pollution. The current review will focus on the most recent proceedings on the interaction mechanisms between VSC regulation and long-distance metal transport.

Keywords: vacuolar sequestration capacity, vacuole, transporter, chelator, metal transport

INTRODUCTION

Mineral nutrients including iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), nickel (Ni), and molybdenum (Mo) are required in different amounts by different plant tissues. Therefore, once taken up into plants, long-distance transport and allocation of these metals play a pivotal role in plant development and adaptation to the environment. To date, studies of long-distance root-to-shoot metal transport within plants focus preferentially on transporters localized to either xylem parenchyma cells or phloem companion cells, as they directly mediate xylem and phloem loading or unloading thus contributing essentially to this metal reallocation process. Moreover, some chelators, including nicotianamine (NA; reviewed by Curie et al., 2009), glutathione (GSH), and phytochelatins (PCs; reviewed by Mendoza-Cozatl et al., 2011), were observed to also act as important players in the process of long-distance transport of metals.

Plant vacuoles are predominant organelles serving as temporary storages for essential and toxic metabolites, mineral nutrients and toxic pollutants. Accumulating evidences indicate that in addition to the direct regulation from those ion transporters and chelators, the Vacuole sequestration capacity (VSC) also contributes substantially to long-distance transport and allocation of metals in plants. Vacuoles function as buffering pools where the sequestration capacities are dynamically adjusted to the changing environmental cues, through the interaction between tonoplast-localized transporters and ion chelators. This mini-review will focus on the regulation of VSC and its interaction with long-distance root-to-shoot transport of either essential or non-essential metals in plants.

REGULATION OF VSC

REGULATION BY TRANSPORTERS

Up to date, most tonoplast-localized metal transporters in plants (reviewed by Martinoia et al., 2012) have been identified to regulate VSC and hence metal allocation between different organs or tissues (**Table 1**).

In response to mineral nutrient deficiency, genes involving in nutrient remobilization from vacuoles to cytosols, such as *NRAMP3* and *NRAMP4* (Thomine et al., 2000, 2003), are up-regulated, thus reducing the VSC to release the stored nutrients and consequently alleviating nutrient deficiency. In contrast, other genes including *MTP3* (*Metal Transporter 3*; Arrivault et al., 2006), *ZIF1* (*Zinc-Induced Facilitator 1*; Haydon and Cobbett, 2007), *VIT2* (*Vacuolar Iron Transporter 2*; Zhang et al., 2012), *CAX4* (*Cation Exchanger 4*; Mei et al., 2009), which regulate metal sequestration into vacuoles, are up-regulated when excessive metals are available in the environment, and the enhanced expression consequently leads to enlarge VSC and metal accumulation in vacuoles. Another group of genes including *VIT1*(Kim et al., 2006; Zhang et al., 2012), *MTP1*(Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Kawachi et al., 2009), *COPT5* (*Copper Transporters 5*; Garcia-Molina et al., 2011; Klaumann et al., 2011), remain unaffected by the metal status in the environment, though functional abortion

Table 1 | Tonoplast-located transporters that have impact on long-distance transport of metal micronutrients (or toxic metals) in plant.

Transporters	Mineral	Reference
MTP1	Zn	reviewed by Ricachenevsky et al. (2013)
MTP3	Zn	Arrivault et al. (2006)
ZIF1	Zn	Haydon and Cobbett (2007), Haydon et al. (2012)
NRAMP3/NRAMP4	Fe	Thomine et al. (2003), Lanquar et al. (2005)
VIT1/VIT2	Fe, Zn	Kim et al. (2006), Zhang et al. (2012)
FPN2(IREG2)	Fe, Co, Ni	Morrissey et al. (2009), Schaaf et al. (2006)
COPT5	Cu	Klaumann et al. (2011)
CAX2/CAX4	Cd	Korenkov et al. (2007)
HMA3	Cd	Ueno et al. (2010, 2011)

of these genes altered metal allocation at either subcellular or tissue levels, suggesting that a stable expression level of those genes enables proper metal allocation.

Metal hyperaccumulators modulate their VSC via a more conservative way than we have imagined, as they tend to utilize some transporters which are also present in their corresponding non-hyperaccumulators but have evolved with more complex regulation mechanisms. MTP1 is a key transporter modulating the VSC of Zn (Sinclair and Krämer, 2012; Ricachenevsky et al., 2013). Piled evidences suggest that the expression level of *MTP1* is greatly enhanced in various metal hyperaccumulators (Assunção et al., 2001; Persans et al., 2001; Becher et al., 2004; Dräger et al., 2004; Hammond et al., 2006; Talke et al., 2006; van de Mortel et al., 2006; Gustin et al., 2009; Shahzad et al., 2010; Zhang et al., 2011), which later on was shown to be attributable to genome amplification of gene copies, in contrast to a single copy in their relative non-hyperaccumulators (Dräger et al., 2004; Shahzad et al., 2010). A similar regulation of *TcHMA3 (Heavy Metal ATPases 3)* was also observed (Ueno et al., 2011), among which gene amplification leads to much higher expression of *TcHMA3* in the accumulating ecotype Ganges of *Thlaspi caerulescens* than in the non-accumulating Prayon, thus VSC of Cd in roots is greatly reduced and more metal is driven to translocate to shoots.

REGULATION BY CHELATORS

Phytochelatins are important chelating molecules in plants, which detoxify heavy metals via chelation and hence forming stable PCs-metal complexes which are subsequently sequestered into vacuoles (Cobbett and Goldsbrough, 2002). In an effort to identify the relative contribution to VSC by PCs and GSH, the authors unexpected found that when ectopically expressing *SpHMT1*, a fission yeast gene encoding vacuolar PC-Cd transporters, the rate of long-distance Cd transport was significantly delayed in *Arabidopsis* (Huang et al., 2012). They assumed that this delay might be due to the enlarged VSC in root vacuoles. To test their hypothesis, SpHMT1 was targeted to roots and as expected, accumulation of Cd, Cu, and As in seeds was reduced up to 70% of the controls. These results show that VSC of heavy metals is essentially regulated by PCs.

Nicotianamine is another kind of metal chelator which is ubiquitously present in higher plants. Recent studies suggest that NA modulates the VSC of Zn and Fe and hence their distribution. In the hyperaccumulator *Arabidopsis halleri*, large amounts of NA is present in the root tissues and was proposed to essentially contribute to long-distance transport of Zn to shoots, as in the *AhNAS2*-RNAi plants with substantially reduced NA contents, much less Zn was observed in the xylem sap and shoots (Deinlein et al., 2012). Another research showed that overexpression of *AtZIF1* in *Arabidopsis* enhanced NA sequestration into vacuoles (Haydon et al., 2012), which consequently led to Fe overaccumulation in shoot tissues. The authors implicated that enhanced vacuolar NA sequestration depleted cytosolic NA, thus affected the intercellular mobility of Fe as Fe-NA complex (Haydon et al., 2012).

The amino acid histidine (His) was also identified to be functional in Ni detoxification and translocation in some Ni hyperaccumulators (Krämer et al., 1996; Kerkeb and Krämer, 2003; Krämer, 2010). Using a tonoplast transport assay strategy, Richau et al. (2009) found that when Ni was supplied as a Ni-His complex, a much higher uptake rate was observed in tonoplast vesicles derived from the hyperaccumulator than those from the non-hyperaccumulator *T. arvense*. Given high concentration of His was mainly found in *T. caerulescens* roots, and the level is about 10 times of that in *T. arvense* roots, they postulated that accumulation of His in *T. caerulescens* roots reduces the VSC of Ni by cytosolic chelation, thus promoting Ni transport and hyperaccumulation in shoots.

In general, VSC regulation by chelators could be classified into two categories: (1) the chelated metals are prone to translocation into vacuoles, thus enlarging VSC of certain metals; or (2) chelation of metals leads to reduced vacuolar sequestration and hence decreasing the VSC of certain metals. Root-targeted expression of *TaPCS1* in *cad1-3* significantly enhanced root-to-shoot long-distance transport of Cd (Gong et al., 2003), and disruption of NA biosynthesis retained Zn in roots (Deinlein et al., 2012). All these studies suggest that chelation of metals by PCs or NA tends to promote long-distance metal transport, though the chelation is supposed to enlarge VSC to some extent. One possible explanation might be that the VSC regulated by chelators is also dependent on relevant transporters, as overexpression of SpHMT1, the PC-Cd complex transporter, successfully trapped Cd in roots (Huang et al., 2012), indicating a complex interaction between chelators and their transporters in the regulation of VSC.

VSC REGULATES LONG-DISTANCE METAL TRANSPORT IN PLANTS

VSC AND TRANSPORT OF MINERAL NUTRIENTS

Consistent with its major function in photosynthesis, most cellular Fe is found in the chloroplast. However, a substantial amount of this mineral nutrient is also stored in leaf vacuoles. Zhang et al. (2012) isolated two tonoplast-localized metal transporters OsVIT1 and OsVIT2 from rice. Ectopic expression of *OsVIT1* and

OsVIT2 genes partially rescued the Fe and Zn sensitive phenotypes and increased vacuolar Fe and Zn accumulation in yeast, suggesting a primary role for *OsVIT1* and *OsVIT2* is sequestering Fe/Zn into vacuoles across the tonoplast. *OsVIT1* and *OsVIT2* are highly expressed in rice flag leaves. Functional disruption of these two genes led to decreased Fe/Zn accumulation in flag leaves while enhanced accumulation in rice grains and phloem exudates of flag leaves and uppermost nodes. Meanwhile, no obvious changes were observed in either uptake or root-to-shoot transport of Fe/Zn between the mutants and the wild-type. These observations suggest that *OsVIT1* and *OsVIT2* play an important role in Fe and Zn long-distance translocation between source (flag leaves) and sink organs (seeds), via the modulation of VSC for Fe and Zn in flag leaves. In *Arabidopsis*, functional disruption of the tonoplast-localized Fe transporters AtVIT1 (Kim et al., 2006), AtNRAMP3 (*Natural Resistance Macrophage Protein 3*) and AtNRAMP4 (Lanquar et al., 2005) resulted in growth arrest especially in Fe deficient condition, providing further support to the hypothesis that modulation of VSC essentially affect Fe mobilization and reallocation between different tissues in plants.

The hypothesis also applies to the modulation of the VSC of Zn in plants. *AtMTP3* mediates Zn transport into vacuoles and expresses preferentially in *Arabidopsis* roots. When exposed to excessive Zn, *AtMTP3* is induced thus enhancing the VSC and consequently Zn sequestration into vacuoles in roots. It is believed that this mechanism helps to protect the most important organelles including chloroplasts through reduced long-distance transport of Zn to aerial tissues (Sinclair and Krämer, 2012). One supportive evidence came from the observation that more Zn accumulated in shoot tissues of the *atmtp3* mutant (Arrivault et al., 2006). Consistent results were obtained in the research of other Zn transporters such as the tonoplast-localized AtZIF1. *AtZIF1* overexpression lines showed decreased Zn accumulation in shoots and increased root-to-shoot ratios of Zn concentrations compared to the wild-type control (Haydon and Cobbett, 2007; Haydon et al., 2012), as would be expected according to the theory that Zn translocation could be mediated by VSC.

Additional supportive evidences came from Cu reallocation. *AtCOPT5* is expressed in root vascular tissues at high levels, and functions as a vacuolar Cu exporter. In the T-DNA-insertion mutant *atcopt5*, remarkably increased copper concentrations were observed in the vacuoles compared with those in the wild-type. Correspondingly, more Cu accumulated in roots and less in siliques and seeds of the mutant plants (Garcia-Molina et al., 2011; Klaumann et al., 2011).

VSC AND TRANSPORT OF TOXIC METALS

Non-essential toxic metal(loid)s including Cd and As may lead to adverse effects on plants, mainly through the derived oxidative injuries or competitive inhibition of essential mineral nutrient. To protect the aerial parts which are active in photosynthesis and other important biological processes, plants have evolved sophisticated machineries in regulating metal distribution between roots and shoots, among which VSC also plays a very important role.

One example is about the rice *OsHMA3*, which has been correlated to Cd accumulation in rice shoots. Specifically, Ueno et al. (2010) found that a functional disrupted *OsHMA3* allele resulted in Cd overaccumulation in rice shoots. Further research revealed that OsHMA3 is localized to the tonoplast in rice roots, and it functions primarily to mediate Cd transport into vacuoles. Overexpression of the functional *OsHMA3* allele significantly decreased Cd accumulation in rice grains, while no apparent effect was observed on the accumulation of other essential micronutrients. These results suggest that the VSC in rice roots mediated by OsHMA3 contributes essentially to the long-distance transport of Cd from roots to shoots.

Another example is about the metal hyperaccumulators. In contrast to non-hyperaccumulators, hyperaccumulators show extraordinarily high accumulation of toxic metals in aerial parts without any visual effects (reviewed by Krämer, 2010). Interestingly, studies have suggested that VSC regulation also gets involved in both the long-distance transport of toxic metals and the detoxification mechanisms that render plants high tolerance to metals. It was shown that the VSC for Zn in hyperaccumulator *T. caerulescens* roots is significantly decreased, compared to the non-hyperaccumulating relative *T. arvense*, which consequently drives more Zn to translocate from roots to shoots as indicated by the enhanced xylem loading of Zn in *T. caerulescens* (Lasat et al., 1998). In different ecotypes of the *T. caerulescens*, it was also found that the efficiency of Cd loading to the xylem is highly correlated to the VSC for Cd in root cells (Xing et al., 2008). A similar correlation between metal translocation efficiency to shoots and its VSC in roots is also found in *Sedum alfredii,* another Zn/Cd hyperaccumulator found specifically in Southern China (Yang et al., 2006). These observations suggest metal transport regulated by VSC modulation might be a common mechanism in hyperaccumulators. In addition to the regulation on long-distance metal transport, VSC also functions on metal detoxification in hyperaccumulators, supportive evidences came from the results that a predominant proportion of shoot Cd was localized to vacuoles (Küpper et al., 1999, 2001; Krämer et al., 2000; Ma et al., 2005).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Vacuoles, occupying over 80% of the cellular volume in vegetative tissues, are the largest organelles undergoing relatively less metabolisms, which makes it perfect for vacuoles to store chemical compounds and metal ions. However, their role in regulating long-distance root-to-shoot transport of metals has been largely ignored, though such a deduction is becoming more and more apparent with accumulating evidences. Here we proposed that VSC plays a role of "buffering pool" to dynamically mediate long-distance metal transport in plants. Generally the VSC of certain metal varies between different plant tissues to ensure proper metal distribution, e.g., the VSC of toxic metals is larger in roots than in shoots, thus an essential proportion of the toxic metals will be trapped in roots as we normally observe. When exposed to high levels of toxic metals, specifically when the VSC of a certain metal is used up an "overflow" mechanism as proposed (Gong et al., 2003) takes over and the excessive metal is subjected to long-distance root-to-shoot transport. In metal hyperaccumulators, however, greatly reduced VSC in roots has evolved, which greatly promotes long-distance metal

transport from roots to shoots. We also reviewed the regulation mechanisms of VSC by tonoplast-localized transporters and ion chelators. we believe that the study of VSC would contribute not only to a better understanding of metal homeostasis and distribution, but also to an efficient biofortification of essential mineral nutrients and phytoremediation of non-essential toxic metals.

As a primary producer, plants provide a lot of mineral nutrients that are essential for human health. However, some of these nutrients in the edible parts of stable crops are often deficient or poorly bioavailable thus resulting in an unbalanced diet. Therefore, nutrient biofortification is of urgent and necessary importance. In addition to the traditional approaches that tackle these concerns by increasing the efficiency of nutrient uptake and bioavailability (reviewed by Hirschi, 2009), modulation of VSC might be a more efficient way, in which nutrient taken into plants are forced to accumulate in targeted tissues, thus enhanced uptake might not be required and the risk of undesired accumulation of toxic metals is greatly reduced (Arrivault et al., 2006; Haydon et al., 2012; Zhang et al., 2012). Furthermore, targeted modulation of VSC could also help to reduce toxic metal accumulation in edible tissues, as demonstrated by the root-specific expression of *SpHMT1* or the natural variation in *OsHMA3* in rice (Ueno et al., 2010; Huang et al., 2012), where a strong correlation between the VSC in roots and metal accumulation in shoots was observed.

On the other side, phytoremediation has been proposed as an efficient way to tackle the widespread heavy metal pollution in many countries, however, the practical application of metal hyperaccumulators to field trial is not so optimistic, mainly because of the relative low biomass and growth rate of those specialized plants. In these specific circumstances, an optimal alternative could be the fine modulation of the VSC in both roots and shoots in some plants with large biomass and growth rate.

REFERENCES

Arrivault, S., Senger, T., and Krämer, U. (2006). The *Arabidopsis* metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *Plant J.* 46, 861–879. doi: 10.1111/j.1365-313X.2006.02746.x

Assunção, A. G. L., Martins, P. D., De Folter, S., Vooijs, R., Schat, H., and Aarts, M. G. M. (2001). Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant Cell Environ.* 24, 217–226. doi: 10.1111/j.1365-3040.2001.00666.x

Becher, M., Talke, I. N., Krall, L., and Krämer, U. (2004). Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant J.* 37, 251–268. doi: 10.1046/j.1365-313X.2003.01959.x

Cobbett, C. S., and Goldsbrough, P. (2002). Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* 53, 159–182. doi: 10.1146/annurev.arplant.53.100301.135154

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T. H., et al. (2012). Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. *Plant Cell* 24, 708–723. doi: 10.1105/tpc.111.095000

Desbrosses-Fonrouge, A. G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U. (2005). *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Lett.* 579, 4165–4174. doi: 10.1016/j.febslet.2005.06.046

Dräger, D. B., Desbrosses-Fonrouge, A. G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., et al. (2004). Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *Plant J.* 39, 425–439. doi: 10.1111/j.1365-313X.2004.02143.x

Garcia-Molina, A., Andrés-Colás, N., Perea-García, A., Del Valle-Tascón, S., Peñarrubia, L., and Puig, S. (2011). The intracellular *Arabidopsis* COPT5 transport protein is required for photosynthetic electron transport under severe copper deficiency. *Plant J.* 65, 848–860. doi: 10.1111/j.1365-313X.2010.04472.x

Gong, J. M., Lee, D. A., and Schroeder, J. I. (2003). Long-distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10118–10123. doi: 10.1073/pnas.1734072100

Gustin, J. L., Loureiro, M. E., Kim, D., Na, G., Tikhonova, M., and Salt, D. E. (2009). MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. *Plant J.* 57, 1116–1127. doi: 10.1111/j.1365-313X.2008.03754.x

Hammond, J. P., Bowen, H. C., White, P. J., Mills, V., Pyke, K. A., Baker, A. J., et al. (2006). A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. *New Phytol.* 170, 239–260. doi: 10.1111/j.1469-8137.2006.01662.x

Haydon, M. J., and Cobbett, C. S. (2007). A novel major facilitator superfamily protein at the tonoplast influences zinc tolerance and accumulation in *Arabidopsis*. *Plant Physiol.* 143, 1705–1719. doi: 10.1104/pp.106.092015

Haydon, M. J., Kawachi, M., Wirtz, M., Hillmer, S., Hell, R., and Krämer, U. (2012). Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in *Arabidopsis*. *Plant Cell* 24, 724–737. doi: 10.1105/tpc.111.095042

Hirschi, K. D. (2009). Nutrient biofortification of food crops. *Annu. Rev. Nutr.* 29, 401–421. doi: 10.1146/annurev-nutr-080508-141143

Huang, J., Zhang, Y., Peng, J. S., Zhong, C., Yi, H. Y., Ow, D. W., et al. (2012). Fission yeast HMT1 lowers seed cadmium through phytochelatin-dependent vacuolar sequestration in *Arabidopsis*. *Plant Physiol.* 158, 1779–1788. doi: 10.1104/pp.111.192872

Kawachi, M., Kobae, Y., Mori, H., Tomioka, R., Lee, Y., and Maeshima, M. (2009). A mutant strain *Arabidopsis thaliana* that lacks vacuolar membrane zinc transporter MTP1 revealed the latent tolerance to excessive zinc. *Plant Cell Physiol.* 50, 1156–1170. doi: 10.1093/pcp/pcp067

Kerkeb, L., and Krämer, U. (2003). The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiol.* 131, 716–724. doi: 10.1104/pp102.010686

Kim, S. A., Punshon, T., Lanzirotti, A., Li, L., Alonso, J. M., Ecker, J. R., et al. (2006). Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science* 314, 1295–1298. doi: 10.1126/science.1132563

Klaumann, S., Nickolaus, S. D., Fürst, S. H., Starck, S., Schneider, S., Ekkehard Neuhaus, H., et al. (2011). The tonoplast copper transporter COPT5 acts as an exporter and is required for interorgan allocation of copper in *Arabidopsis thaliana*. *New Phytol.* 192, 393–404. doi: 10.1111/j.1469-8137.2011.03798.x

Kobae, Y., Uemura, T., Sato, M. H., Ohnishi, M., Mimura, T., Nakagawa, T., et al. (2004). Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant Cell Physiol.* 45, 1749–1758. doi: 10.1093/pcp/pci015

Korenkov, V., Hirschi, K., Crutchfield, J. D., and Wagner, G. J. (2007). Enhancing tonoplast Cd/H antiport activity increases Cd, Zn, and Mn tolerance, and impacts root/shoot Cd partitioning in *Nicotiana tabacum* L. *Planta* 226, 1379–1387. doi: 10.1007/s00425-007-0577-0

Krämer, U. (2010). Metal hyperaccumulation in plants. *Annu. Rev. Plant Biol.* 61, 517–534. doi: 10.1146/annurev-arplant-042809-112156

Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. M., and Smith, J. A. C. (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379, 635–638. doi: 10.1038/379635a0

Krämer, U., Pickering, I. J., Prince, R. C., Raskin, I., and Salt, D. E. (2000). Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. *Plant Physiol.* 122, 1343–1353. doi: 10.1104/pp.122.4.1343

Küpper, H., Lombi, E., Zhao, F. J., Wieshammer, G., and McGrath S. P. (2001). Cellular compartmentation of nickel in the hyperaccumulators *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*. *J. Exp. Bot.* 52, 2291–2300. doi: 10.1093/jexbot/52.365.2291

Küpper, H., Zhao, F. J., and McGrath S. P. (1999). Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol.* 119, 305–312. doi: 10.1104/pp.119.1.305

Lanquar, V., Lelièvre, F., Bolte, S., Hamès, C., Alcon, C., Neumann, D., et al. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24, 4041–4051. doi: 10.1038/sj.emboj.7600864

Lasat, M. M., Baker, A. J., and Kochian, L. V. (1998). Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiol.* 118, 875–883. doi: 10.1104/pp.118.3.875

Ma, J. F., Ueno, D., Zhao, F. J., and McGrath S. P. (2005). Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Planta* 220, 731–736. doi: 10.1007/s00425-004-1392-5

Martinoia, E., Meyer, S., De Angeli, A., and Nagy, R. (2012). Vacuolar transporters in their physiological context. *Annu. Rev. Plant Biol.* 63, 183–213. doi: 10.1146/annurev-arplant-042811-105608

Mei, H., Cheng, N. H., Zhao, J., Park, S., Escareno, R. A., Pittman, J. K., et al. (2009). Root development under metal stress in *Arabidopsis thaliana* requires the H+/cation antiporter CAX4. *New Phytol.* 183, 95–105. doi: 10.1111/j.1469-8137.2009.02831.x

Mendoza-Cozatl, D. G., Jobe, T. O., Hauser, F., and Schroeder, J. I. (2011). Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmiumand arsenic. *Curr. Opin. Plant Biol.* 14, 554–562. doi: 10.1016/j.pbi.2011.07.004

Morrissey, J., Baxter, I. R., Lee, J., Li, L., Lahner, B., Grotz, N., et al. (2009), The ferroportin metal efflux proteins function in iron and cobalt homeostasis in *Arabidopsis*. *Plant Cell.* 21, 3326–3338. doi: 10.1105/tpc.109.069401

Persans, M. W., Nieman, K., and Salt, D. E. (2001). Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9995–10000. doi: 10.1073/pnas.171039798

Ricachenevsky, F. K., Menguer, P. K., Sperotto, R. A., Williams, L. E., and Fett, J. P. (2013). Roles of plant metal tolerance proteins (MTP) in metal storage and potential use in biofortification strategies. *Front. Plant Sci.* 4:144. doi: 10.3389/fpls.2013.00144

Richau, K. H., Kozhevnikova, A. D., Seregin, I. V., Vooijs, R., Koevoets, P. L., Smith, J. A., et al. (2009). Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator *Thlaspi caerulescens*. *New Phytol.* 183, 106–116. doi: 10.1111/j.1469-8137.2009.02826.x

Schaaf, G., Honsbein, A., Meda, A. R., Kirchner, S., Wipf, D., and von Wirén, N. (2006). AtIREG2 encodes a tonoplast transport protein involved in iron-dependent nickel detoxification in *Arabidopsis thaliana* roots. *J. Biol. Chem.* 281, 25532–25540. doi: 10.1074/jbc.M601062200

Shahzad, Z., Gosti, F., Frérot, H., Lacombe, E., Roosens, N., Saumitou-Laprade, P., et al. (2010). The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *PLoS Genet.* 6:e1000911. doi: 10.1371/journal.pgen.1000911

Sinclair, S. A., and Krämer, U. (2012). The zinc homeostasis network of land plants. *Biochim. Biophys. Acta.* 1823, 1553–1567. doi: 10.1016/j.bbamcr.2012.05.016

Talke, I. N., Hanikenne, M., and Krämer, U. (2006). Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiol.* 142, 148–167. doi: 10.1104/pp.105.076232

Thomine, S., Lelièvre, F., Debarbieux, E., Schroeder, J. I., and Barbier-Brygoo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant J.* 34, 685–695. doi: 10.1046/j.1365-313X.2003.01760.x

Thomine, S., Wang, R., Ward, J. M., Crawford, N. M., and Schroeder, J. I. (2000). Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 991–996. doi: 10.1073/pnas.97.9.4991

Ueno, D., Milner, M. J., Yamaji, N., Yokosho, K., Koyama, E., Clemencia Zambrano, M., et al. (2011). Elevated expression of TcHMA3 plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Plant J.* 66, 852–862. doi: 10.1111/j.1365-313X.2011.04548.x

Ueno, D., Yamaji, N., Kono, I., Huang, C. F., Ando, T., Yano, M., et al. (2010). Gene limiting cadmium accumulation in rice. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16500–16205. doi: 10.1073/pnas.1005396107

van de Mortel, J. E., Almar Villanueva, L., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P. D., et al. (2006). Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol.* 142, 1127–1147. doi: 10.1104/pp.106.082073

Xing, J. P., Jiang, R. F., Ueno, D., Ma, J. F., Schat, H., McGrath S. P., et al. (2008). Variation in root-to-shoot translocation of cadmium and zinc among different accessions of the hyperaccumulators *Thlaspi caerulescens* and *Thlaspi praecox*. *New Phytol.* 178, 315–325. doi: 10.1111/j.1469-8137.2008.02376.x

Yang, X. E., Li, T. Q., Long, X. X., Xiong, X. H., He, Z. H., and Stoffella, P. J. (2006). Dynamics of zinc uptake and accumulation in the hyperaccumulating and non-hyperaccumulating ecotypes of *Sedum alfredii* Hance. *Plant and Soil.* 284,109–119. doi: 10.1007/s11104-006-0033-0

Zhang, M., Senoura, T., Yang, X., and Nishizawa, N. K. (2011). Functional analysis of metal tolerance proteins isolated from Zn/Cd hyperaccumulating ecotype and non-hyperaccumulating ecotype of *Sedum alfredii* Hance. *FEBS Lett.* 585, 2604–2609. doi: 10.1016/j.febslet.2011.07.013

Zhang, Y., Xu, Y. H., Yi, H. Y., and Gong, J. M. (2012). Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. *Plant J.* 72, 400–410. doi: 10.1111/j.1365-313X.2012.05088.x

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Molybdenum metabolism in plants and crosstalk to iron

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In the form of molybdate the transition metal molybdenum is essential for plants as it is required by a number of enzymes that catalyze key reactions in nitrogen assimilation, purine degradation, phytohormone synthesis, and sulfite detoxification. However, molybdate itself is biologically inactive and needs to be complexed by a specific organic pterin in order to serve as a permanently bound prosthetic group, the molybdenum cofactor, for the socalled molybdo-enyzmes. While the synthesis of molybdenum cofactor has been intensively studied, only little is known about the uptake of molybdate by the roots, its transport to the shoot and its allocation and storage within the cell. Yet, recent evidence indicates that intracellular molybdate levels are tightly controlled by molybdate transporters, in particular during plant development. Moreover, a tight connection between molybdenum and iron metabolisms is presumed because (i) uptake mechanisms for molybdate and iron affect each other, (ii) most molybdo-enzymes do also require iron-containing redox groups such as iron-sulfur clusters or heme, (iii) molybdenum metabolism has recruited mechanisms typical for iron-sulfur cluster synthesis, and (iv) both molybdenum cofactor synthesis and extramitochondrial iron-sulfur proteins involve the function of a specific mitochondrial ABC-type transporter.

Keywords: aldehyde oxidase, iron, molybdenum, molybdate transporter, molybdo-enzymes, nitrate reductase, sulfite oxidase, xanthine dehydrogenase

INTRODUCTION

Molybdenum is a transition metal, which occurs in the lithosphere at an average abundance of 1.2 mg kg^{-1} and represents one of the scarcest trace elements in biological systems (Kaiser et al., 2005). In the soil, molybdenum exists predominantly in the form of the oxyanion molybdate, which serves as an essential micronutrient in all kingdoms of life. Yet, molybdate alone does not exhibit biological activity, but is bound to an organic pterin backbone, which upon binding of molybdate is converted into the molybdenum cofactor (Moco). Once being incorporated as prosthetic group, Moco becomes part of the active site of molybdo-enzymes, where molybdenum can vary its oxidation state between Mo(IV), Mo(V), and Mo(VI), thereby enabling the respective protein to transfer electrons, and in most cases also oxygen, from or to a substrate (Hille, 2013). Due to its special importance for plants, another molybdenum-containing cofactor exclusively found in certain bacteria is mentioned. This cofactor is part of the unique enzyme nitrogenase that catalyzes the fixation of nitrogen by reduction of atmospheric N_2 to NH_3 in free-living, but also symbiotic bacteria in the nodules of legumes (Kneip et al., 2007). Unlike Moco however, the nitrogenase cofactor is constituted of molybdenum ligated to a complex iron-sulfur cluster and homocitrate and therefore is named FeMoco (Hu and Ribbe, 2013).

In soil, a critical point concerns the bioavailability of molybdate, which is favored above pH 5.5 and impaired at lower pH due to the adsorption of molybdate to soil oxides (Kaiser et al., 2005). Under low-pH conditions, molybdate assimilation is therefore limited resulting in molybdenum deficiency associated with reduced molybdo-enzyme activities and reductions in plant growth and yield. Fortunately, this type of molybdenum deficiency can be compensated by fertilization with molybdate or by increasing the soil pH by liming. In contrast, molybdenum toxicity by oversupply of plants with molybdate is extremely rare and characterized by relatively mild symptoms such as yellowish leaves (Kaiser et al., 2005) or reduced seedling growth and increased anthocyanin concentrations (Kumchai et al., 2013).

In consideration of the present knowledge of molybdate uptake, transport and storage, the specific functions of the molybdenum-dependent enzymes and the interrelation between molybdenum and iron, this review focuses on the current understanding of the molybdenum homeostasis network in plants and points to some hitherto unidentified factors (**Table 1**; **Figure 1**).

UPTAKE, TRANSPORT, AND STORAGE OF MOLYBDATE

Early in plant molybdenum research Kannan and Ramani (1978) described the active uptake of exogenously applied molybdate by roots and its transport to the shoot. However, molybdate levels reached a maximum in the shoot already 6 h after application, indicating that uptake of molybdate and sensing of intracellular molybdate levels are well controlled processes. Notably, when molybdate is applied solely to leaves transport also occurs downward to the stem and roots, demonstrating that molybdate is a highly mobile compound translocated between various plant tissues. Furthermore, the finding that sulfate is a potent inhibitor of molybdate uptake (Stout et al., 1951; Kannan and Ramani, 1978) and that in turn low sulfate concentrations in the soil promote molybdate uptake (Shinmachi et al., 2010) pioneered speculations on the nature of molybdate transporters. In fact,

Table 1 | Components of molybdenum metabolism in higher plants (*Arabidopsis thaliana*).

Protein names	Agi code	Known / proposed function
MOT1/SULTR 5;2	AT2G25680	Molybdate transport
MOT2/SULTR 5;1	AT1G80310	Molybdate transport / export from vacuole
CNX1	AT5G20990	Moco biosynthesis step 3
CNX2	AT2G31955	Moco biosynthesis step 1
CNX3	AT1G01290	Moco biosynthesis step 1
CNX5	AT5G55130	Moco biosynthesis step 2
CNX6	AT2G43760	Moco biosynthesis step 2
CNX7	AT4G10100	Moco biosynthesis step 2
Nia1/NR1	AT1G77760	Nitrate reductase (minor form)
Nia2/NR2	AT1G37130	Nitrate reductase (main form)
SO	AT3G01910	Oxidation/elimination of cytotoxic sulfite
mARC1/MOSC1	AT5G44720	Unknown (reduction of *N*-hydroxylated compounds ?)
mARC2/MOSC2	AT1G30910	Unknown (reduction of *N*-hydroxylated compounds ?)
AAO1	AT5G20960	Unknown (IAA biosynthesis ?)
AAO2	AT3G43600	Unknown (IAA biosynthesis ?)
AAO3	AT2G27150	ABA biosynthesis
AAO4	AT1G04580	Synthesis of benzoic acid
AtXDH1	AT4G34890	Purine degradation
AtXDH2	AT4G34900	Pseudogene ?
ABA3/LOS5	AT1G16540	Mocosulfuration and activation of AO and XDH proteins
ATM3/ABCB25	AT5G58270	Transporter involved in cytosolic Fe-S assembly and Moco synthesis

sulfate and molybdate share a high degree of similarity as they both possess a double negative charge (SO_4^{2-}, MoO_4^{2-}), are similar in size, and have tetrahedral structures. It was thus proposed that molybdate import and distribution are facilitated by sulfate transporters or related systems. The first identified molybdate-specific transporters from *Chlamydomonas reinhardtii* (Tejada-Jimenez et al., 2007) and *Arabidopsis thaliana* (Tomatsu et al., 2007; Baxter et al., 2008) indeed turned out to belong to the family of sulfate transporters, yet lacking the otherwise typical STAS domain essential for sulfate transport activity (Shibagaki and Grossman, 2006). Unfortunately, the two reports on the *Arabidopsis* transporter, referred to as MOT1, differ in the subcellular localization of the transporter with Tomatsu et al. (2007) primarily showing endomembrane localization and Baxter et al. (2008) reporting a mitochondrial localization. Ascribing a precise function to MOT1 is therefore difficult and it remains to be proven in future studies whether MOT1 is involved in the intra- and/or the inter-cellular translocation of molybdate. In addition to MOT1, another molydate transporter of the sulfate transporter family, MOT2, has been identified in *Arabidopsis* (Gasber et al., 2011). MOT2 localizes to the vacuole and *MOT2*-deficient plants are characterized by accumulation of molybdenum in leaves and reduced molybdenum contents in seeds. Along with the finding that *MOT2* transcripts accumulate in senescing leaves, this suggests a function of MOT2 in exporting stored molybdate from the vacuole into the cytosol for translocation into maturing seeds. Moreover, total molybdenum contents were found to correlate with the levels of Moco in wildtype and *MOT2* mutant leaves, indicating plants adjust Moco synthesis to the cellular levels of molybdate.

Just recently a novel molybdate transporter, also denoted as MOT2, has been identified in *Chlamydomonas* (Tejada-Jiménez et al., 2011). In contrast to *Arabidopsis* MOT2 however, this transporter is a member of the major facilitator superfamily which is completely unrelated to the sulfate transporter family. It is tempting to speculate that the homolog of this transporter in higher plants represents the as yet unidentified transporter involved in the uptake of molybdate at the root:soil interface.

MOLYBDENUM COFACTOR AND MOLYBDENUM-DEPENDENT ENZYMES

The only process known to require molybdenum/molybdate directly by plants is the biosynthesis of Moco, which is initiated in mitochondria and finalized in the cytosol. However, a detailed description of Moco biosynthesis is out of the scope of this work and is comprehensively covered by Bittner and Mendel (2010) and Mendel (2013). Even though Moco biosynthesis is well understood only little knowledge exists about the genetic and enzymatic regulation of Moco biosynthesis in plants. For instance, it is largely unknown whether the expression levels of Moco synthesis genes are affected by molybdate, and whether the endogenous levels of Moco vary, e.g., during plant development or in response to stress conditions. The only available information in this respect concerns the expression of molybdenum metabolism-related genes in the *Arabidopsis MOT1* mutant that was grown without additional molybdate supply to induce endogenous molybdate deficiency (Ide et al., 2011). In fact, the Moco biosynthesis genes *CNX2* and *CNX6* were demonstrated to be upregulated under molybdate deficiency, which was likewise true for the molybdo-enzymes *NR1* and *NR2*, the Moco sulfurase *ABA3* and the transporter *MOT2*. Apart from these genes, molybdate deficiency also affected the expression of many genes involved in transport, stress responses, signal tranduction and in the metabolisms of nitrogen, sulfur, and phosphate, but also the levels of amino acids, sugars, organic acids, and purine metabolites were significantly altered, indicating that molybdate nutrition has global impact on plants.

Among the molybdo-enzymes, nitrate reductase (NR) represents the cytosolic key enzyme of nitrogen assimilation that reduces nitrate to nitrite (Campbell, 2001). In addition to Moco, NR also depends on heme and FAD as prosthetic groups and strictly requires NADH or NADPH for enzymatic activity. A deficiency in NR results in the inability of the plant to mobilize nitrogen, which is inseparably associated with the loss of plant viability in the absence of alternative nitrogen sources. Under low-oxygen conditions, NR is capable of reducing nitrite to nitric oxide (NO), and NR-derived NO appears to be among the major sources of NO in plants with impact on plant development, protection against reactive oxygen species, phytoalexin accumulation, and pathogen resistance (Rockel et al., 2002).

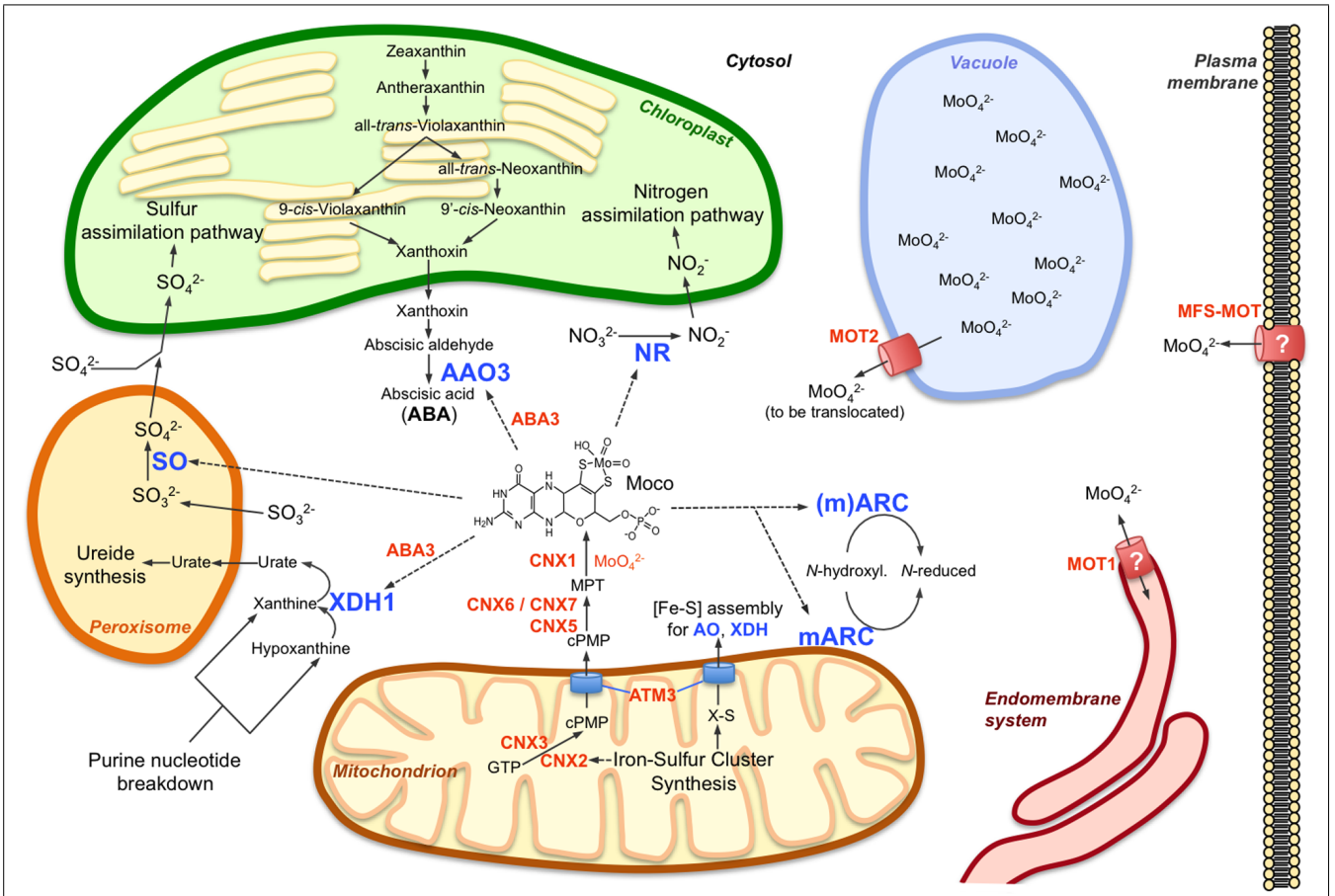

FIGURE 1 | Molybdenum metabolism in higher plant cells. The main components of molybdenum metabolism in plants are shown including the Moco biosynthetic pathway (CNX proteins) in mitochondria and cytosol, the Moco user enzymes and their respective main functions in nitrogen assimilation (NR), ABA synthesis (AAO3), purine catabolism (XDH1), and sulfite detoxification (SO). mARC enzymes are proposed to function in reduction of certain *N*-hydroxylated substrates, which have not yet been identified. While one of the two mARC isoforms (mARC2) contains an NH_2-terminal mitochondrial targeting sequence, such targeting sequence is absent at the second isoform, which therefore is assumed to act in the cytosol. In contrast to the molybdate transporter MOT2, which functions at the vacuolar membrane as a molybdate exporter, MOT1 might localize to the endomembrane system, possibly to the endoplasmic reticulum. A mitochondrial localization of MOT1 has also been reported but appears less likely as no obvious reason exists for import or export of molybdate into or out of the mitochondria, respectively. The plant homolog (MFS-MOT) of the major facilitator superfamily molybdate transporter MOT2 from *Chlamydomonas* might be required for molybdate import across the plasma membrane. The function of the Moco sulfurase ABA3 in activation of AO and XDH is indicated, just as the functions of the mitochondrial ABC transporter ATM3 in export of cPMP from mitochondria and in cytosolic iron-sulfur cluster ([Fe-S]) assembly for AO and XDH (and other extra-mitochondrial proteins). Further details are given in the main text and are reviewed by Bittner and Mendel (2010) and Mendel (2013). Molybdo-enzymes are indicated by blue letters, other components of molybdenum metabolism by orange letters; dotted arrows indicate requirement for Moco by molybdo-enzymes.

Plant sulfite oxidase (SO) is a peroxisomal enzyme (Nowak et al., 2004), which exclusively consists of a Moco-binding domain required for oxidizing sulfite to sulfate. In this process, substrate-derived electrons are transferred to molecular oxygen with formation of superoxide anions (Byrne et al., 2009) and hydrogen peroxides (Haensch et al., 2006). In the physiological context, SO represents a part of an intracellular sulfite homeostasis network required to prevent plant cells from the toxic effects of endogenously arising sulfite (Brychkova et al., 2007; Lang et al., 2007).

Xanthine dehydrogenase (XDH) requires Moco, FAD, and two iron-sulfur clusters (Hille et al., 2011), and its main function is associated to purine degradation by oxidizing hypoxanthine to xanthine and xanthine to uric acid in the cytosol. Electrons released from the substrate are preferably transferred to NAD^+. At extremely low concentrations of NAD^+, molecular oxygen can serve as alternative electron acceptor with simultaneous generation of superoxides (Hesberg et al., 2004; Yesbergenova et al., 2005; Zarepour et al., 2010). As indicated by XDH-deficient plants, the function of XDH is crucial for plant growth, senescence, and fertility (Nakagawa et al., 2007; Brychkova et al., 2008). Independent from other substrates, XDH exhibits strong intrinsic NADH oxidase activity, which is accompanied by the use of oxygen as electron acceptor and simultaneous formation of superoxide anions (Yesbergenova et al., 2005; Zarepour et al., 2010). It is speculated that this activity is of importance in the response to biotic and abiotic stresses.

Aldehyde oxidase (AO) has derived from XDH by gene duplication and neo-functionalization (Rodríguez-Trelles et al., 2003) and therefore shares catalytical and structural similarities with XDH. In contrast to XDH however, AO proteins preferably oxidize aldehydes to the respective carboxylic acid. Moreover, molecular oxygen is the exclusive electron acceptor during catalysis and its consumption is obligatorily linked with the generation of hydrogen peroxide and superoxide anions (Yesbergenova et al., 2005; Zarepour et al., 2012). Obviously, the genome of most, if not all, plant species harbors several AO genes, which indicates a specific need of plants for several independent aldehyde oxidation activities. The number of AO proteins and their specific functions might therefore relate to the specific metabolic and environmental demands of different plant species. For instance, the *Arabidopsis* genome encodes the genes *AAO1 – AAO4* and the respective proteins form homo- and hetero-dimers with overlapping, but also distinct substrate specificities (Akaba et al., 1999; Koiwai et al., 2000; Seo et al., 2000a,b). The most important isoform is AAO3, which catalyzes the oxidation of abscisic aldehyde to abscisic acid (ABA) in the last cytosolic step of ABA synthesis. Due to the function of ABA in many aspects of plant growth and development, and in adaptation to a variety of abiotic stresses, *AAO3*-deficient plants with reduced ABA levels are characterized by a high transpiration rate, reduced stress tolerance, and impaired seed dormancy (Seo and Koshiba, 2011). For AAO1 and AAO2, a function in one of the multiple biosynthesis routes of indole-3-acetic acid is suggested as they both efficiently catalyze the oxidation of indole acetaldehyde to indole-3-acetic acid *in vitro*. AAO4 is expressed preferably in siliques and catalyzes the oxidation of benzaldehyde into benzoic acid, the latter being incorporated into glucosinolates that likely serve as herbivore defense compounds (Ibdah et al., 2009).

The mitochondrial amidoxime reducing component (mARC) has been identified in mitochondria of mammals and catalyzes the reduction of *N*-hydroxylated substances (Havemeyer et al., 2011). Like mammals, plant genomes encode two mARC isoforms, which have not yet been investigated in detail. The physiological role of mARC proteins is therefore still enigmatic, even though previous studies in *Chlamydomonas* and on recombinant human proteins suggest a function in the detoxification of *N*-hydroxylated base analogs (Chamizo-Ampudia et al., 2011; Krompholz et al., 2012) and/or in the regulation of L-arginine-dependent NO synthesis (Kotthaus et al., 2011).

Although the physiological functions of some molybdo-enzymes are as yet not fully understood, it is obvious that others hold key positions in essential or at least important metabolic pathways. Any factor that affects one of these enzymes will thus also affect the respective pathway, with effects on Moco biosynthesis and molybdate supply resulting in the pleiotropic impairment of all molybdo-enzyme activities, associated with severe reduction of plant viability or even death of the plant.

CROSSTALK BETWEEN MOLYBDENUM AND IRON METABOLISM

An interrelation between molybdenum and iron has been reported by Berry and Reisenauer (1967) who found that molybdate supply significantly increases the capacity of tomato plants to absorb Fe^{2+}. The inverse phenomenon, an influence of Fe^{2+} on molybdate uptake, has later been investigated in excised rice roots, which showed increased molybdate uptake capacity in the presence of $FeSO_4$ (Kannan and Ramani, 1978). This interrelation was confirmed in an ionomics study involving iron-deficient plants, in which molybdenum contents were shown to be reduced (Baxter, 2009). However, the link between the two metals in this approach is not fully understood and might be related either to acidification of the rhizosphere during iron-deficiency, which would result in molybdenum becoming less available to the plant, or to down-regulation of MOT1, which might affect molybdate uptake. Interestingly, several key genes involved in molybdenum metabolism were found to respond to iron-deficiency in *Arabidopsis* roots with the *MOT1* gene indeed showing down-regulation, and the genes *CNX2*, *CNX3*, and *ABA3* showing up-regulation (www.genevestigator.com). By contrast, under molybdate-deficient conditions as induced by Ide et al. (2011) none of the known key genes of iron uptake and transport such as *FRO2*, *IRT1*, or *ferritins* were found to be altered in expression. Instead, only the genes of the ferric-chelate reductases oxidases FRO6 and FRO7 and the iron-regulated protein IREG1 showed down-regulation. It can thus be assumed that iron availability is a crucial regulatory element for plant molybdenum metabolism, while molybdate availability is of subordinated importance for iron metabolism.

Apart from metal uptake and gene regulation, crosstalk between molybdenum and iron is observed on the levels of Moco biosynthesis and molybdo-enyzmes. As previously mentioned, some of the latter require iron-containing prosthetic groups such as iron-sulfur clusters (AO and XDH) or heme (NR) besides Moco and FAD. Indeed, when looking in more detail on the more than 50 molybdo-enzymes known in all organisms, then the vast majority also depends on such iron-containing groups (Hille, 2013). It seems therefore reasonable to assume that this co-occurence of molybdenum and iron is due to the beneficial influence on each other's redox properties. In addition, the Moco biosynthesis enzyme CNX2 requires two [4Fe-4S] clusters for activity and the crosstalk between molybdenum and iron is further substantiated by the fact that *CNX2* gene expression is controlled by both, molybdate and iron availability.

Molybdenum and iron metabolisms also merge at the function of ATM3, a mitochondrial ABC-type transporter that is crucial for the maturation of extra-mitochondrial iron-sulfur proteins (Bernard et al., 2009; Balk and Pilon, 2011). ATM3 exports an as yet unknown sulfur-containing compound from the mitochondria into the cytosol, where this compound is used for assembly of iron-sulfur clusters. Surprisingly, ATM3 has been found to play an important role in enabling the export of the Moco biosynthesis intermediate cPMP from mitochondria into the cytosol as ATM3-deficient plants present reduced levels of Moco accompanied by increased cPMP levels in mitochondria (Teschner et al., 2010). Since the two molecules requiring ATM3 for transport are likely to differ markedly, one can hypothesize that both are co-transported with glutathione, which has been speculated earlier to be a substrate of ATM3 (Kim et al., 2006) and to stimulate the activity of this type of transporter (Kuhnke et al., 2006).

Finally, an evolutionary overlap between molybdenum and iron metabolisms exists by the function of the Moco sulfurase ABA3,

which is essentially required for the final activation of AO and XDH. ABA3 is a two-domain protein with a N-terminal L-cysteine desulfurase domain that decomposes L-cysteine to L-alanine and sulfur (Bittner et al., 2001; Heidenreich et al., 2005). The sulfur is bound as a persulfide to a strictly conserved cysteine residue of the protein (ABA3-$Cys_{430}SH + \underline{S}^{2-} => ABA3\text{-}Cys_{430}S\text{-}\underline{S}H$) and transferred to the Moco-binding C-terminal domain (Wollers et al., 2008). On the C-terminal domain, the persulfide sulfur is transformed into a molybdenum-bound sulfido ligand by replacing an oxygen ligand [$pterin\text{-}MoO_2(OH) + \underline{S}^{2-} => pterin\text{-}MoO\underline{S}(OH) + O^{2-}$]. With receiving this type of Moco, the target proteins of ABA3, AO, and XDH, finally gain activity. Interestingly, L-cysteine desulfurases originated from the more ancient iron-sulfur cluster biosynthesis, in which they abstract sulfur from L-cysteine and deliver it to various scaffold proteins, onto which iron-sulfur clusters are assembled prior to insertion into apo-enzymes (Lill et al., 2012). In case of ABA3 and its orthologs however, a cysteine desulfurase must have been fused to a selected scaffold to evolve into a new activator protein highly specific and exclusive for AO and XDH.

Recently, an interesting observation has been made concerning the effect of sulfur supply on AO and XDH proteins (Cao et al., 2013). In this work, sulfate deficiency has been found to impair endogenous cysteine levels and simultaneously, the biosynthesis of the phytohormone ABA. This effect has been ascribed to reduced activity of AAO3 and it was concluded that AAO3, and in addition also XDH, cannot properly be sulfurated by the Moco sulfurase ABA3 due to the limited availability of its substrate L-cysteine. This might indeed be one possible explanation for reduced activities of AO and XDH proteins in sulfate-deprived plants; yet, an effect on the biosynthesis of iron-sulfur clusters as required by AO and XDH proteins appears even more likely due to the higher demand of this process for L-cysteine (Forieri et al., 2013). As sulfur is embedded into molybdenum metabolism also at several other steps (e.g., Moco synthesis, sulfite detoxification, molybdate uptake), the three nutrients molybdenum, iron and sulfur appear to interact closely on various levels within a common metabolic network that needs to be elucidated in future studies.

CONCLUSIONS AND PERSPECTIVES

Many players in the molybdenum homeostasis network have meanwhile been identified and characterized with the enzymes of Moco synthesis and the molybdo-enzymes representing the most intensively-studied topics, even though also here many aspects still remain to be solved. Understanding of the function of molybdate transporters has just begun and a lot more effort needs to be invested to depict the import and export routes in plants and to identify the candidate(s) responsible for uptake of molybdate at the root:soil interface. A point, which has not been considered before is whether molybdate-binding and/or -storage proteins exist in plants that might shuttle molybdate between organelles and tissues or store it for specific requirements, respectively. Moreover, very little is known about factors controlling the expression of genes and interaction of proteins involved in molybdenum homeostasis, thus requiring further in-depth analysis on the transcriptome, proteome, interactome, metabolome, and ionome level.

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REFERENCES

Akaba, S., Seo, M., Dohmae, N., Takio, K., Sekimoto, H., Kamiya, Y., et al. (1999). Production of homo- and hetero-dimeric isozymes from two aldehyde oxidase genes of *Arabidopsis thaliana*. *J. Biochem.* 126, 395–401.

Balk, J., and Pilon, M. (2011). Ancient and essential: the assembly of iron-sulfur clusters in plants. *Trends Plant Sci.* 16, 218–226. doi: 10.1016/j.tplants.2010.12.006

Baxter, I. (2009). Ionomics: studying the social network of mineral nutrients. *Curr. Opin. Plant Biol.* 12, 381–386. doi: 10.1016/j.pbi.2009.05.002

Baxter, I., Muthukumar, B., Park, H. C., Buchner, P., Lahner, B., Danku, J., et al. (2008). Variation in molybdenum content across broadly distributed populations of *Arabidopsis thaliana* is controlled by a mitochondrial molybdenum transporter (MOT1). *PLoS Genet.* 4:e1000004. doi: 10.1371/journal.pgen.1000004

Bernard, D. G., Cheng, Y., Zhao, Y., and Balk, J. (2009). An allelic mutant series of ATM3 reveals its key role in the biogenesis of cytosolic iron-sulfur proteins in *Arabidopsis*. *Plant Physiol.* 151, 590–602. doi: 10.1104/pp.109.143651

Berry, J. A., and Reisenauer, H. M. (1967). The influence of molybdenum on iron nutrition of tomato. *Plant Soil* 27, 303–313.

Bittner, F., and Mendel, R. R. (2010). "Cell Biology of Molybdenum," in *Cell Biology of Metals and Nutrients*, eds. R. Hell and R. R. Mendel (Springer-Verlag Berlin Heidelberg 2010), *Plant Cell Monogr.* 17, 119–143. doi: 10.1007/978-3-642-10613-2_6

Bittner, F., Oreb, M., and Mendel, R. R. (2001). ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana*. *J. Biol. Chem.* 276, 40381–40384. doi: 10.1074/jbc.C100472200

Brychkova, G., Alikulov, Z., Fluhr, R., and Sagi, M. (2008). A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the Atxdh1 *Arabidopsis* mutant. *Plant J.* 54, 496–509. doi: 10.1111/j.1365-313X.2008.03440.x

Brychkova, G., Xia, Z., Yang, G., Yesbergenova, Z., Zhang, Z., Davydov, O., et al. (2007). Sulfite oxidase protects plants against sulfur dioxide toxicity. *Plant J.* 50, 696–709. doi: 10.1111/j.1365-313X.2007.03080.x

Byrne, R. S., Haensch, R., Mendel, R. R., and Hille, R. (2009). Oxidative half-reaction of *Arabidopsis thaliana* sulfite oxidase: generation of superoxide by a peroxisomal enzyme. *J. Biol. Chem.* 284, 35479–35484. doi: 10.1074/jbc.M109.067355

Campbell, W. H. (2001). Structure and function of eukaryotic NAD(P)H:nitrate reductase. *Cell Mol. Life Sci.* 58,194–204. doi: 10.1007/PL00000847

Cao, M. J., Wang, Z., Zhao, Q., Mao, J. L., Speiser, A., Wirtz, M., et al. (2013). Sulfate availability affects ABA levels and germination response to ABA and salt stress in *Arabidopsis thaliana*. *Plant J.* doi: 10.1111/tpj.12407[Epub ahead of Print].

Chamizo-Ampudia, A., Galvan, A., Fernandez, E., and Llamas, A. (2011). The *Chlamydomonas reinhardtii* molybdenum cofactor enzyme crARC has a Zn-dependent activity and protein partners similar to those of its human homologue. *Eukaryot. Cell* 10, 1270–1282. doi: 10.1128/EC.05096-11

Forieri, I., Wirtz, M., and Hell, R. (2013). Toward new perspectives on the interaction of iron and sulfur metabolism in plants. *Front. Plant Sci.* 4:357. doi: 10.3389/fpls.2013.00357.

Gasber, A., Klaumann, S., Trentmann, O., Trampczynska, A., Clemens, S., Schneider, S., et al. (2011). Identification of an *Arabidopsis* solute carrier critical for intracellular transport and inter-organ allocation of molybdate. *Plant Biol. (Stuttg).* 13, 710–718. doi: 10.1111/j.1438-8677.2011.00448.x

Haensch, R., Lang, C., Riebeseel, E., Lindigkeit, R., Gessler, A., Rennenberg, H., et al. (2006). Plant sulfite oxidase as novel producer of H2O2: combination of enzyme catalysis with a subsequent non-enzymatic reaction step. *J. Biol. Chem.* 281, 6884–6888. doi: 10.1074/jbc.M513054200

Havemeyer, A., Lang, J., and Clement, B. (2011). The fourth mammalian molybdenum enzyme mARC: current state of research. *Drug Metab. Rev.* 43, 524–539. doi: 10.3109/03602532.2011.608682

Heidenreich, T., Wollers, S., Mendel, R. R., and Bittner, F. (2005). Characterization of the NifS-like domain of ABA3 from *Arabidopsis thaliana* provides insight into the mechanism of molybdenum cofactor sulfuration. *J. Biol. Chem.* 280, 4213–4218. doi: 10.1074/jbc.M411195200

Hesberg, C., Haensch, R., Mendel, R. R., and Bittner, F. (2004). Tandem orientation of duplicated xanthine dehydrogenase genes from *Arabidopsis thaliana*:

differential gene expression and enzyme activities. *J. Biol. Chem.* 279, 13547–13554. doi: 10.1074/jbc.M312929200

Hille, R. (2013). The molybdenum oxotransferases and related enzymes. *Dalton Trans.* 42, 3029–3042. doi: 10.1039/c2dt32376a

Hille, R., Nishino, T., and Bittner, F. (2011). Molybdenum enzymes in higher organisms. *Coord. Chem. Rev.* 255, 1179–1205. doi: 10.1016/j.ccr.2010.11.034

Hu, Y., and Ribbe, M. W. (2013). Nitrogenase assembly. *Biochim. Biophys. Acta* 1827, 1112–1122. doi: 10.1016/j.bbabio.2012.12.001

Ibdah, M., Chen, Y.T., Wilkerson, C.G., and Pichersky, E. (2009). An aldehyde oxidase in developing seeds of *Arabidopsis* converts benzaldehyde to benzoic acid. *Plant Physiol.* 150, 416–423. doi: 10.1104/pp.109.135848

Ide, Y., Kusano, M., Oikawa, A., Fukushima, A., Tomatsu, H., Saito, K., et al. (2011). Effects of molybdenum deficiency and defects in molybdate transporter MOT1 on transcript accumulation and nitrogen/sulphur metabolism in *Arabidopsis thaliana*. *J. Exp. Bot.* 62, 1483–1497. doi: 10.1093/jxb/erq345

Kaiser, B. N., Gridley, K. L., Ngaire Brady, J., Phillips, T., and Tyerman, S. D. (2005). The role of molybdenum in agricultural plant production. *Ann. Bot.* 96, 745–754. doi: 10.1093/aob/mci226

Kannan, S., and Ramani, S. (1978). Studies on molybdenum absorption and transport in bean and rice. *Plant Physiol.* 62, 179–181. doi: 10.1104/pp.62.2.179

Kim, D. Y., Bovet, L., Kushnir, S., Noh, E. W., Martinoia, E., and Lee, Y. (2006). AtATM3 is involved in heavy metal resistance in *Arabidopsis*. *Plant Physiol.* 140, 922–932. doi: 10.1104/pp.105.074146

Kneip, C., Lockhart, P., Voss, C., and Maier, U. G. (2007). Nitrogen fixation in eukaryotes-new models for symbiosis. *BMC Evol. Biol.* 7:55. doi: 10.1186/1471-2148-7-55

Koiwai, H., Akaba, S., Seo, M., Komano, T., and Koshiba, T. (2000). Functional expression of two *Arabidopsis* aldehyde oxidases in the yeast Pichia pastoris. *J. Biochem.* 127, 659–664.

Kotthaus, J., Wahl, B., Havemeyer, A., Kotthaus, J., Schade, D., Garbe-Schoenberg, D., et al. (2011). Reduction of N(ω)-hydroxy-L-arginine by the mitochondrial amidoxime reducing component (mARC). *Biochem. J.* 433, 383–391. doi: 10.1042/BJ20100960

Krompholz, N., Krischkowski, C., Reichmann, D., Garbe-Schoenberg, D., Mendel, R. R., Bittner, F., et al. (2012). The mitochondrial amidoxime reducing component (mARC) is involved in detoxification of N-hydroxylated base analogues. *Chem. Res. Toxicol.* 25, 2443–2450. doi: 10.1021/tx300298m

Kuhnke, G., Neumann, K., Muehlenhoff, U., and Lill, R. (2006). Stimulation of the ATPase activity of the yeast mitochondrial ABC transporter Atm1p by thiol compounds. *Mol. Membr. Biol.* 23, 173–184.

Kumchai, J., Huang, J. Z., Lee, C. Y., Chen, F. C., and Chin, S. W. (2013). Proline partially overcomes excess molybdenum toxicity in cabbage seedlings grown in vitro. *Genet. Mol. Res.* 12, 5589–5601. doi: 10.4238/2013.November.18.8

Lang, C., Popko, J., Wirtz, M., Hell, R., Herschbach, C., Kreuzwieser, J., et al. (2007). Sulfite oxidase as key enzyme for protecting plants against sulfur dioxide. *Plant Cell Environ.* 30, 447–455. doi: 10.1111/j.1365-3040.2006.01632.x

Lill, R., Hoffmann, B., Molik, S., Pierik, A. J., Rietzschel, N., Stehling, O., et al. (2012). The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. *Biochim. Biophys. Acta* 1823, 1491–1508. doi: 10.1016/j.bbamcr.2012.05.009

Mendel, R. R. (2013). The molybdenum cofactor. *J. Biol. Chem.* 288, 13165–13172. doi: 10.1074/jbc.R113.455311

Nakagawa, A., Sakamoto, S., Takahashi, M., Morikawa, H., and Sakamoto, A. (2007). The RNAi-mediated silencing of xanthine dehydrogenase impairs growth and fertility and accelerates leaf senescence in transgenic *Arabidopsis* plants. *Plant Cell Physiol.* 48, 1484–1495. doi: 10.1093/pcp/pcm119

Nowak, K., Luniak, N., Witt, C., Wuestefeld, Y., Wachter, A., Mendel, R. R., et al. (2004). Peroxisomal localization of sulfite oxidase separates it from chloroplast-based sulfur assimilation. *Plant Cell Physiol.* 45, 1889–1894. doi: 10.1093/pcp/pch212

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W. M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J. Exp. Bot.* 53, 103–110. doi: 10.1093/jexbot/53.366.103

Rodríguez-Trelles, F., Tarrío, R., and Ayala, F. J. (2003). Convergent neofunctionalization by positive darwinian selection after ancient recurrent duplications of the xanthine dehydrogenase gene. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13413–13417. doi: 10.1073/pnas.1835646100

Seo, M., Koiwai, H., Akaba, S., Komano, T., Oritani, T., Kamiya, Y., et al. (2000a). Abscisic aldehyde oxidase in leaves of *Arabidopsis thaliana*. *Plant J.* 23, 481–488. doi: 10.1046/j.1365-313x.2000.00812.x

Seo, M., Peeters, A. J., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J. A., et al. (2000b). The *Arabidopsis* aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. *Proc. Natl. Acad. Sci. U.S.A.* 97, 12908–12913. doi: 10.1073/pnas.220426197

Seo, M., and Koshiba, T. (2011). Transport of ABA from the site of biosynthesis to the site of action. *J. Plant Res.* 124, 501–507. doi: 10.1007/s10265-011-0411-4

Shibagaki, N., and Grossman, A. R. (2006). The role of the STAS domain in the function and biogenesis of a sulfate transporter as probed by random mutagenesis. *J. Biol. Chem.* 281, 22964–22973. doi: 10.1074/jbc.M603462200

Shinmachi, F., Buchner, P., Stroud, J. L., Parmar, S., Zhao, F. J., McGrath, S. P., et al. (2010). Influence of sulfur deficiency on the expression of specific sulfate transporters and the distribution of sulfur, selenium, and molybdenum in wheat. *Plant Physiol.* 153, 327–336. doi: 10.1104/pp.110.153759

Stout, P. R., Meagher, W. R., Pearson, G. A., and Johnson, C. M. (1951). Molybdenum nutrition of crop plants. I. The influence of phosphate and sulfate on the absorption of molybdenum from soils and solution cultures. *Plant Soil* 3, 51–87.

Tejada-Jiménez, M., Galván, A., and Fernández, E. (2011). Algae and humans share a molybdate transporter. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6420–6425. doi: 10.1073/pnas.1100700108

Tejada-Jimenez, M., Llamas, A., Sanz-Luque, E., Galvan, A., and Fernandez, E. (2007). A high-affinity molybdate transporter in eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20126–20130. doi: 10.1073/pnas.0704646104

Teschner, J., Lachmann, N., Geisler, M., Selbach, K., Balk, J., Mendel, R. R., et al. (2010). A novel role for mitochondrial ABC transporter ATM3 from *Arabidopsis* in molybdenum cofactor biosynthesis. *Plant Cell* 22, 468–480. doi: 10.1105/tpc.109.068478

Tomatsu, H., Takano, J., Takahashi, H., Watanabe-Takahashi, A., Shibagaki, N., and Fujiwara, T. (2007). An *Arabidopsis thaliana* high-affinity molybdate transporter required for efficient uptake of molybdate from soil. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18807–18812. doi: 10.1073/pnas.070637310

Wollers, S., Heidenreich, T., Zarepour, M., Zachmann, D., Kraft, C., Zhao, Y., et al. (2008). Binding of sulfurated molybdenum cofactor to the C-terminal domain of ABA3 from *Arabidopsis thaliana* provides insight into the mechanism of molybdenum cofactor sulfuration. *J. Biol. Chem.* 283, 9642–9650. doi: 10.1074/jbc.M708549200

Yesbergenova, Z., Yang, G., Oron, E., Soffer, D., Fluhr, R., and Sagi, M. (2005). The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. *Plant J.* 42, 862–876. doi: 10.1111/j.1365-313X.2005.02422.x

Zarepour, M., Kaspari, K., Stagge, S., Rethmeier, R., Mendel, R. R., and Bittner, F. (2010). Xanthine dehydrogenase AtXDH1 from *Arabidopsis thaliana* is a potent producer of superoxide anions via its NADH oxidase activity. *Plant Mol. Biol.* 72, 301–310. doi: 10.1007/s11103-009-9570-2

Zarepour, M., Simon, K., Wilch, M., Nielaender, U., Koshiba, T., Seo, M., et al. (2012). Identification of superoxide production by *Arabidopsis thaliana* aldehyde oxidases AAO1 and AAO3. *Plant Mol. Biol.* 80, 659–671. doi: 10.1007/s11103-012-9975-1

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Many rivers to cross: the journey of zinc from soil to seed

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An important goal of micronutrient biofortification is to enhance the amount of bioavailable zinc in the edible seed of cereals and more specifically in the endosperm. The picture is starting to emerge for how zinc is translocated from the soil through the mother plant to the developing seed. On this journey, zinc is transported from symplast to symplast via multiple apoplastic spaces. During each step, zinc is imported into a symplast before it is exported again. Cellular import and export of zinc requires passage through biological membranes, which makes membrane-bound transporters of zinc especially interesting as potential transport bottlenecks. Inside the cell, zinc can be imported into or exported out of organelles by other transporters. The function of several membrane proteins involved in the transport of zinc across the tonoplast, chloroplast or plasma membranes are currently known. These include members of the ZIP (ZRT-IRT-like Protein), and MTP (Metal Tolerance Protein) and heavy metal ATPase (HMA) families. An important player in the transport process is the ligand nicotianamine that binds zinc to increase its solubility in living cells and in this way buffers the intracellular zinc concentration.

Keywords: plants, seeds, apoplastic barrier, zinc, nicotianamine

INTRODUCTION

It is estimated that one-third of the world's population suffers from zinc deficiency with serious consequences (Sandstead, 1991; Welch and Graham, 1999, 2004; World Health Organization, 2003). Zinc deficiency is especially pronounced in areas where people are relying on a plant-based diet, as the edible parts of plants have a low content of bioavailable micronutrients. In cereal grains, high concentrations of zinc are found in the embryo and the aleurone layer of the endosperm (Lombi et al., 2011; Lu et al., 2013a). The localization of zinc is of concern since the embryo and aleurone layer are removed during the polishing of the grains. It is therefore of interest to produce grains with a higher concentration of zinc in the edible endosperm.

Plants, like humans and any other organism, rely on a sufficient zinc supply to drive cellular functions. More than a thousand different proteins have been found to be associated with zinc for functionality in *Arabidopsis thaliana* (Krämer et al., 2007). Zinc serves catalytic, regulatory, and structural roles for a great number of proteins and enzymes with one of the biggest classes of zinc-requiring proteins being the zinc-finger transcription factors (Broadley et al., 2007). Enzymes involved in the synthesis and maintenance of DNA and RNA also requires zinc and the copper/zinc superoxide dismutase in the chloroplast stroma is another example (Hansch and Mendel, 2009). However, in excess amounts zinc is able to replace other metals or bind to undesired proteins and enzymes resulting in their inactivation. Thus, zinc is essential for cellular functions but is toxic at high concentrations. Therefore a tightly controlled homeostatic network consisting of import, trafficking, sequestration and export is needed for the plant to survive (Clemens, 2001; Clemens et al., 2002; Hall, 2002).

Once zinc is taken up into roots it enters a symplast, a living interconnected networks of cells. However, the long way for zinc to the developing seed requires multiple steps where zinc has to move from symplast to symplast. During this process it first has to leave the symplast and enter dead space outside cells, the apoplast, before it can be taken up in a new symplast (**Figure 1**). This transport into and out of the apoplast seems to be the major bottleneck in the process of nutrient translocation within the plant (Palmgren et al., 2008).

A major feature of the plasma membrane of living cells is the presence of a membrane potential, negative on the inside. This membrane potential is maintained by the plasma membrane H^+-ATPase (Sondergaard et al., 2004), and is a main driving force behind passive cellular uptake of positively charged cations. In *Arabidopsis*, membrane potentials of root cells vary typically between −100 and −115 mV (Bose et al., 2010; Kenderesová et al., 2012) and have been reported as high as above −200 mV (Hirsch et al., 1998). According to Nernst equation (and assuming standard conditions) a membrane potential in this range can sustain a hundred-fold concentration difference of a monovalent cation between the outside and the inside of a cell, and a remarkable ten thousand-fold concentration difference of a divalent cation. With 30 μM of Zn^{2+} in the growth medium (as in Murashige and Skoog medium; Murashige and Skoog, 1962) and an inside membrane potential of −115 mV, the cells would in theory by passive uptake be able to establish a cytoplasmic concentration of around 300 mM Zn^{2+}, which would be far above the cellular requirement and in fact highly toxic. It is therefore evident that uptake of zinc is not an energetic problem and that this process does not require active transport. What is an advantage for uptake of Zn^{2+}, however, becomes a major disadvantage when Zn^{2+} has to be exported from cells. Now the membrane potential, positive on the outside, is a strong opposing factor for outward transport of a divalent cation, and a primary active transport system is required.

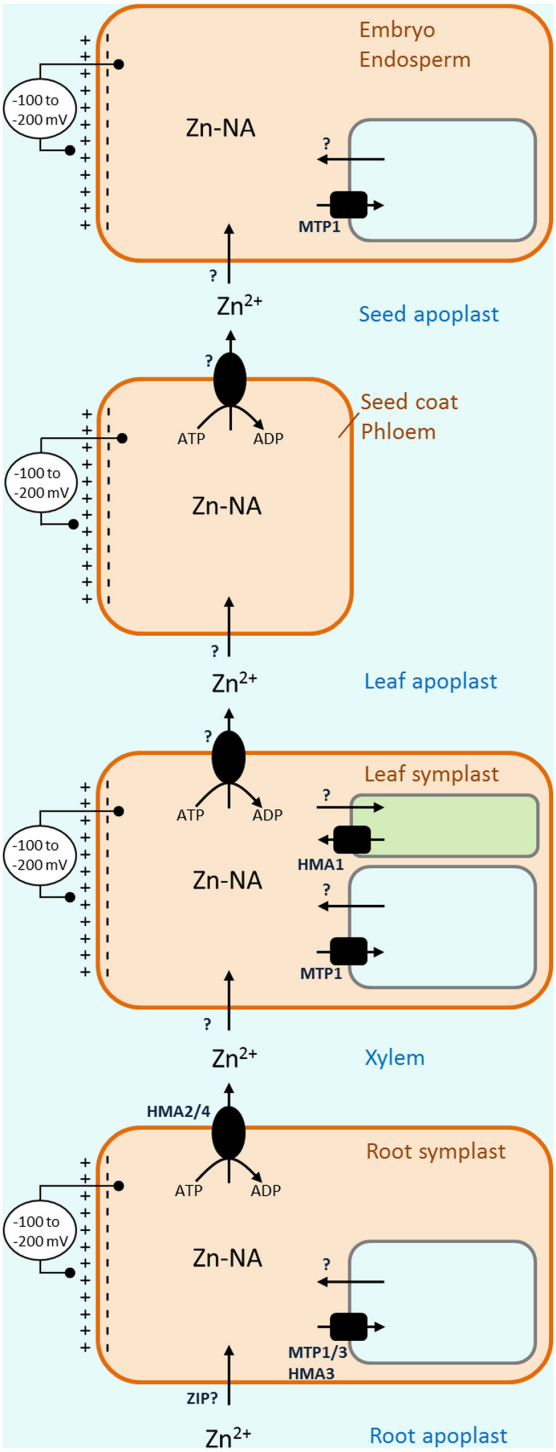

FIGURE 1 | Overview of transport barriers leading to loading of zinc into seeds. Following uptake of zinc into the root symplast, at least three apoplastic barriers have to be crossed on the way to the seed. Substantial membrane potentials cross the membranes of the plasma membrane, vacuole and chloroplast, transport of zinc into the cytoplasm, out of the vacuole or into the chloroplast is energetically favorable, requiring passive transporters only (arrows). On the other hand, transport out of the cytosol, into the vacuole or out of the chloroplast requires active transporters or secondary active transporters (round and square symbols, respectively).

The vacuole has an inside positive membrane potential requiring active transport systems for import and making export from the vacuole energetically favorable.

ZINC UPTAKE INTO ROOTS

The first symplastic domain zinc has to enter is that of the root cortex. Uptake into this symplast is likely to be carried out by transport proteins in the plasma membrane of root epidermal or cortical cells. In *Arabidopsis*, IRT1 is a broad substrate range metal ion transporter localized to the plasma membrane that can transport iron (Fe^{2+}), zinc (Zn^{2+}), manganese (Mn^{2+}), cobalt (Co^{2+}) and cadmium (Cd^{2+}) (Korshunova et al., 1999) and is subject to intricate metal-dependent post-translational regulation (Shin et al., 2013). This transporter is expressed in the plasma membrane of root epidermal cells (Vert et al., 2002) and is therefore likely to be involved in zinc uptake. Excess Zn causes reduced Fe uptake and excess Fe alleviates Zn toxicity under these conditions, which supports the view that Zn and Fe compete for the same uptake system(s) (Fukao et al., 2011; Shanmugam et al., 2011, 2013). A closely related broad affinity metal transporter, IRT2, is also expressed in epidermal cells (Vert et al., 2001) but in internal membranes (Vert et al., 2009), which excludes a role in direct uptake. Other members of the ZIP (*Z*RT-*I*RT-like *P*rotein) family of metal transporters, to which IRT1 and IRT2 belong, have been proposed to import zinc into roots (Grotz et al., 1998; Lin et al., 2009) but this assumption has recently been questioned as functional studies with plants mutated in the major root *ZIP* genes, At*ZIP1*, and At*ZIP2*, point to a role of these transporters in root to shoot transport (Milner et al., 2013). The barley transporter HvZIP7 is important for zinc uptake but is localized in root vascular tissue suggesting a similar role in promoting root to shoot transport (Tiong et al., 2013). In rice, OsZIP1 and OsZIP3 have been proposed to be involved in root zinc uptake whereas OsZIP 4, OsZIP5, and OsZIP8 could be involved in root to shoot translocation (Bashir et al., 2013).

After uptake, zinc is in living cells with a neutral pH, a condition in which zinc is prone to bind to a multitude of organic molecules present, which severely restricts its free mobility. Zinc therefore travels between living cells strongly bound to a symplastic metal chelator, which at least in *A. halleri* is the metabolite nicotianamine (Deinlein et al., 2012). The zinc-nicotianamine complex is transportable and can diffuse between cells in the root symplast, which are interconnected by plasmodesmal bridges, toward the xylem, the dead vascular tissue leading to the shoot. The Casparian strip is an impermeable diffusion barrier present in the root apoplast. In the dicot *Arabidopsis* it is present as a layer of lignin (Naseer et al., 2012), which by surrounding the cells of the root endodermis divides the root apoplast in two, an outer apoplast which includes the cell walls of the cortex and extends to the soil solution, and an inner apoplast, which includes the xylem of the central stele. In other plants, such as in monocot cereals, an additional diffusion barrier is present in the exodermis above the cortex and below the endodermis. Cereals thus possess basically two layers of Casparian strips that divide the root into three apoplasts. In the root symplast the transport of zinc is restricted by sequestration for storage into the vacuole, an import requiring active transporters. Two MTPs (*M*etal *T*olerance *P*rotein) AtMTP1 and AtMTP3 have been implicated in this process (Desbrosses-Fonrouge et al.,

2005; Arrivault et al., 2006; Kawachi et al., 2009). The HMA AtHMA3 also seems to function in the vacuole to sequester zinc (Morel et al., 2009).

XYLEM LOADING IN ROOTS

In order to enter the xylem, the dead vascular tissue leading to the shoot, zinc has to be exported from the symplast, which requires active transporters. *A. thaliana* HMA 2 and 4 (AtHMA2 and AtHMA4, respectively), which are primary active zinc pumps, are involved in such loading of the root xylem (Hussain et al., 2004; Verret et al., 2004; Sinclair et al., 2007). AtHMA4 is equipped with an intracellularly exposed zinc-binding domain that may signify post-translational regulation of pump activity in response to a cytoplasmic zinc sensor (Baekgaard et al., 2010). Also, AtHMA2 is known to be regulated at the transcriptional level in response to zinc availability (van de Mortel et al., 2006); both kind of regulation could ensure a tightly controlled xylem loading step. The importance of HMA4 in root-to-shoot translocation of zinc is clearly seen in *A. halleri* where the zinc hyperaccumulation trait strictly depends on AhHMA4 pumping zinc into root xylem vessels (Hanikenne et al., 2008). In rice, the homolog OsHMA2 has also been shown to be involved in root-to-shoot translocation of zinc (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012). In *A. thaliana* other transporters also play a role in xylem loading. At least one member of the Plant Cadmium Resistance family, AtPCR2, is involved in root-to-shoot translocation of zinc (Song et al., 2010) and recently AtZIP1 and AtZIP2 have been implicated in this process (Milner et al., 2013).

Once in the xylem, where pH is slightly acidic (around pH 5.5), zinc can be transported as a free cation. In the zinc hyperaccumulator *Sedum alfredii*, zinc is predominantly transported in the xylem as aqueous zinc. Noteworthy, however, in this plant a significant proportion (around 40%) is associated in the xylem sap with its predominant organic acid, citric acid (Lu et al., 2013b). In *A. halleri*, the zinc mimic cadmium is transported in the free ionic form only (Ueno et al., 2008). In rice, deoxymugineic acid has been associated with long-distance transport of zinc (Suzuki et al., 2008), but it is not known whether the deoxymugineic acid-zinc complex is present in the xylem.

XYLEM UNLOADING

Zinc is not transported directly to the developing seed by the xylem. Typically, in order to reach the seed, zinc first makes a detour to the leaves. Here it is taken up from the xylem into living xylem parenchyma cells of the leaf symplast. This process is mediated by transport proteins yet to be characterized. Subsequently, zinc is exported from mesophyll cells into the leaf apoplast from where it is loaded into the phloem, which provides the vascular route to the developing seeds. Because of their expression in the vasculature, AtIRT3 (Lin et al., 2009) and OsZIP4 (Ishimaru et al., 2005) may be implied in xylem unloading and/or the phloem loading processes (see below). In grasses, such as rice, a complicated network of vascular bundles in the nodes of the stem allows for a more direct transfer of zinc from the xylem to phloem strands leading to the panicle (Yamaguchi et al., 2012).

PHLOEM LOADING

The vascular tissue of the phloem is the only route for zinc leading to the developing seeds. Loading of zinc into the phloem typically takes places in leaves. During senescence, zinc is remobilized from withering leaves in order to be allocated to reproductive tissues where demand becomes high. This process requires zinc transport out of mesophyll cells into the leaf apoplast and subsequent transport into the phloem. Before zinc can leave the mesophyll it has to leave the chloroplast, where it plays an important process in photosystem II, and the vacuole, where excess zinc is stored. In *Arabidopsis* HMA1 is localized to the chloroplast envelope and involved in the export of zinc (Kim et al., 2009). This function has also been shown for HvHMA1, where HMA1 has been implied in the remobilization of zinc (Mikkelsen et al., 2012). During zinc remobilization, active transport is not required for zinc in order to leave the vacuole because of the inside positive membrane potential across the tonoplast. In cereals, the remobilization of zinc in the plant during the period of grain loading is not restricted to leaves but also occurs from stems, peduncles, florets and rachis (Walker and Waters, 2011). How zinc is transported out of leaf mesophyll cells is not known.

How zinc enters the phloem is also not known. Transport proteins of the Yellow Stripe-Like (YSL) family, which appear to transport complexes of zinc and nicotianamine across membranes, are highly expressed in senescing leaves (Waters et al., 2006; Curie et al., 2009), and might play a role in transporting zinc into the phloem. Among these, at least AtYSL1 and AtYSL3 are required for allocation of zinc to the developing grain (Chu et al., 2010). Other members of the YSL family might also be involved in the translocation of zinc. AtYSL4 and AtYSL6 have been described as iron transporters localized to organelles, either the chloroplast (Divol et al., 2013) or other endomembrane systems (Conte et al., 2013).

The pH of the phloem is slightly alkaline (Dinant et al., 2010), and therefore zinc has to travel in the phloem bound to a metal chelator, which often appear to be nicotianamine (Curie et al., 2009; Nishiyama et al., 2012).

PHLOEM UNLOADING IN DEVELOPING SEEDS

The mechanisms involved in phloem unloading and post-phloem movement of zinc in the developing seed are not well understood, but a model has been proposed based on the findings of several apoplastic barriers in the *Arabidopsis* seed. The seed consists of three genetically distinct tissues: the maternal seed coat, the zygotic embryo and the triploid zygotic endosperm. It is anticipated that such diverse tissues do not contain any symplastic connections and apoplastic barriers are likely to be separating these three tissues in *Arabidopsis* (Stadler et al., 2005). Phloem unloading in the developing seed is believed to be symplastic into a phloem-unloading domain, which has symplastic connections to the entire seed coat (Stadler et al., 2005; Werner et al., 2011). Another apoplastic barrier seems to be present in the seed coat where the outer two cell layers (the outer integument) lacks symplastic connectivity with the three inner cell layers of the seed coat (the inner integument) (Stadler et al., 2005). This gives rise to three apoplastic barriers in the *Arabidopsis* seed where active transport system(s) yet to be identified must be present for translocation of zinc to the embryo:

(1) between the outer and inner integuments in the seed coat, (2) between the seed coat and the endosperm, and (3) between the endosperm and the embryo. As active transport is required for zinc in order to escape living cells having a membrane potential highly negative on the inside, zinc efflux from symplasts in the seeds resemble the situation of xylem loading in the root.

The architecture of a cereal grain differs substantially from an *Arabidopsis* seed. In barley nutrients are also unloaded symplastically from the phloem, but they do not end up in the seed coat as in *Arabidopsis*. Instead the nutrients flow to highly specialized nucellar projection transfer cells. In the developing barley grain, apparently a single apoplastic barrier is present between the maternal transfer cells and the filial endosperm (Borg et al., 2009). In the wheat grain, an additional apoplastic barrier for Zn has been identified between the stem tissue rachis and the developing grain, which most likely is linked to unloading from the xylem of the stem and subsequent loading of the phloem leading to the grain (Wang et al., 2011). Whether this is a general feature of cereal grains remains to be shown. In a microarray study from laser capture microdissection of the developing barley grain it was found that *HvHMA2* was almost exclusively expressed in the transfer cells in the developing barley grain (Tauris et al., 2009). The expression of *HvHMA2* in the transfer cells makes HvHMA2 a prime candidate for export of zinc from the mother plant into the endosperm cavity (Tauris et al., 2009). The zinc sensitivity of the *Saccharomyces cerevisiae zrc cot1* mutant is alleviated following heterologous expression the heterologous expression of *HvHMA2,* supporting the notion that HvHMA2 functions as a zinc export pump (Mills et al., 2012). Transporters of the MTP family might also be involved in controlling the amount of zinc crossing the apoplastic barriers in the developing seed by sequestering zinc into the vacuole on either side. Several MTPs are expressed in barley (Tauris et al., 2009) and *Arabidopsis* (Desbrosses-Fonrouge et al., 2005) seeds.

NICOTIANAMINE – THE INTERCELLULAR AND PHLOEM MOBILE ZINC-LIGAND

Apart from the transport across apoplastic barriers in the root and seed requiring membrane transporters, the intercellular and long-distance transport of zinc influences the loading of zinc to the seed. Due to its low solubility at neutral and alkaline pH, zinc is not present as free ions, but will be bound to a ligand. Nicotianamine, in particular, has emerged as a major zinc chelator in plants although it also chelates other metals such as iron and copper (Clemens et al., 2013).

In the zinc hyperaccumulator *A. halleri*, nicotianamine synthase (NAS) is highly expressed and nicotianamine has been shown to be involved in root-to-shoot translocation of zinc with a fivefold decrease in *NAS2*-RNAi lines. This has been ascribed to a role for nicotianamine in the symplastic mobility of zinc in the root toward the pericycle cells (Deinlein et al., 2012). The phenotypes of the *A. halleri NAS2*-RNAi lines (Deinlein et al., 2012) and the *HMA4*-RNAi lines (Hanikenne et al., 2008) are somewhat similar. Both show reduced root-to-shoot translocation and accumulation of zinc in the root pericycle cells due to reduced xylem loading. This makes it very interesting to look further into the connection between nicotianamine and HMA4.

Nicotianamine seems to be highly important for the intercellular mobility of zinc but might also be involved in enhancing the mobility of zinc in the phloem essential for getting zinc to the developing seed. Several reports have shown that the constitutive overexpression of *NAS* genes in rice is able to increase the zinc concentration in the rice grain by in average twofold, also in the polished grain (Lee et al., 2009; Masuda et al., 2009; Johnson et al., 2011; Lee et al., 2011). This probably happens because the enhanced level of nicotianamine increases the phloem mobility of zinc, thus enhancing the flow of zinc to the nucellar projection in the rice grain. Likewise, overexpression of *NAS* in combination with ferritin affects Fe accumulation in the rice endosperm (Wirth et al., 2009). Nicotianamine has been shown to be the major zinc-ligand in the leaf phloem sap of wild-type rice (Nishiyama et al., 2012) and one of the main limitations in getting zinc to the developing seed has been identified as being its transport in the phloem (White and Broadley, 2011). Also, it was found that zinc bound to nicotianamine in the endosperm is highly bioavailable (Lee et al., 2011). A significant problem concerning zinc biofortification is that transporters for zinc often co-transport the zinc mimic cadmium. However, nicotianamine seems to be highly specific for zinc over cadmium and, accordingly, rice plants overexpressing *OsNAS* do not show an increase in cadmium (Lee et al., 2009, 2011).

CONCLUSION

Taken together, the transport of zinc from the root to the shoot and further to the developing seed results from an interplay not only between different membrane transporters but also between the mobility of zinc both intercellularly and between organs by binding to ligands. The presence of multigenic factors that control the amount of zinc ending up in the edible parts of the seed makes it a challenging future to increase bioavailable zinc in the edible parts of cereal grains. The substantial increase in the grain zinc concentration found in plants overexpressing *NAS* genes makes nicotianamine an interesting target for zinc biofortification.

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REFERENCES

Arrivault, S., Senger, T., and Kramer, U. (2006). The *Arabidopsis* metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *Plant J.* 46, 861–879. doi: 10.1111/j.1365-313X.2006.02746.x

Baekgaard, L., Mikkelsen, M. D., Sorensen, D. M., Hegelund, J. N., Persson, D. P., Mills, R. F., et al. (2010). A combined zinc/cadmium sensor and zinc/cadmium export regulator in a heavy metal pump. *J. Biol. Chem.* 285, 31243–31252. doi: 10.1074/jbc.M110.111260

Bashir, K., Takahashi, R., Nakanishi, H., and Nishizawa, N. (2013). The road to micronutrient biofortification of rice: progress and prospects. *Front. Plant Sci.* 4:15. doi: 10.3389/fpls.2013.00015

Borg, S., Brinch-Pedersen, H., Tauris, B., and Holm, P. B. (2009). Iron transport, deposition and bioavailability in the wheat and barley grain. *Plant Soil* 325, 15–24. doi: 10.1007/s11104-009-0046–46

Bose, J., Babourina, O., Shabala, S., and Rengel, Z. (2010). Aluminium-induced ion transport in *Arabidopsis*: the relationship between Al tolerance and root ion flux. *J. Exp. Bot.* 61, 3163–3175. doi: 10.1093/jxb/erq143

Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., and Lux, A. (2007). Zinc in plants. *New Phytol.* 173, 677–702. doi: 10.1111/j.1469-8137.2007.01996.x

Chu, H.-H., Chiecko, J., Punshon, T., Lanzirotti, A., Lahner, B., Salt, D., et al. (2010). Successful reproduction requires the function of *Arabidopsis* Yellow Stripe-like1 and Yellow Stripe-like3 metal-nicotianamine transporters in both vegetative and reproductive structures. *Plant Physiol.* 154, 197–210. doi: 10.1104/pp.110.159103

Clemens, S. (2001). Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* 212, 475–486. doi: 10.1007/s004250000458

Clemens, S., Deinlein, U., Ahmadi, H., Höreth, S., and Uraguchi, S. (2013). Nicotianamine is a major player in plant Zn homeostasis. *Biometals* 26, 623–632. doi: 10.1007/s10534-013-9643–9641

Clemens, S., Palmgren, M. G., and Kramer, U. (2002). A long way ahead: Understanding and engineering plant metal accumulation. *Trends Plant Sci.* 7, 309–315. doi: 10.1016/S1360-1385(02)02295-1

Conte, S., Chu, H., Rodriguez, D., Punshon, T., Vasques, K., Salt, D., et al. (2013). *Arabidopsis thaliana* Yellow Stripe1-Like4 and Yellow Stripe1-Like6 localize to internal cellular membranes and are involved in metal ion homeostasis. *Front. Plant Sci.* 4:283. doi: 10.3389/fpls.2013.00283

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and Yellow Stripe1-Like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T., et al. (2012). Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. *Plant Cell* 24, 708–723. doi: 10.1105/tpc.111.095000

Desbrosses-Fonrouge, A.-G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U. (2005). *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Lett.* 579, 4165–4174. doi: 10.1016/j.febslet.2005.06.046

Dinant, S., Bonnemain, J. L., Girousse, C., and Kehr, J. (2010). Phloem sap intricacy and interplay with aphid feeding. *C. R. Biol.* 333, 504–515. doi: 10.1016/j.crvi.2010.03.008

Divol, F., Couch, D., Conéjéro, G., Roschzttardtz, H., Mari, S., and Curie, C. (2013). The *Arabidopsis* Yellow Stripe Like4 and 6 transporters control iron release from the chloroplast. *Plant Cell* 25, 1040–1055. doi: 10.1105/tpc.112.107672

Fukao, Y., Ferjani, A., Tomioka, R., Nagasaki, N., Kurata, R., Nishimori, Y., et al. (2011). iTRAQ analysis reveals mechanisms of growth defects due to excess zinc in *Arabidopsis*. *Plant Physiol.* 155, 1893–1907. doi: 10.1104/pp.110.169730

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., and Eide, D. (1998). Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7220–7224. doi: 10.1073/pnas.95.12.7220

Hall, J. L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.* 53, 1–11. doi: 10.1093/jexbot/53.366.1

Hanikenne, M., Talke, I. N., Haydon, M. J., Lanz, C., Nolte, A., Motte, P., et al. (2008). Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature* 453, 391–396. doi: 10.1038/nature 06877

Hansch, R., and Mendel, R. R. (2009). Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Curr. Opin. Plant Biol.* 12, 259–266. doi: 10.1016/j.pbi.2009.05.006

Hirsch, R., Lewis, B., Spalding, E., and Sussman, M. (1998). A role for the AKT1 potassium channel in plant nutrition. *Science* 280, 918–921. doi: 10.1126/science.280.5365.918

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., et al. (2004). P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell* 16, 1327–1339. doi: 10.1105/tpc.020487

Ishimaru, Y., Suzuki, M., Kobayashi, T., Takahashi, M., Nakanishi, H., Mori, S., et al. (2005). OsZIP4, a novel zinc-regulated zinc transporter in rice. *J. Exp. Bot.* 56, 3207–3214 doi: 10.1093/jxb/eri317

Johnson, A., Kyriacou, B., Callahan, D. L., Carruthers, L., Stangoulis, J., Lombi, E., et al. (2011). Constitutive overexpression of the OsNAS gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS ONE* 6:e24476. doi: 10.1371/journal.pone.0024476

Kawachi, M., Kobae, Y., Mori, H., Tomioka, R., Lee, Y., and Maeshima, M. (2009). A mutant strain *Arabidopsis thaliana* that lacks vacuolar membrane zinc transporter MTP1 revealed the latent tolerance to excessive zinc. *Plant Cell Physiol.* 50, 1156–1170. doi: 10.1093/pcp/pcp067

Kenderesová, L., Stanová, A., Pavlovkin, J., Durisová, E., Nadubinská, M., Ciamporová, M., et al. (2012). Early Zn2+-induced effects on membrane potential account for primary heavy metal susceptibility in tolerant and sensitive *Arabidopsis* species. *Ann. Bot.* 110, 445–459. doi: 10.1093/aob/mcs111

Kim, Y.-Y., Choi, H., Segami, S., Cho, H.-T., Martinoia, E., Maeshima, M., et al. (2009). AtHMA1 contributes to the detoxification of excess Zn(II) in *Arabidopsis*. *Plant J.* 58, 737–753. doi: 10.1111/j.1365-313X.2009.03818.x

Korshunova, Y., Eide, D., Clark, W., Guerinot, M., and Pakrasi, H. (1999). The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Mol. Biol.* 40, 37–44. doi: 10.1023/a:1026438615520

Krämer, U., Talke, I. N., and Hanikenne, M. (2007). Transition metal transport. *FEBS Lett.* 581, 2263–2272. doi: 10.1016/j.febslet.2007.04.010

Lee, S., Jeon, U., Lee, S., Kim, Y.-K., Persson, D., Husted, S., et al. (2009). Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22014–22019. doi: 10.1073/pnas.0910950106

Lee, S., Persson, D., Hansen, T., Husted, S., Schjoerring, J., Kim, Y.-S., et al. (2011). Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. *Plant Biotech. J.* 9, 865–873. doi: 10.1111/j.1467-7652.2011.00606.x

Lin, Y.-F., Liang, H.-M., Yang, S.-Y., Boch, A., Clemens, S., Chen, C.-C., et al. (2009). *Arabidopsis* IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New Phytol.* 182, 392–404. doi: 10.1111/j.1469-8137.2009.02766.x

Lombi, E., Smith, E., Hansen, T., Paterson, D., De Jonge, M., Howard, D., et al. (2011). Megapixel imaging of (micro)nutrients in mature barley grains. *J. Exp. Bot.* 62, 273–282. doi: 10.1093/jxb/erq270

Lu, L., Tian, S., Liao, H., Zhang, J., Yang, X., Labavitch, J., et al. (2013a). Analysis of metal element distributions in rice (*Oryza sativa* L.) seeds and relocation during germination based on X-ray fluorescence imaging of Zn, Fe, K, Ca, and Mn. *PLoS ONE* 8:e57360. doi: 10.1371/journal.pone.0057360

Lu, L., Tian, S., Zhang, J., Yang, X., Labavitch, J., Webb, S., et al. (2013b). Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant species *Sedum alfredii*. *New Phytol.* 198, 721–731. doi: 10.1111/nph.12168

Masuda, H., Kanako, U., Takanori, K., Yasuhiro, I., Yusuke, K., Michiko, T., et al. (2009). Overexpression of the barley nicotianamine synthase gene HvNAS1 increases iron and zinc concentrations in rice grains. *Rice* 2, 155–166. doi: 10.1007/s12284-009-9031–9031

Mikkelsen, M., Pedas, P., Schiller, M., Vincze, E., Mills, R., Borg, S., et al. (2012). Barley HvHMA1 is a heavy metal pump involved in mobilizing organellar Zn and Cu and plays a role in metal loading into grains. *PLoS ONE* 7:e49027. doi: 10.1371/journal.pone.0049027

Mills, R. F., Peaston, K. A., Runions, J., and Williams, L. E. (2012). HvHMA2, a P1B-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport. *PLoS ONE* 7:e42640. doi: 10.1371/journal.pone.0042640

Milner, M., Seamon, J., Craft, E., and Kochian, L. (2013). Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. *J. Exp. Bot.* 64, 369–381. doi: 10.1093/jxb/ers315

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., et al. (2009). AtHMA3, a P(1B)-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in *Arabidopsis*. *Plant Physiol.* 149, 894–904. doi: 10.1104/pp.108.130294

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures *Physiol. Plant* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Naseer, S., Lee, Y., Lapierre, C., Franke, R., Nawrath, C., and Geldner, N. (2012). Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10101–10106. doi: 10.1073/pnas.1205726109

Nishiyama, R., Kato, M., Nagata, S., Yanagisawa, S., and Yoneyama, T. (2012). Identification of Zn-nicotianamine and Fe-2'-deoxymugineic acid in the phloem sap from rice plants (*Oryza sativa* L.). *Plant Cell Physiol.* 53, 381–390. doi: 10.1093/pcp/pcr188

Palmgren, M. G., Clemens, S., Williams, L. E., Kraemer, U., Borg, S., Schjorring, J. K., et al. (2008). Zinc biofortification of cereals: problems and solutions. *Trends Plant Sci.* 13, 464–473. doi: 10.1016/j.tplants.2008.06.005

Sandstead, H. H. (1991). Zinc deficiency. A public health problem? *Am. J. Dis. Child.* 145, 853–859. doi: 10.1001/archpedi.1991.02160080029016

Satoh-Nagasawa, N., Mori, M., Nakazawa, N., Kawamoto, T., Nagato, Y., Sakurai, K., et al. (2012). Mutations in Rice (*Oryza sativa*) Heavy Metal ATPase 2 (OsHMA2)

Restrict the Translocation of Zinc and Cadmium. *Plant Cell Physiol.* 53, 213–224. doi: 10.1093/pcp/pcr166

Shanmugam, V., Lo, J. C., Wu, C. L., Wang, S. L., Lai,C. C., Connolly, E. L., et al. (2011). Differential expression and regulation of iron-regulated metal transportersin *Arabidopsis halleri* and *Arabidopsis thaliana* – the role in zinc tolerance. *New Phytol.* 190, 125–137.doi:10.1111/j.1469-8137.2010.03606.x

Shanmugam, V., Lo, J. C., and Yeh, K. C. (2013). Control of Zn uptake in *Arabidopsis halleri*: a balance between Zn and Fe. *Front. Plant Sci.* 4:281 doi: 10.3389/fpls.2013.00281

Shin, L.-J., Lo, J.-C., Chen, G.-H., Callis, J., Fu, H., and Yeh, K.-C. (2013). IRT1 degradation factor1, a ring E3 ubiquitin ligase, regulates the degradation of iron-regulated transporter1 in *Arabidopsis*. *Plant Cell* 25, 3039–3051. doi: 10.1105/tpc.113.115212

Sinclair, S. A., Sherson, S. M., Jarvis, R., Camakaris, J., and Cobbett, C. S. (2007). The use of the zinc-fluorophore, Zinpyr-1, in the study of zinc homeostasis in *Arabidopsis* roots. *New Phytol.* 174, 39–45. doi: 10.1111/j.1469-8137.2007.02030.x

Sondergaard, T. E., Schulz, A., and Palmgren, M. G. (2004). Energization of transport processes in plants. Roles of the plasma membrane H+-ATPase. *Plant Physiol.* 136, 2475–2482. doi: 10.1104/pp.104.048231

Song, W.-Y., Choi, K. S., Kim, D. Y., Geisler, M., Park, J., Vincenzetti, V., et al. (2010). *Arabidopsis* PCR2 is a zinc exporter involved in both zinc extrusion and long-distance zinc transport. *Plant Cell* 22, 2237–2252. doi: 10.1105/tpc.109.070185

Stadler, R., Lauterbach, C., and Sauer, N. (2005). Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in *Arabidopsis* seeds and embryos. *Plant Physiol.* 139, 701–712. doi: 10.1104/pp.105.065607

Suzuki, M., Tsukamoto, T., Inoue, H., Watanabe, S., Matsuhashi, S., Takahashi, M., et al. (2008). Deoxymugineic acid increases Zn translocation in Zn-deficient rice plants. *Plant Mol. Biol.* 66, 609–617. doi: 10.1007/s11103-008-9292-x

Takahashi, R., Ishimaru, Y., Shimo, H., Ogo, Y., Senoura, T., Nishizawa, N., et al. (2012). The OsHMA2 transporter is involved in root-to-shoot translocation of Zn and Cd in rice. *Plant Cell Environ.* 35, 1948–1957. doi: 10.1111/j.1365-3040.2012.02527.x

Tauris, B., Borg, S., Gregersen, P. L., and Holm, P. B. (2009). A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. *J. Exp. Bot.* 60, 1333–1347. doi: 10.1093/jxb/erp023

Tiong, J., Mcdonald, G., Genc, Y., Pedas, P., Hayes, J., Toubia, J., et al. (2013). HvZIP7 mediates zinc accumulation in barley (*Hordeum vulgare*) at moderately high zinc supply. *New Phytol.* 201, 131–143. doi: 10.1111/nph.12468 doi: 10.1111/nph.12468

Ueno, D., Iwashita, T., Zhao, F.-J., and Ma, J. (2008). Characterization of Cd translocation and identification of the Cd form in xylem sap of the Cd-hyperaccumulator *Arabidopsis halleri*. *Plant Cell Physiol.* 49, 540–548. doi: 10.1093/pcp/pcn026

van de Mortel, J., Almar Villanueva, L., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P., et al. (2006). Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol.* 142, 1127–1147. doi: 10.1104/pp.106.082073

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., et al. (2004). Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Lett.* 576, 306–312. doi: 10.1016/j.febslet.2004.09.023

Vert, G., Barberon, M., Zelazny, E., Séguéla, M., Briat, J.-F., and Curie, C. (2009). *Arabidopsis* IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta* 229, 1171–1179. doi: 10.1007/s00425-009-0904-908

Vert, G., Briat, J., and Curie, C. (2001). *Arabidopsis* IRT2 gene encodes a root-periphery iron transporter. *Plant J.* 26, 181–189. doi: 10.1046/j.1365-313x.2001.01018.x

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M., Briat, J.-F., et al. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

Walker, E., and Waters, B. (2011). The role of transition metal homeostasis in plant seed development. *Curr. Opin. Plant Biol.* 14, 318–324. doi: 10.1016/j.pbi.2011.03.025

Wang, Y., Specht, A., and Horst, W. (2011). Stable isotope labelling and zinc distribution in grains studied by laser ablation ICP-MS in an ear culture system reveals zinc transport barriers during grain filling in wheat. *New Phytol.* 189, 428–437. doi: 10.1111/j.1469-8137.2010.03489.x

Waters, B., Chu, H.-H., Didonato, R., Roberts, L., Eisley, R., Lahner, B., et al. (2006). Mutations in *Arabidopsis* Yellow Stripe-Like1 and Yellow Stripe-Like3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds. *Plant Physiol.* 141, 1446–1458. doi: 10.1104/pp.106.082586

Welch, R. M., and Graham, R. D. (1999). A new paradigm for world agriculture: meeting human needs: Productive, sustainable, nutritious. *Field Crops Res.* 60, 1–10. doi: 10.1016/S0378-4290(98)00129-4

Welch, R. M., and Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* 55, 353–364. doi: 10.1093/jxb/erh064

Werner, D., Gerlitz, N., and Stadler, R. (2011). A dual switch in phloem unloading during ovule development in *Arabidopsis*. *Protoplasma* 248, 225–235. doi: 10.1007/s00709-010-0223–228

White, P. J., and Broadley, M. R. (2011). Physiological limits to zinc biofortification of edible crops. *Front. Plant Sci.* 2:80. doi: 10.3389/fpls.2011.00080

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., et al. (2009). Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotech. J.* 7, 631–644. doi: 10.1111/j.1467-7652.2009.00430.x

World Health Organization. (2003). *Summary and Conclusion of the Sixty-first Meeting of the Joint FAO/WHO Expert Committee on Food Additives.* Geneva, Switzerland: World Health Organization, 16–18.

Yamaguchi, N., Ishikawa, S., Abe, T., Baba, K., Arao, T., and Terada, Y. (2012). Role of the node in controlling traffic of cadmium, zinc, and manganese in rice. *J. Exp. Bot.* 63, 2729–2737. doi: 10.1093/jxb/err455

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Fixating on metals: new insights into the role of metals in nodulation and symbiotic nitrogen fixation

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Symbiotic nitrogen fixation is one of the most promising and immediate alternatives to the overuse of polluting nitrogen fertilizers for improving plant nutrition. At the core of this process are a number of metalloproteins that catalyze and provide energy for the conversion of atmospheric nitrogen to ammonia, eliminate free radicals produced by this process, and create the microaerobic conditions required by these reactions. In legumes, metal cofactors are provided to endosymbiotic rhizobia within root nodule cortical cells. However, low metal bioavailability is prevalent in most soils types, resulting in widespread plant metal deficiency and decreased nitrogen fixation capabilities. As a result, renewed efforts have been undertaken to identify the mechanisms governing metal delivery from soil to the rhizobia, and to determine how metals are used in the nodule and how they are recycled once the nodule is no longer functional. This effort is being aided by improved legume molecular biology tools (genome projects, mutant collections, and transformation methods), in addition to state-of-the-art metal visualization systems.

Keywords: symbiotic nitrogen fixation (SNF), metals, legume, rhizobia, nodule, iron, zinc, copper

INTRODUCTION

Substantial effort has been put forth to increase legume growth and production of seeds with enhanced nutritional content and bioavailability. In non-legume crops, investigators have used plant breeding and biotechnology to successfully increase metal uptake from soil and delivery to the shoot (Bashir et al., 2013). However, in legumes, in addition to the metabolic processes common to all plants, metals are also required the unique process of symbiotic nitrogen fixation (SNF). SNF heightens the demand for these same metal nutrients in legumes, often exhibiting signs of nutritional stress when undergoing nodulation and SNF (Terry et al., 1991; Slatni et al., 2012). Moreover, nodules, specialized root structures in which SNF occurs, and the process of nodulation itself, increase sensitivity to metal nutrient availability. This review highlights early and more recent studies that provide insight into the role of the nutritional metals in the various stages of the rhizobium–legume symbiosis.

STAGES OF NODULE DEVELOPMENT

Nodulation begins with the recognition of host plant-induced rhizobial nod factors by receptors within the membranes of plant root epidermal cells. This triggers calcium oscillations, membrane depolarization, and signal transduction cascades that lead to root hair curling and root cell wall degradation at the site of infection (for review, see Oldroyd and Downie, 2004). Rhizobia can then enter the root via invagination of the epidermal cell plasma membrane, forming an infection thread that grows and eventually releases rhizobia into the cytosol of nodule primordial cells within the root cortex. The rhizobia and their surrounding peribacteroid membrane (PBM), together known as the symbiosome, continue to divide until cells are filled with thousands of symbiosomes (for review, see Udvardi and Poole, 2013). Eventually, the bacteria within the symbiosomes stop dividing and differentiate into nitrogen-fixing bacteroids.

Many legumes from tropical and subtropical regions (soybean, bean) and some from temperate regions (*Lotus japonicus*) develop determinate nodules, in which meristem activity halts, causing the formation of a spherical nodule. In contrast, most other temperate legumes, including those in the genera *Medicago*, *Trifolium*, and *Pisum*, develop indeterminate nodules. Indeterminate nodules maintain a nodule meristem at the growing tip (zone I), followed by a zone of infection where rhizobia are released from the infection thread (zone II), a nitrogen fixation zone (zone III), and finally, a senescence zone in which bacteroids are degraded and nitrogen fixation ceases (zone IV; Vasse et al., 1990). Nutrient exchange at the nodule is facilitated by vascular vessels that surround cortical infected and uninfected cells.

METAL FUNCTIONS IN THE ESTABLISHMENT OF SNF

Metals are key elements of all living organisms (Frausto da Silva and Williams, 1991) and are an integral part of 30–50% of the proteome of a typical cell (Waldron and Robinson, 2009). They are also involved in every biological process, including the legume-specific stages of SNF from rhizobia infection to nodule

senescence. During the initial stages of infection, manganese and calcium facilitate rhizobial colonization of the root by mediating rhizobial lectin binding to the root hair tips (Kijne et al., 1988). Later stages of SNF signaling are mediated by calcium-spiking in the perinuclear region. As a result, calcium-calmodulin dependent kinases (CCaMKs) are activated (Singh and Parniske, 2012). CCaMKs induce the expression of genes mediating nodulation via the transcription factors NSP1/2 or NIN (Kalo et al., 2005; Andriankaja et al., 2007). Additionally, high levels of potassium have been detected in the apical region of indeterminate nodules, where it might play a role in cell growth (Rodríguez-Haas et al., 2013). Potassium also acts as the counter-ion compensating calcium in earlier signaling processes, via transport processes putatively mediated by channels such as CASTOR, POLLUX or DMI1 (Peiter et al., 2007; Charpentier et al., 2008, 2013). Also, cobalt, a component of cobalamin in ribonucleotide reductases, is essential for rhizobia endoreduplication, which occurs during differentiation into bacteroids (Taga and Walker, 2010).

Iron is the key cofactor of many metabolic reactions involved in SNF. In the early stages of nodulation, heminic iron is critical for catalase-mediated free radical detoxification (Jamet et al., 2003). Upon nodule maturation, iron is required for nitrogenase and leghemoglobin activity. The bacterial nitrogenase complex is necessary for the actual production of a usable nitrogen source (ammonia) from atmospheric nitrogen. NifH and NifDK, components of the nitrogenase complex, require iron–sulfur clusters and the iron–molybdenum cofactor, respectively. In contrast, leghemoglobin, the most abundant protein in the nodule cytosol, contains heminic iron. It effectively buffers oxygen content in the nodule, allowing for levels sufficient for bacteroid respiration without inactivating nitrogenase. Heminic iron is also critical for energy transduction by rhizobia cytochromes (Reedy and Gibney, 2004). Free iron, directly coordinated by amino acids, is involved in free radical detoxification as part of Fe-superoxide dismutases (Fe-SOD; Rubio et al., 2007).

Copper also plays a critical role in nitrogen fixation. Increased copper application results in elevated nitrogen fixation per nodule and increased nitrogen content in plant tissue (Hallsworth et al., 1960; van der Elst et al., 1961; Snowball et al., 1980; Seliga, 1990, 1993). This element is a cofactor of some of the high affinity cytochromes mediating energy transduction in the bacteroid (Preisig et al., 1996). It is also part of several superoxide dismutase systems detoxifying abundant free radicals produced as side products of SNF (Rubio et al., 2007). Additionally, zinc and manganese are integral elements of many superoxide dismutases (Rubio et al., 2007). Zinc is also involved in gene regulation as part of the zinc-finger motif of many transcription factors (Klug, 2010).

Some rhizobia are able to conserve energy by oxidizing H_2 produced during nitrogen fixation via the uptake hydrogenase enzyme, Hup (Emerich et al., 1979). Hup contains nickel, and alterations in nickel supply have been shown to affect Hup activity and formation in soybean and pea (Klucas et al., 1983; Stults et al., 1984; Brito et al., 1994, 1997). Therefore, although not required for nitrogen fixation, nickel can enhance the effectiveness of SNF in those species containing hydrogenase-encoding species, and may prove more critical if breeding and engineering efforts focused around this enzyme are successful.

TRANSITION METAL UPTAKE AND DISTRIBUTION IN SNF

Transition metal transport processes in the nodule are summarized in **Figure 1**. Legumes are Strategy I plants (Andaluz et al., 2009), i.e., iron is incorporated after acidification of the soil, which increases Fe^{3+} solubility. Ferroreductase can then reduce Fe^{3+} to Fe^{2+}, which is finally transported into the epidermal cell by ZIP or NRAMP family members (Curie et al., 2000; Morrissey and Guerinot, 2009; **Figure 1A**). Copper is likely incorporated in a similar manner. Cu^{2+} is reduced to Cu^{+} and a member of the copper transporter (Ctr) family translocates the metal across the epidermal cell plasma membrane. Zn^{2+} and other divalent ions are possibly directly introduced by ZIP or NRAMP family members. Many dicots will also secrete organic acids, phenolics, flavins, and flavonoid compounds under iron deficiency (Rodríguez-Celma et al., 2013). Phenolic compounds have recently been shown to modify the rhizosphere microbial community, leading to increased synthesis of the metal chelators and siderophores in red clover (Jin et al., 2006), which may also facilitate root metal uptake.

Symbiotic nitrogen fixation exerts a heavy toll on plant metal reserves, eliciting up-regulation of metal uptake systems (Terry et al., 1991). Recently, Slatni et al. (2011) measured the overall contribution of nodule versus root in rhizospheric iron uptake, noticing increased ferroreductase activity in the nodule. This could be due to the nodule epidermis playing a major role in iron uptake. Alternatively, since the ferroreductase activity was measured in the microsomal fraction of nodule extracts, some of this increased activity could result from ferroreductases working within the nodule cortex. This alternative hypothesis would take into account that the Fe^{3+}, transported as a citrate complex (Rellán-Álvarez et al., 2010), would have to be reduced to Fe^{2+} in the nodule cortex in order to be incorporated by the vasculature cells. In addition, H^{+}-ATPases and ZIP family members are expressed in the epidermal layer, in close proximity to the vasculature, and in the nitrogen fixation zone of the nodule (Slatni et al., 2012). These observations indicate that iron is present in the apoplast of the nodule cortex and, therefore, cortical cells use these proteins to uptake apoplastic iron.

Synchrotron-based X-ray fluorescence (S-XRF) studies indicate that some legumes with indeterminate nodules deliver iron, and likely other metals, to the nodule through the vasculature rather than using an epidermal pathway (Rodríguez-Haas et al., 2013). The metal distribution throughout different regions of the nodule indicates that there is a massive accumulation of iron around the vascular cells in zone II extending into the nodule cortical cells. Interestingly, this is where symbiosis is established and the symbiosomes differentiate. Iron accumulation in the apoplast would require plasma membrane metal transporters to introduce this element into the cytosolic compartment to synthesize ferroproteins. This transport system seems to be very efficient, since very little or no apoplastic iron is observed in the nitrogen fixation zone of the nodule. Researchers speculate that a ZIP or NRAMP transporter, both involved in divalent metal uptake (Nevo and Nelson, 2006; Lin et al., 2010), are the likely candidates (**Figures 1A,B**).

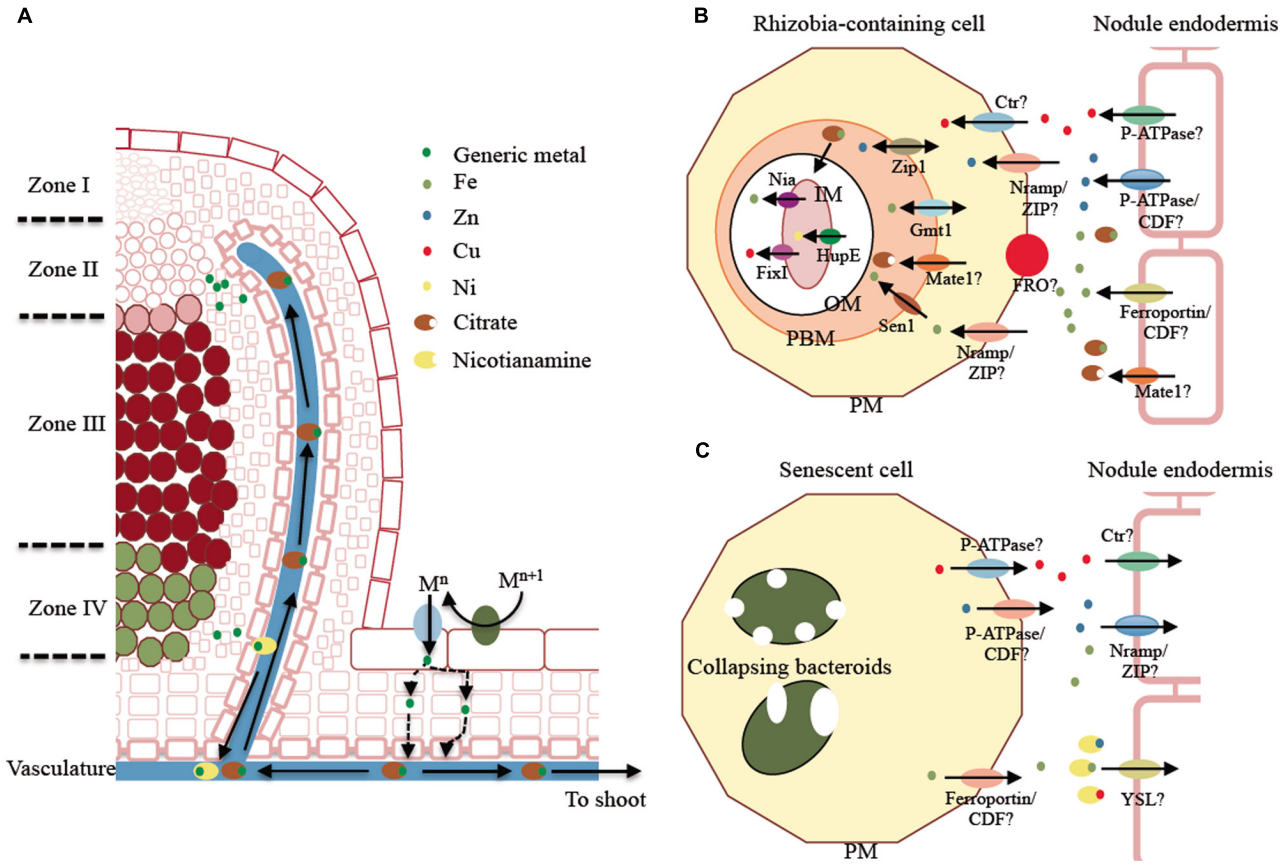

FIGURE 1 | Model of transition metal transport in indeterminate type nodules. (A) Overview of the metal delivery mechanism. Metals are typically incorporated following Strategy I, i.e., reduction by a plasma membrane ferroreductase and uptake by plasma membrane metal transporters. This process occurs over the entire epidermal surface of the root, including the nodule. Metals reach the vasculature where they bind metal chelators as citrate. The metal citrate complex is delivered to zone II of the nodule (infection/differentiation zone) and the metal is released into the apoplast and subsequently incorporated into the infected cell. In zone IV (senescence zone), metals are released to the apoplast, associated with nicotianamine, and translocated into the vasculature. **(B)** Proposed model of metal delivery from the vasculature to zone II. Proposed transporter families to carry out ion transport are indicated with a question mark. **(C)** Proposed model of metal recovery from the senescent zone of the nodule. Proposed transporter families are indicated with a question mark. PM indicates plasma membrane; PBM, peribacteroid membrane; OM, bacterial outer membrane; and IM, bacterial inner membrane.

ZIP family members have been immunodetected in the nodule (Slatni et al., 2012). However, the only ZIP family member characterized in this organ, GmZIP1, seems to be localized in the PBM (Moreau et al., 2002), where it is predicted to play a role in Zn^{2+} transport. In this membrane, an Fe^{2+}-transporting NRAMP member, GmDMT1, is also present (Kaiser et al., 2003; **Figure 1B**). These transporters may be involved in metal transport to the symbiosome. However, biochemical analysis of members of these two families and yeast complementation studies indicate that neither transporter could play a role in metal transfer to the symbiosome (Nevo and Nelson, 2006; Lin et al., 2010), instead they appear to transport metals towards the cytosol. The localization observed could be the result of the endocytic process mediating rhizobia release into the host cell (Leborgne-Castel et al., 2010), which would also carry plasma membrane associated transporters.

A more likely candidate for iron transport into the symbiosome is SEN1 (**Figure 1B**). The *sen1* mutant in *L. japonicus* has a number of alterations in nodulation that are associated with the lack of nitrogenase activity, most likely due to a deficiency in iron loading of the symbiosomes (Hakoyama et al., 2012). The proposed role of SEN1 in iron transport is due to its close sequence similarity to *Saccharomyces cerevisiae* CCC1 and *Arabidopsis thaliana* VIT1 proteins, both involved in divalent metal ion transport into organelles (Li et al., 2001; Kim et al., 2006). However, more detailed analyses, such as subcellular localization of the transporter, characterization of iron distribution, or the restoration of the phenotype by the addition of external iron, would be required to conclude this with certainty

Once metals cross the PBM, they are incorporated and used by the bacteroid. However, in spite of the huge number of genomic sequences available from rhizobia, very little is known about which transporters are involved in metal uptake and usage (**Figure 1B**). One of the first studies indicates that a P_{1b}-type Cu^+-ATPase, FixI, is essential for nitrogen fixation (Kahn et al., 1989). FixI is responsible for transporting Cu^+ to the bacteroid periplasm. Within this compartment Cu^+

is integrated into membrane-bound cytochrome cbb3 oxidase (Preisig et al., 1996), which is responsible for energy transduction in microaerobic environments. The Ni^{2+} importers HupE1 and HupE2 play a similar role in providing metal for the assembly of the Ni–Fe cofactor of hydrogenase (Brito et al., 2010). No direct evidence for an iron importer is available, but there is evidence of protective mechanisms against the local accumulation of toxic concentrations of this element. For example, the P_{1b}-type ATPase, Nia, is responsible for detoxifying excess Fe^{2+} that accumulates upon the massive entry of iron utilized to synthesize nitrogenase and other ferroproteins (Zielazinski et al., 2013).

The role of citrate in iron transport is important, although its role in SNF has not been completely elucidated. The citrate transporter FRD3, a multidrug and toxic compound extrusion (MATE) protein family member, has been shown in *A. thaliana* to be essential for iron transport across symplastically disconnected tissues (Roschzttardtz et al., 2011). Differences in the expression of citrate transporter *GmFRD3* is a major contributing factor that distinguishes iron efficient soybean cultivars from iron inefficient cultivars. This finding suggests that iron efficient varieties exhibit increased tolerance to low iron due to increased solubility of ferric iron, which is facilitated by increased xylem citrate (Rogers et al., 2009). However, the function of GmFRD3 has not been examined in nodulating roots, therefore the role of this putative transporter within the context of SNF is still unknown. At the PBM a citrate transporter is also likely be important, since it has been shown that some rhizobia have a preference for citrate as their siderophore (LeVier et al., 1996). Takanashi et al. (2013) recently reported nodule-specific expression of a *L. japonicus* MATE family member, LjMATE1 (**Figure 1B**). LjMATE1 appears to have a substantial effect on iron distribution and nitrogenase activity in this organ (Takanashi et al., 2013). However, no precise localization of this transporter has been provided to date, and as a result the role of this transporter (long distance iron transport versus PBM translocation) could not be discerned.

NODULE SENESCENCE AND SEED SET

Nodule senescence is a programmed process coupled with the entry into the reproductive stage of the host plant life cycle (Van de Velde et al., 2006). Given that iron is a growth-limiting nutrient (Grotz et al., 1998), it has to be recycled from the senescent nodule. A number of studies indicate that this is the case (Burton et al., 1998; Rodríguez-Haas et al., 2013; **Figure 1C**). In young plants, some of this recycled iron might be redirected to younger parts of the nodule, but it would be predominantly transported to the shoot through the vasculature as the plant enters its reproductive stage. Burton et al. (1998) estimated that around 50% of the total nodular iron is recycled in the seed, in a process that is likely to be reminiscent of leaf senescence (Shi et al., 2012). Although no senescence-upregulated metal transporter has been identified, a senescent nodule-specific nicotianamine (NA) synthase has been cloned (Hakoyama et al., 2009). The synthesis of NA, the molecule responsible for intracellular and phloem metal transport (Curie et al., 2009), indicates that the released metals are transported within the phloem using an unknown Yellow Stripe-like (YSL) transporter, since YSLs are responsible for NA-metal transport (Curie et al., 2009).

The steps leading to cell death during senescence include degradation of plant tissue via free radical oxidation (Thompson et al., 1987). Free radical production *in planta* can be catalyzed by transition metals in the Fenton reaction (Stohs and Bagchi, 1995). Given the high concentration of iron in the nodule, it is likely that it is responsible for accelerating free radical production and eventual senescence (Bhattacharjee, 2005), as evidenced by the strong reduction in nodular deoxyribose degradation and linolenic acid peroxidation in the presence of the iron chelator, desferrioxamine (Becana and Klucas, 1992). No evidence exists for the involvement of other metals in this process, especially given the fact that concentrations of other catalytic metals are likely too low to contribute.

FUTURE DIRECTIONS

Although we have learned a great deal about the developmental and signaling processes involved in SNF in recent years, many questions remain about the molecular mechanisms by which nutrients, metals in particular, are transported to and from developing and mature nodules. Recent advances in high-resolution elemental analysis have been used to show changes in iron localization in indeterminate nodules (Rodríguez-Haas et al., 2013). Continued use of these and other elemental localization techniques (for review, see Samira et al., 2013; Zhao et al., 2014) such as energy-dispersive X-ray analysis or nanoSIMS to detect other elements should facilitate great strides in understanding metal distribution and translocation in SNF in the near future.

Recent transcriptomic approaches have also been particularly useful for identifying whole-genome responses involved in nutrient translocation and assimilation during various developmental stages of SNF. Transcriptional profiles at the onset of *Mesorhizobium loti* infection, during nodule primordia initiation and nodule organogenesis, and at the onset of nitrogen fixation indicate that there is little overlap between transcripts present at the earlier stages of infection and those present in fully developed nodules (Takanashi et al., 2012). This same study led to the discovery of LjMATE1, the putative dicarboxylic acid (citrate) transporter described above. Additionally, transcripts involved in the transport of carbohydrates, metals, and peptides are abundant at all stages of nodule development. Transcriptomic analysis of *L. japonicus* nodules of primary and lateral roots, followed by non-biased metabolic profiling, revealed that the majority of nodule-specific genes are involved in carbon and amino acid metabolism, while 5% are involved in transport of metabolites or inorganic ions (Colebatch et al., 2004). Molecular genetic analysis of candidate genes identified by these studies should greatly enhance our understanding of regulatory processes that facilitate nutrient transport for SNF.

Overall, few studies have examined genotypic difference in response to metal availability in the context of SNF. So the question remains: what part of the changes in the nodule metallo-transcriptome are metal dependent and which are symbiosis-dependent? Time course and split root experiments of nodulating and non-nodulating roots under nutrient-sufficient and -deficient conditions followed by cell-specific transcriptional, proteomic,

and metabolic profiling could begin to answer the question above. Furthermore, the application of modeling approaches and comparative studies will allow for the identification of metal homeostasis factors that are specific to symbiotic interactions and enable efforts for increased production of leguminous crops.

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REFERENCES

Andaluz, S., Rodríguez-Celma, J., Abadía, A., Abadía, J., and López-Millán, A.-F. (2009). Time course induction of several key enzymes in *Medicago truncatula* roots in response to Fe deficiency. *Plant Physiol. Biochem.* 47, 1082–1088. doi: 10.1016/j.plaphy.2009.07.009

Andriankaja, A., Boisson-Dernier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D. G., et al. (2007). AP2-ERF transcription factors mediate Nod factor dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif. *Plant Cell* 19, 2866–2885. doi: 10.1105/tpc.107.052944

Bashir, K., Takahashi, R., Nakanishi, H., and Nishizawa, N. K. (2013). The road to micronutrient biofortification of rice: progress and prospects. *Front Plant Sci.* 4:15. doi: 10.3389/fpls.2013.00015

Becana, M., and Klucas, R. V. (1992). Transition metals in legume root nodules: iron-dependent free radical production increases during nodule senescence. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8958–8962. doi: 10.1073/pnas.89.19.8958

Bhattacharjee, S. (2005). Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plants. *Curr. Sci.* 89, 1113–1121.

Brito, B., Martínez, M., Fernández, D., Rey, L., Cabrera, E., Palacios, J. M., et al. (1997). Hydrogenase genes from *Rhizobium leguminosarum* bv. viciae are controlled by the nitrogen fixation regulatory protein nifA. *Proc. Natl. Acad. Sci. U.S.A.* 94, 6019–6024. doi: 10.1073/pnas.94.12.6019

Brito, B., Palacios, J., Hidalgo, E., Imperial, J., and Ruiz-Argüeso, T. (1994). Nickel availability to pea (*Pisum sativum L.*) plants limits hydrogenase activity of i bv. viciae bacteroids by affecting the processing of the hydrogenase structural subunits. *J. Bacteriol.* 176, 5297.

Brito, B., Prieto, R. I., Cabrera, E., Mandrand-Berthelot, M. A., Imperial, J., Ruiz-Argueso, T., et al. (2010). *Rhizobium leguminosarum* hupE encodes a nickel transporter required for hydrogenase activity. *J. Bacteriol.* 192, 925–935. doi: 10.1128/JB.01045-09

Burton, J. W., Harlow, C., and Theil, E. C. (1998). Evidence for reutilization of nodule iron in soybean seed development. *J. Plant Nutr.* 21, 913–927. doi: 10.1080/01904169809365453

Charpentier, M., Bredemeier, R., Wanner, G., Takeda, N., Schleiff, E., and Parniske, M. (2008). *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. *Plant Cell* 20, 3467–3479. doi: 10.1105/tpc.108.063255

Charpentier, M., Vaz Martins, T., Granqvist, E., Oldroyd, G. E., and Morris, R. J. (2013). The role of DMI1 in establishing Ca (2+) oscillations in legume symbioses. *Plant Signal. Behav.* 8, e22894. doi: 10.4161/psb.22894

Colebatch, G., Desbrosses, G., Ott, T., Krusell, L., Montanari, O., Kloska, S., et al. (2004). Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant J.* 39, 487–512. doi: 10.1111/j.1365-313X.2004.02150.x

Curie, C., Alonso, J. M., Le Jean, M., Ecker, J. R., and Briat, J. F. (2000). Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem. J.* 347, 749–755. doi: 10.1042/0264-6021:3470749

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Emerich, D. W., Ruiz-Argüeso, T., Ching, T. M., and Evans, H. J. (1979). Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. *J. Bacteriol.* 137, 153–160.

Frausto da Silva, J. J. R., and Williams, R. J. P. (eds). (1991). *The Biological Chemistry of the Elements, the Inorganic Chemistry of Life*. Oxford: Clarendon Press. doi: 10.1016/0307-4412(92)90039-O

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., and Eide, D. (1998). Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7220–7224. doi: 10.1073/pnas.95.12.7220

Hakoyama, T., Niimi, K., Yamamoto, T., Isobe, S., Sato, S., Nakamura, Y., et al. (2012). The integral membrane protein SEN1 is required for symbiotic nitrogen fixation in *Lotus japonicus* nodules. *Plant Cell Physiol.* 53, 225–236. doi: 10.1093/pcp/pcr167

Hakoyama, T., Watanabe, H., Tomita, J., Yamamoto, A., Sato, S., Mori, Y., et al. (2009). Nicotianamine synthase specifically expressed in root nodules of *Lotus japonicus*. *Planta* 230, 309–317. doi: 10.1007/s00425-009-0944-0

Hallsworth, E. G., Wilson, S. B., and Greenwood, E. A. (1960). Copper and cobalt in nitrogen fixation. *Nature* 187, 79–80. doi: 10.1038/187079a0

Jamet, A., Sigaud, S., Van De Sype, G., Puppo, A., and Herouart, D. (2003). Expression of the bacterial catalase genes during *Sinorhizobium meliloti–Medicago sativa* symbiosis and their crucial role during the infection process. *Mol. Plant Microbe Interact.* 16, 217–225. doi: 10.1094/MPMI.2003.16.3.217

Jin, C. W., He, Y. F., Tang, C. X., Wu, P., and Zheng, S. J. (2006). Mechanisms of microbially enhanced Fe acquisition in red clover (*Trifolium pratense L.*). *Plant Cell Environ.* 29, 888–897. doi: 10.1111/j.1365-3040.2005.01468.x

Kahn, D., David, M., Domergue, O., Daveran, M. L., Ghai, J., Hirsch, P. R., et al. (1989). *Rhizobium meliloti* fixGHI sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. *J. Bacteriol.* 171, 929–939.

Kaiser, B. N., Moreau, S., Castelli, J., Thomson, R., Lambert, A., Bogliolo, S., et al. (2003). The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. *Plant J.* 35, 295–304. doi: 10.1046/j.1365-313X.2003.01802.x

Kalo, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R. M., Hirsch, S., et al. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science* 308, 1786–1789. doi: 10.1126/science.1110951

Kijne, J. W., Smit, G., Diaz, C. L., and Lugtenberg, B. J. (1988). Lectin-enhanced accumulation of manganese-limited *Rhizobium leguminosarum* cells on pea root hair tips. *J. Bacteriol.* 170, 2994–3000.

Kim, S. A., Punshon, T., Lanzirotti, A., Li, L., Alonso, J. M., Ecker, J. R., et al. (2006). Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science* 314, 1295–1298. doi: 10.1126/science.1132563

Klucas, R. V., Hanus, F. J., Russell, S. A., and Evans, H. J. (1983). Nickel: a micronutrient element for hydrogen-dependent growth of *Rhizobium japonicum* and for expression of urease activity in soybean leaves. *Proc. Natl. Acad. Sci. U.S.A.* 80, 2253. doi: 10.1073/pnas.80.8.2253

Klug, A. (2010). The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annu. Rev. Biochem.* 79, 213–231. doi: 10.1146/annurev-biochem-010909-095056

Leborgne-Castel, N., Adam, T., and Bouhidel, K. (2010). Endocytosis in plant–microbe interactions. *Protoplasma* 247, 177–193. doi: 10.1007/s00709-010-0195-8

LeVier, K., Day, D. A., and Guerinot, M. L. (1996). Iron uptake by symbisomes from soybean root nodules. *Plant Physiol.* 111, 893–900. doi: 10.1104/pp.111.3.893

Li, L., Chen, O. S., McVey Ward, D., and Kaplan, J. (2001). CCC1 is a transporter that mediates vacuolar iron storage in yeast. *J. Biol. Chem.* 276, 29515–29519. doi: 10.1074/jbc.M103944200

Lin, W., Chai, J., Love, J., and Fu, D. (2010). Selective electrodiffusion of zinc ions in a Zrt-, Irt-like protein, ZIPB. *J. Biol. Chem.* 285, 39013–39020. doi: 10.1074/jbc.M110.180620

Moreau, S., Thomson, R. M., Kaiser, B. N., Trevaskis, B., Guerinot, M. L., Udvardi, M. K., et al. (2002). GmZIP1 encodes a symbiosis-specific zinc transporter in soybean. *J. Biol. Chem.* 277, 4738–4746. doi: 10.1074/jbc.M106754200

Morrissey, J., and Guerinot, M. L. (2009). Iron uptake and transport in plants: the good, the bad, and the ionome. *Chem. Rev.* 109, 4553–4567. doi: 10.1021/cr900112r

Nevo, Y., and Nelson, N. (2006). The NRAMP family of metal-ion transporters. *Biochim. Biophys. Acta* 1763, 609–620. doi: 10.1016/j.bbamcr.2006.05.007

Oldroyd, G. E. D., and Downie, J. A. (2004). Calcium, kinases and nodulation signalling in legumes. *Nat. Rev. Mol. Cell Biol.* 5, 566–576. doi: 10.1038/nrm1424

Peiter, E., Sun, J., Heckmann, A. B., Venkateshwaran, M., Riely, B. K., Otegui, M. S., et al. (2007). The *Medicago truncatula* DMI1 protein modulates cytosolic calcium signaling. *Plant Physiol.* 145, 192–203. doi: 10.1104/pp.107.097261

Preisig, O., Zufferey, R., Thony-Meyer, L., Appleby, C. A., and Hennecke, H. (1996). A high-affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *J. Bacteriol.* 178, 1532–1538.

Reedy, C. J., and Gibney, B. R. (2004). Heme protein assemblies. *Chem. Rev.* 104, 617–649. doi: 10.1021/cr0206115

Rellán-Álvarez, R., Giner-Martínez-Sierra, J., Orduna, J., Orera, I., Rodríguez-Castrillón, J. A., García-Alonso, J. I., et al. (2010). Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol.* 51, 91–102. doi: 10.1093/pcp/pcp170

Rodríguez-Celma, J., Lin, W. D., Fu, G. M., Abadía, J., López-Millán, A.-F., and Schmidt, W. (2013). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by *Arabidopsis* and *Medicago truncatula*. *Plant Physiol.* 162, 1473–1485. doi: 10.1104/pp.113.220426

Rodríguez-Haas, B., Finney, L. A., Vogt, S., González-Melendi, P., Imperial, J., and González-Guerrero, M. (2013). Iron distribution through the developmental stages of *Medicago truncatula* nodules. *Metallomics* 5, 1247–1253. doi: 10.1039/c3mt00060e

Rogers, E. E., Wu, X., Stacey, G., and Nguyen, H. T. (2009). Two MATE proteins play a role in iron efficiency in soybean. *J. Plant Physiol.* 166, 1453–1459. doi: 10.1016/j.jplph.2009.02.009

Roschzttardtz, H., Séguéla-Arnaud, M., Briat, J. F., Vert, G., and Curie, C. (2011). The FRD3 citrate effluxer promotes iron nutrition between symplastically disconnected tissues throughout *Arabidopsis* development. *Plant Cell* 23, 2725–2737. doi: 10.1105/tpc.111.088088

Rubio, M. C., Becana, M., Sato, S., James, E. K., Tabata, S., and Spaink, H. P. (2007). Characterization of genomic clones and expression analysis of the three types of superoxide dismutases during nodule development in *Lotus japonicus*. *Mol. Plant Microbe Interact.* 20, 262–275. doi: 10.1094/MPMI-20-3-0262

Samira, R., Stallmann, A., Massenburg, L. N., and Long, T. A. (2013). Ironing out the issues: integrated approaches to understanding iron homeostasis in plants. *Plant Sci.* 210, 250–259. doi: 10.1016/j.plantsci.2013.06.004

Seliga, H. (1990). The effect of copper nutrition on symbiotic nitrogen fixation in yellow lupine (*Lupinus luteus* L.) plants. *Acta Physiol. Plant.* 12, 287–291.

Seliga, H. (1993). The role of copper in nitrogen fixation in *Lupinus luteus L. Plant Soil* 155–156, 349–352. doi: 10.1007/BF00025054

Shi, R., Weber, G., Koster, J., Reza-Hajiresaei, M., Zou, C., Zhang, F., et al. (2012). Senescence-induced iron mobilization in source leaves of barley (*Hordeum vulgare*) plants. *New Phytol.* 195, 372–383. doi: 10.1111/j.1469-8137.2012.04165.x

Singh, S., and Parniske, M. (2012). Activation of calcium- and calmodulin-dependent protein kinase (CCaMK), the central regulator of plant root endosymbiosis. *Curr. Opin. Plant Biol.* 15, 444–453. doi: 10.1016/j.pbi.2012.04.002

Slatni, T., Vigani, G., Salah, I. B., Kouas, S., Dell'Orto, M., Gouia, H., et al. (2011). Metabolic changes of iron uptake in N2-fixing common bean nodules during iron deficiency. *Plant Sci.* 181, 151–158. doi: 10.1016/j.plantsci.2011.04.015

Slatni, T., Dell'Orto, M., Ben Salah, I., Vigani, G., Smaoui, A., Gouia, H., et al. (2012). Immunolocalization of H^+-ATPase and IRT1 enzymes in N_2-fixing common bean nodules subjected to iron deficiency. *J. Plant Physiol.* 169, 242–248. doi: 10.1016/j.jplph.2011.10.003

Snowball, K., Robson, A. D., and Loneragan, J. F. (1980). The effect of copper on nitrogen fixation in subterranean clover (*Trifolium subterraneum*). *New Phytol.* 85, 63–72. doi: 10.1111/j.1469-8137.1980.tb04448.x

Stohs, S. J., and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* 18, 321–336. doi: 10.1016/0891-5849(94)00159-H

Stults, L. W., O'Hara, E. B., and Maier, R. J. (1984). Nickel is a component of hydrogenase in *Rhizobium japonicum*. *J. Bacteriol.* 159, 153–158.

Taga, M. E., and Walker, G. C. (2010). *Sinorhizobium meliloti* requires a cobalamin-dependent ribonucleotide reductase for symbiosis with its plant host. *Mol. Plant Microbe interact.* 23, 1643–1654. doi: 10.1094/MPMI-07-10-0151

Takanashi, K., Takahashi, H., Sakurai, N., Sugiyama, A., Suzuki, H., Shibata, D., et al. (2012). Tissue-specific transcriptome analysis in nodules of *Lotus japonicus*. *Mol. Plant Microbe Interact.* 25, 869–876. doi: 10.1094/MPMI-01-12-0011-R

Takanashi, K., Yokosho, K., Saeki, K., Sugiyama, A., Sato, S., Tabata, S., et al. (2013). LjMATE1: a citrate transporter responsible for iron supply to the nodule infection zone of *Lotus japonicus*. *Plant Cell Physiol.* 54, 585–594. doi: 10.1093/pcp/pct019

Terry, R. E., Soerensen, K. U., Jolley, V. D., and Brown, J. C. (1991). The role of active *Bradyrhizobium japonicum* in iron stress response of soy-beans. *Plant Soil* 130, 225–230. doi: 10.1007/BF00011877

Thompson, J. E., Legge, R. L., and Barber, R. F. (1987). The role of free radicals in senescence and wounding. *New Phytol.* 105, 317–344. doi: 10.1111/j.1469-8137.1987.tb00871.x

Udvardi, M., and Poole, P. S. (2013). Transport and metabolism in legume–rhizobia symbioses. *Annu. Rev. Plant Biol.* 64, 781–805. doi: 10.1146/annurev-arplant-050312-120235

Van de Velde, W., Guerra, J. C. P. R., Keyser, A. D., De Rycke, R., Rombauts, S. P., Maunoury, N., et al. (2006). Aging in legume symbiosis. A molecular view on nodule senescence in *Medicago truncatula*. *Plant Physiol.* 141, 711–720. doi: 10.1104/pp.106.078691

van der Elst, F. H., McNaught, K. J., and Rolt, W. F. (1961). Effect of copper deficiency in white clover on nitrogen fixation. *Nature* 192, 1315–1315. doi: 10.1038/1921315a0

Vasse, J., De Billy, F., Camut, S., and Truchet, G. (1990). Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.* 172, 4295–4306.

Waldron, K. J., and Robinson, N. J. (2009). How do bacterial cells ensure that metalloproteins get the correct metal? *Nat. Rev. Microbiol.* 7, 25–35. doi: 10.1038/nrmicro2057

Zhao, F. J., Moore, K. L., Lombi, E., and Zhu, Y. G. (2014). Imaging element distribution and speciation in plant cells. *Trends Plant Sci.* doi: 10.1016/j.tplants.2013.12.001 [Epub ahead of print].

Zielazinski, E. L., González-Guerrero, M., Subramanian, P., Stemmler, T. L., Argüello, J. M., and Rosenzweig, A. C. (2013). *Sinorhizobium meliloti* Nia is a P-ATPase expressed in the nodule during plant symbiosis and is involved in Ni and Fe transport. *Metallomics* 5, 1614–1623. doi: 10.1039/c3mt00195d

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Moving toward a precise nutrition: preferential loading of seeds with essential nutrients over non-essential toxic elements

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Plants and seeds are the main source of essential nutrients for humans and livestock. Many advances have recently been made in understanding the molecular mechanisms by which plants take up and accumulate micronutrients such as iron, zinc, copper and manganese. Some of these mechanisms, however, also facilitate the accumulation of non-essential toxic elements such as cadmium (Cd) and arsenic (As). In humans, Cd and As intake has been associated with multiple disorders including kidney failure, diabetes, cancer and mental health issues. Recent studies have shown that some transporters can discriminate between essential metals and non-essential elements. Furthermore, sequestration of non-essential elements in roots has been described in several plant species as a key process limiting the translocation of non-essential elements to aboveground edible tissues, including seeds. Increasing the concentration of bioavailable micronutrients (biofortification) in grains while lowering the accumulation of non-essential elements will likely require the concerted action of several transporters. This review discusses the most recent advances on mineral nutrition that could be used to preferentially enrich seeds with micronutrients and also illustrates how precision breeding and transport engineering could be used to enhance the nutritional value of crops by re-routing essential and non-essential elements to separate sink tissues (roots and seeds).

Keywords: food security, heavy metals, long distance transport, seed loading, mineral nutrition

INTRODUCTION

Plants and seeds are the main dietary source of micronutrients (i.e., zinc, copper, manganese, and iron). Over the last 50 years, the production of cereals has tripled and the demand is expected to rise due to the constantly increasing population (Gregory and George, 2011). Any effort devoted to increase yield has to ensure that the nutritional value of seeds and grains is retained or, preferably, improved. The consumption of grains with low quantity of micronutrients has been associated with mineral deficiencies in humans (Burchi et al., 2011; Murgia et al., 2012). More than 2 billion people are affected by at least one type of micronutrient deficiency (White and Broadley, 2009; Waters and Sankaran, 2011). Another concern that could impact food security is the consumption of non-essential elements such as cadmium (Cd) and arsenic (As) through contaminated food. More than 80% of Cd intake in humans comes from cereals and vegetables (Olsson et al., 2002; Egan et al., 2007). Natural occurrence of As in groundwater used for irrigation has been found around the world (Nordstrom, 2002; Rodriguez-Lado et al., 2013) potentially exposing more than 100 million people to As. Chronic exposure to low levels of Cd and As has been associated with cancer, diabetes, renal failure and neurological disorders (Satarug et al., 2010; Cho et al., 2013; Clemens et al., 2013).

Uptake and distribution of nutrients from the soil and within the plant is a dynamic process driven by root uptake transporters, root-to-shoot translocation (xylem transport) and source-to-sink transport through the phloem, which includes seed loading (**Figure 1** and **Table 1**) (Mendoza-Cozatl et al., 2011; Waters and Sankaran, 2011; Sinclair and Kramer, 2012). Non-essential elements like As and Cd are taken up by plants and distributed between plant tissues by the same transporters that mobilize nutrients such as Fe^{2+}, Zn^{2+}, Mn^{2+}, or PO_4^{2-}. Therefore, each of the steps required to move nutrients from the soil and into seeds (root uptake, translocation, xylem and phloem transport) represent an opportunity to increase the specificity towards essential nutrients and re-route non-essential elements to non-edible parts of the plant (**Figure 1**). Understanding the molecular mechanisms of each transport process and the contribution of different transporters to the overall allocation of essential and non-essential elements within plant tissues will help in developing crops that yield grains of higher nutritional value with lower content of non-essential elements. This review discusses the most recent discoveries in plant nutrition that could help achieving the goal of safe nutritional enrichment by means of precision breeding or transport engineering (Collard and Mackill, 2008; Nour-Eldin and Halkier, 2013).

ROOT UPTAKE, VACUOLAR SEQUESTRATION, AND ROOT-TO-SHOOT TRANSLOCATION OF Cd AND As

Root uptake represents the first step where selectivity towards essential elements can be improved and evidence suggest that it is possible to increase the selectivity of transporters towards

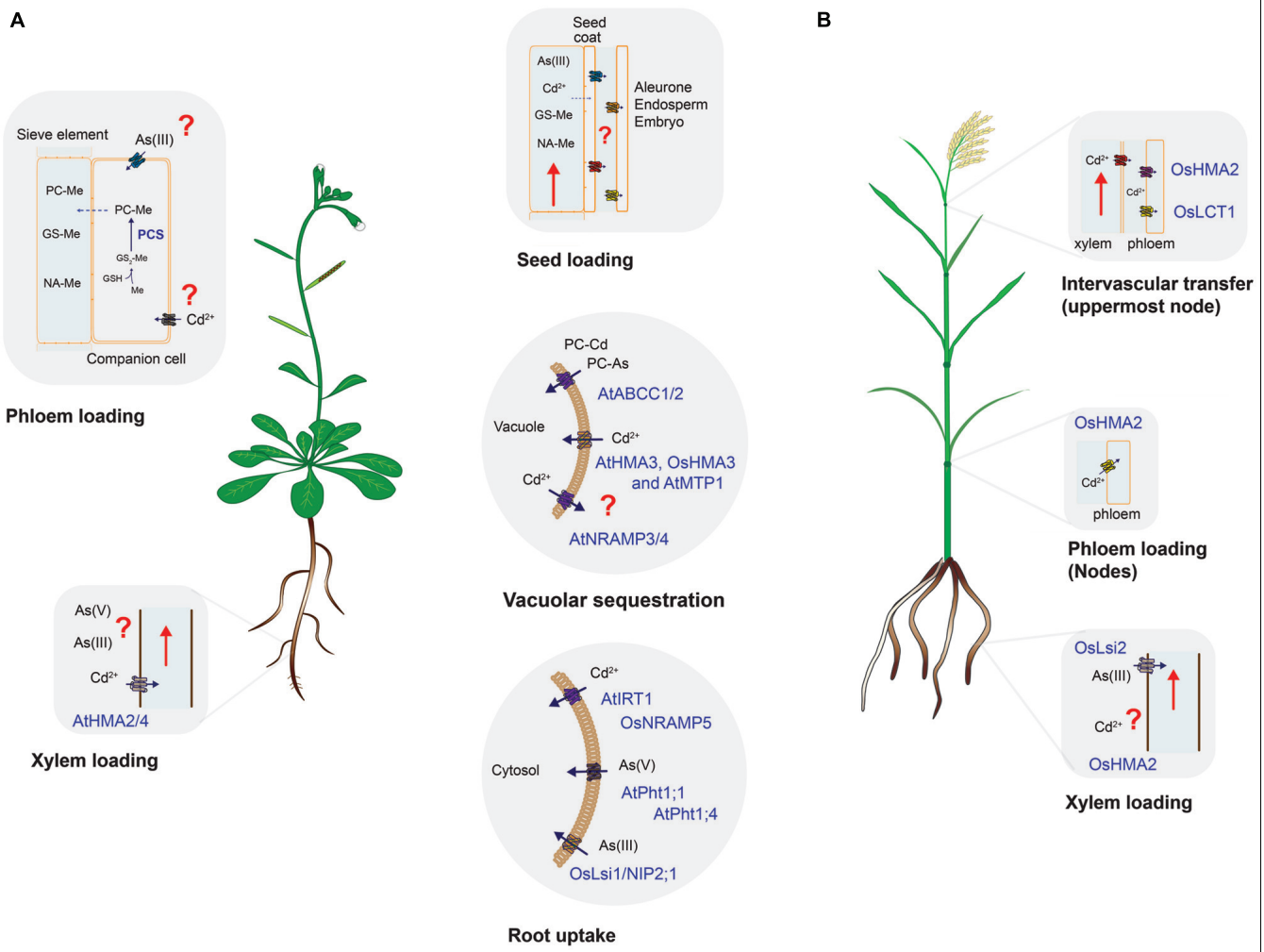

FIGURE 1 | Uptake and distribution of cadmium and arsenic in (A) *Arabidopsis* and (B) rice. Processes conserved in both species (root uptake, vacuolar sequestration, and seed loading) are shown in the middle panels. Species-specific transporters are denoted by At (*Arabidopsis*) or Os (rice). Processes where information is scarce, absent or that require further research are labeled with red question marks. The embryo and the maternal tissues (seed coat) are symplastically isolated, thus molecules have to be unloaded from the phloem to later be taken up by embryo-related tissues. Unloading and uptake of molecules in reproductive tissues is thought to be mediated by specific transporters but the identity of these proteins has not been established. Only proteins characterized *in vitro* and *in planta* are shown. For simplicity, co-substrates such as H^+ or ATP-Mg were omitted. GSH, glutathione; Me, metal(loid) such as Cd or As(III); PCS, phytochelatin synthase; PC–Me, phytochelatin–metal(loid) complex; GS–Me, glutathione–metal(loid) complex; NA–Me, NA–metal complex.

essential elements. In *Arabidopsis*, uptake of Fe^{2+}, Zn^{2+}, Mn^{2+}, and Cd^{2+} is mediated by the ZIP family of transporters (Palmer and Guerinot, 2009; Mendoza-Cozatl et al., 2011). IRT1 is one of the best characterized members of this family and despite its broad specificity, previous studies have shown that changes in specific amino acid residues can modify its substrate specificity (Rogers et al., 2000). These studies have shown that discrimination between Fe^{2+}/Mn^{2+} against Cd^{2+} is feasible. Selectivity of Zn^{2+} over Cd^{2+} is more challenging due to the highly similar electron configuration between these two elements. Selectivity of Zn^{2+} over Cd^{2+}, however, has been demonstrated for mammalian ZnT transporters and bacterial P-type ATPases (Hoch et al., 2012). Conversely, selectivity of Cd^{2+} over Zn^{2+} has been found in the rice vacuolar P1B-type ATPase OsHMA3 and this gene has been identified as a major determinant limiting Cd accumulation in rice grains (see below; Ueno et al., 2010). The molecular basis and amino acid residues required for this metal selectivity have not been determined and could potentially be used in other crop species to limit the accumulation of Cd in seeds. In rice, the natural resistance-associated macrophage protein 5 (OsNRAMP5) has been identified as the main Mn^{2+} and Cd^{2+} transporter at the root level (**Figure 1** and **Table 1**; Sasaki et al., 2012). OsIRT1 and OsIRT2 also mobilize Fe^{2+}, Zn^{2+}, and Cd^{2+} into roots but these transporters seem to play a minor role in Cd uptake compared to OsNRAMP5 (Sasaki et al., 2012). Substrate specificity studies could help identifying OsNRAMP5 natural variants with preference for Mn^{2+} over Cd^{2+} that can be introgressed into high-yield varieties of rice.

In contrast to Cd, As can occur in soils in several oxidation states, being arsenate (As^V) and arsenite (As^{III}) the most common ones (Ma et al., 2008; Ali et al., 2009; Zhao et al., 2009). In *Arabidopsis*, As^V is taken up from aerobic soils by the phosphate transporters AtPht1;1 and 1;4 (Shin et al., 2004). The striking similarities between phosphate and arsenate in terms of p*K*a values, charged oxygen atoms and thermochemical radii differing only by 4% make a substrate specificity approach very challenging (Elias et al., 2012). Some bacterial phosphate binding proteins are able to discriminate between phosphate and arsenate and the structural basis for this discrimination has recently been established (Elias et al., 2012). Similar structural studies with plant transporters could help engineering phosphate transporters with high selectivity for phosphate over As^V. Once inside the cells, As^V is quickly reduced to As^{III}, which is the predominant form of inorganic arsenic in plant cells (Ali et al., 2009; Zhao et al., 2009). Arsenite also occurs mostly in anaerobic soils and can be taken up by plants thus affecting flooded crops like rice. OsNIP2;1, a member of the Nod26-like major intrinsic protein (NIP) has been identified as a major pathway for As^{III} uptake into rice roots (Ma et al., 2008; Zhao et al., 2009). The physiological function of OsNIP2;1, also known as Lsi1, is silicon uptake. Lsi2 on the other hand, functions as a silicon and As^{III} exporter and both Lsi1 and Lsi2 are the main transporters controlling As^{III} uptake and translocation to shoots (**Figure 1** and **Table 1**). Recently, there have been some efforts to identify amino acid residues affecting the substrate selectivity of OsNIP2;1/Lsi1 but so far no selectivity of essential nutrients over As^{III} has been found (Mitani-Ueno et al., 2011).

Sequestration in root vacuoles has been demonstrated as the major process limiting the translocation of As and Cd to shoots and seeds (Ueno et al., 2010; Kopittke et al., 2013). Similar to Cd^{2+}, As^{III} has a strong affinity for thiol-containing molecules such as cysteine, glutathione and phytochelatins (PCs). PCs are glutathione-derived peptides synthesized in response to As^{III} or Cd^{2+} exposure (Mendoza-Cozatl et al., 2005). PC–metal(loid) complexes are transported into vacuoles by ATP-binding cassette transporters and, in *Arabidopsis*, ABCC1 and ABCC2 have been identified as the main transporters mediating PC uptake into vacuoles (Song et al., 2010b). Orthologs of AtABCC1 have been identified in grasses including rice, maize and barley and kinetic analysis using vacuoles isolated from barley suggest that the transport mechanism is conserved across species (Song et al., 2013). Furthermore, a recent analysis using six rice varieties showing low and high accumulation of arsenic in grains suggest that PCs play a key role trapping inorganic As in roots, limiting the transfer of As to shoots and grains (Batista et al., 2013). PCs are usually considered as a mechanism to detoxify non-essential elements; however, there is evidence to suggest that PCs also play a role in the homeostasis of essential metals such as Zn and Mn (Tennstedt et al., 2009; Song et al., 2013). Therefore, more studies are needed to explore whether increasing vacuolar sequestration of PCs in roots could have a negative impact on the content of essential elements in aboveground edible tissues.

P1B-type ATPases have also been shown to play a key role sequestering Cd in root vacuoles (Ueno et al., 2010). OsHMA3, a vacuolar P1B-type ATPase was found to be responsible for 85.6% of the variance in Cd content between low- and high-cadmium

Table 1 | Transporters mediating the uptake and mobilization of arsenic or cadmium (discussed in this review).

Transporter	Localization	Function/substrate	Reference
AtIRT1	Root (PM)	Uptake of Fe, Zn, Mn and Cd	Rogers et al. (2000)
OsHMA3	Root (Vac)	Cd sequestration in root vacuoles	Ueno et al. (2010)
AhHMA3	Root/shoot (Vac)	Zn sequestration in root vacuoles	Becher et al. (2004)
AtHMA3	Vascular tissues and root apex (Vac)	Transport of Zn, Cd, Co and Pb	Morel et al. (2009)
OsNRAMP5	Root (PM)	Uptake of Mn and Cd	Sasaki et al. (2012)
AtPht1;1/1;4	Root hair cells, root cap (PM)	Uptake of PO_4^{3-} and arsenate (As^V)	Shin et al. (2004)
OsNip2;1 (OsLsi1)	Roots, exodermis, and endodermis (PM)	Uptake of arsenate (As^{III})	Ma et al. (2008)
OsLsi2	Roots, exodermis, and endodermis (PM)	Efflux of arsenite (As^{III})	Ma et al. (2008)
AtABCC1/2	Root/shoot (Vac)	Arsenite–PC transporter	Song et al. (2010b)
AtHMA2/4	Roots, vascular tissue, and leaves (PM)	Xylem loading of Cd/Zn	Hussain et al. (2004)
OsHMA9	Vascular bundles and anthers (PM)	Cu, Zn and Cd detoxification	Lee et al. (2007)
OsHMA2	Roots and vascular bundles (PM)	Zn/Cd delivery to developing tissues	Yamaji et al. (2013)
AtPCR2	Roots, epidermal cells, and xylem (PM)	Zn homeostasis and Cd transport	Song et al. (2004, 2010a)
OsNRAMP1	Root and shoot (PM)	Cd uptake	Takahashi et al. (2011)
AtMTP1	Roots and leaves (Vac)	Zn and Cd transporter	Desbrosses-Fonrouge et al. (2005)
AtZIF1	Roots and leaves (Vac)	Mobilization of nicotianamine	Haydon and Cobbett (2007)
AtNRAMP3/4	Vascular bundles, roots, and leaves (Vac)	Fe efflux transporter	Lanquar et al. (2005); Thomine et al. (2003)

The first two letters denote the organism of origin: At, Arabidopsis thaliana; Ah, Arabidopsis halleri; Os, Oryza sativa. Subcellular localization is shown in parenthesis: PM, plasma membrane; Vac, vacuole (tonoplast). See also **Figure 1** *and text for further details.*

accumulation varieties of rice (Ueno et al., 2010). OsHMA3 sequesters Cd in root vacuoles, thus preventing it from reaching shoots and grains. Of particular interest is the fact that OsHMA3 seems to be highly specific for Cd while the *Arabidopsis halleri* HMA3 has preference for Zn and the *A. thaliana* HMA3 shows broad substrate specificity being able to transport Co, Pb, Cd, and Zn (Becher et al., 2004; Morel et al., 2009). Structural studies using OsHMA3, AhHMA3, and AtHMA3 could help identifying which residues give the high specificity of OsHMA3 for Cd over essential elements such as Zn or Mn.

Xylem loading is the next step where translocation of non-essential elements can be blocked allowing only essential elements to reach leaves and seeds. In *Arabidopsis* Cd and Zn are loaded into the xylem by two P1B-type ATPases, HMA2, and HMA4 (Hussain et al., 2004). It would be interesting to determine whether it is possible to alter the selectivity of HMA2 and 4 to favor the translocation of Zn over Cd. PCR2 is another *Arabidopsis* protein that has been implicated in the long-distance transport of Cd and Zn (Song et al., 2004, 2010a). However, PCR2 expression is not restricted to xylem parenchyma suggesting that PCR2 may have other roles beside root-to-shoot translocation of Cd and Zn (Song et al., 2004, 2010a). Less information is known about the HMA family in rice, OsHMA2 has been proposed to mediate the loading of Cd and Zn into the xylem (Takahashi et al., 2012), together with OsHMA9 and proteins from the NRAMP family of transporters (OsNRAMP1; Lee et al., 2007; Takahashi et al., 2011). OsHMA5 has also been localized in root pericycle cells and xylem but OsHMA5 seems to be a copper-specific transporter with little or no effect on the accumulation of Fe, Mn, or Zn in rice tissues (Deng et al., 2013).

Metal(loid)–ligand chemistry is another process that is key to understand how elements are mobilized throughout the plant. For instance, Fe and Cd are taken up by IRT1, but once inside the cell, Fe and Cd will form complexes with different ligands; iron will prefer oxygen-containing molecules such as citrate and nicotianamine while Cd will bind to thiol- and nitrogen-containing compounds such as glutathione, PCs and histidine (Meda et al., 2007; Mendoza-Cozatl et al., 2008; Rellán-Álvarez et al., 2008). This metal–ligand interaction explains why Cd and Zn, but not Fe, compete for the same ligands and transporters.

Inorganic arsenic can reach the xylem as As^{V} or As^{III} but fluorescence-X-ray absorption near-edge spectroscopy (fluorescence-XANES) has recently shown that As^{III} is the predominant form of arsenic in plant cells (Kopittke et al., 2013). Similar to Cd, As^{III} has a strong affinity for thiol groups and, consequently, most of the As^{III} found in the cortex and stele was identified as As(III)–thiol complexes (Kopittke et al., 2013). In rice, Lsi2 has been identified as the transporter mediating As^{III} loading into the xylem (Ma et al., 2008). In *Arabidopsis*, it is not clear which transporter mediates this process but transporters of the NIP family are possible candidates that are currently being evaluated (Ali et al., 2009; Kamiya et al., 2009).

INTRACELLULAR TRANSPORT AND PHLOEM LOADING

In contrast to natural As and Cd hyperaccumulators, *Arabidopsis*, rice and wheat accumulate only a minor fraction of As and Cd in leaves. In *Arabidopsis*, MTP1 has been described as a Cd/Zn vacuolar transporter and structure–function analyses suggest that it is possible to increase the selectivity of AtMTP1 towards Zn (Desbrosses-Fonrouge et al., 2005; Kawachi et al., 2012; Podar et al., 2012). OsMTP8.1 has been described as a vacuolar Mn-specific transporter in rice but its role on Cd uptake has not been evaluated (Chen et al., 2013). ZIF1 is a tonoplast-localized transporter that mobilizes nicotianamine into *Arabidopsis* vacuoles thus affecting Fe and Zn homeostasis (Haydon et al., 2012). Deletion of ZIF1 leads to Cd hypersensitivity in *Arabidopsis* but it is not clear whether this sensitivity is the result of an impaired Fe/Zn homeostasis or whether ZIF1-mediated transport of nicotianamine plays a role in sequestering Cd in shoot vacuoles. *Arabidopsis* NRAMP3 and 4 are transporters that export Fe from vacuoles (Lanquar et al., 2005). When expressed in yeast, NRAMP3 and 4 can mobilize Cd and it has recently been shown that photosynthesis in the *Arabidopsis nramp3 nramp4* double mutant is particularly sensitive to Cd (Molins et al., 2013). NRAMP proteins, like ZIP transporters, have broad substrate specificity and more structural analyses are needed to determine how NRAMP proteins could be used to restrict the accumulation of non-essential elements while preserving the homeostasis of essential metals.

The low accumulation of As and Cd in shoots observed in non-hyperaccumulator species was thought to be solely the result of limited translocation to shoots but recent evidence suggest that low-retention in leaves together with phloem-mediated transport to roots could play an important role re-allocating non-essential elements from shoots to roots (Mendoza-Cozatl et al., 2008, 2011; Ye et al., 2010). Nutrients and non-essential molecules are loaded into seeds through the phloem, which is a vascular system made up of two highly specialized cells, companion cells and sieve elements (Moore et al., 2013; Slewinski et al., 2013). Companion cells transfer molecules into sieve elements for long-distance transport, thus transporters expressed in companion cells are critical proteins that could impact phloem sap and seed composition. In contrast to xylem transport, phloem loading of micronutrients and non-essential elements remains largely unexplored. However, the phloem plays a key role in delivering nutrients to developing seeds, where xylem transport is limited. *Arabidopsis* YSL1 and YSL3 are nicotianamine–metal transporters required to mobilize Mn, Zn, Cu and Fe out of senescing leaves but their role in Cd mobilization, if any, has not been evaluated. Similarly, rice OsYSL16 has been characterized as a Cu–NA transporter while OsYSL6 is required to detoxify excess of Mn but neither OsYSL16 or OsYSL6 seem to participate in Cd mobilization to developing grains (Sasaki et al., 2011; Chen et al., 2013).

Phloem loading mechanisms may vary across species and in the case of rice, xylem-to-phloem transfer, particularly at the node connected to the flag leaf and panicle, appears to be the major route for Cd loading into the phloem. Recently, OsLCT1 was identified as a strong candidate mediating the intervascular transfer of Cd into the phloem. OsLCT1 is strongly expressed in nodes during grain ripening and RNAi-mediated knockdown of OsLCT1 resulted in 50% less Cd in grains without affecting the concentration of essential micronutrients or grain yield (Uraguchi et al., 2011). OsHMA2, besides being expressed in roots, is also expressed in

the nodes (phloem) at reproductive stage and insertional mutants showed less Cd and Mn in the upper nodes compared to wild-type (Yamaji et al., 2013). Thus, OsLCT1 and OsHMA2 could be the target for further manipulation to limit the translocation of Cd into rice grains.

TOWARD A PRECISE NUTRITION

Several reviews have recently discussed how QTL mapping, GWAS, transgenic approaches and conventional breeding have successfully been used to increase the concentration of micronutrients in seeds (White and Broadley, 2005, 2009; Sperotto et al., 2012). From these studies it is clear that several processes regulating the movement of elements into seeds are still largely unknown and that the mechanisms mediating long-distance transport of nutrients vary widely across species. Moreover, because non-essential elements such as As and Cd use the transport systems for essential nutrients to move within the plant, efforts to increase the content of micronutrients in crops could also increase the concentration of toxic elements in seeds. This is a serious threat to food security particularly in places where the occurrence of non-essential toxic elements in groundwater used for irrigation is above safety limits.

A successful precision breeding or transport engineering approach to produce crops able to accumulate micronutrients over non-essential metals should consider: (i) high substrate selectivity of transporters for essential metals, (ii) tissue-specific expression and subcellular localization, (iii) the developmental stage at which the transporter is needed, and (iv) the need of ligand molecules in sink tissues to keep micronutrients bioavailable and prevent toxicity. Research suggests that successful approaches will likely require the simultaneous modification of more than one step in different tissues. Cross-species studies of transporters of the same family, crystallography and site-directed mutagenesis have shown to be extremely useful to identify amino acid residues critical to enhance the selectivity of transporters (Becher et al., 2004; Courbot et al., 2007; Zimmermann et al., 2009). The lower cost of next-generation sequencing together with natural variant accessions available for some crops makes feasible the identification of transporters that can later be introgressed into high yield varieties to obtain safer and more nutritious grains. While transgenic approaches have a faster turnaround compared to traditional breeding, other technologies such as precision breeding or genome editing are non-transgenic alternatives that could achieve similar results in comparable time frames. Also, the development of tissue-specific translatome analysis in *Arabidopsis* offers the opportunity, for the first time, to study the regulation of transporters at tissue-specific level (Mustroph et al., 2009). Phloem unloading and re-uptake of nutrients by seed tissues is the last barrier where the accumulation of non-essential metals in seeds could be blocked. The identity and regulation of transporters mediating phloem unloading and seed loading are largely unknown and phloem-specific translatome analysis in seed loading tissues could help identifying such transporters. Translatome analyses are also currently being established in crop plants (Mendoza-Cozatl and Stacey, unpublished) and will likely provide more details about the mechanisms mediating the mobilization of nutrients and toxic elements within the plant and ultimately into seeds.

The urgent need of providing more nutritious food to a rapidly growing population is challenging considering environmental issues such as climate change, contamination of soil and water, and land available, but sustainable solutions to these global challenges are more likely to come from cross-disciplinary approaches between farmers, breeders, biologists, geneticists and bioengineers.

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REFERENCES

Ali, W., Isayenkov, S. V., Zhao, F. J., and Maathuis, F. J. (2009). Arsenite transport in plants. *Cell. Mol. Life Sci.* 66, 2329–2339. doi: 10.1007/s00018-009-0021-7

Batista, B. L., Nigar, M., Mestrot, A., Alves Rochad, B., Barbosa, F., Price, A. H., et al. (2013). Identification and quantification of phytochelatins in roots of rice at long-term exposure: evidence of individual role on arsenic accumulation. *J. Exp. Bot.* [in press].

Becher, M., Talke, I. N., Krall, L., and Kramer, U. (2004). Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant J.* 37, 251–268. doi: 10.1046/j.1365-313X.2003.01959.x

Burchi, F., Fanzo, J., and Frison, E. (2011). The role of food and nutrition system approaches in tackling hidden hunger. *Int. J. Environ. Res. Public Health* 8, 358–373. doi: 10.3390/ijerph8020358

Chen, Z., Fujii, Y., Yamaji, N., Masuda, S., Takemoto, Y., Kamiya, T., et al. (2013). Mn tolerance in rice is mediated by MTP8.1, a member of the cation diffusion facilitator family. *J. Exp. Bot.* 64, 4375–4387. doi: 10.1093/jxb/ert243

Cho, Y. A., Kim, J., Woo, H. D., and Kang, M. (2013). Dietary cadmium intake and the risk of cancer: a meta-analysis. *PLoS ONE* 8:e75087. doi: 10.1371/journal.pone.0075087

Clemens, S., Aarts, M. G., Thomine, S., and Verbruggen, N. (2013). Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci.* 18, 92–99. doi: 10.1016/j.tplants.2012.08.003

Collard, B. C., and Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 557–572. doi: 10.1098/rstb.2007.2170

Courbot, M., Willems, G., Motte, P., Arvidsson, S., Roosens, N., Saumitou-Laprade, P., et al. (2007). A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase. *Plant Physiol.* 144, 1052–1065. doi: 10.1104/pp.106.09513

Deng, F., Yamaji, N., Xia, J., and Ma, J. F. (2013). A member of the heavy metal P-type ATPase OsHMA5 is involved in xylem loading of copper in rice. *Plant Physiol.* 163, 1353–1362. doi: 10.1104/pp.113.226225

Desbrosses-Fonrouge, A. G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U. (2005). *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Lett.* 579, 4165–4174 doi: 10.1016/j.febslet.2005.06.046

Egan, S. K., Bolger, P. M., and Carrington, C. D. (2007). Update of US FDA's Total Diet Study food list and diets. *J. Expo. Sci. Environ. Epidemiol.* 17, 573–582. doi: 10.1038/sj.jes.7500554

Elias, M., Wellner, A., Goldin-Azulay, K., Chabriere, E., Vorholt, J. A., Erb, T. J., et al. (2012). The molecular basis of phosphate discrimination in arsenate-rich environments. *Nature* 491, 134–137. doi: 10.1038/nature11517

Gregory, P. J., and George, T. S. (2011). Feeding nine billion: the challenge to sustainable crop production. *J. Exp. Bot.* 62, 5233–5239. doi: 10.1093/jxb/err232

Haydon, M. J., and Cobbett, C. S. (2007). A novel major facilitator superfamily protein at the tonoplast influences zinc tolerance and accumulation in *Arabidopsis*. *Plant Physiol.* 143, 1705–1719. doi: 10.1104/pp.106.092015

Haydon, M. J., Kawachi, M., Wirtz, M., Hillmer, S., Hell, R., and Kramer, U. (2012). Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in *Arabidopsis*. *Plant Cell* 24, 724–737. doi: 10.1105/tpc.111.095042

Hoch, E., Lin, W., Chai, J., Hershfinkel, M., Fu, D., and Sekler, I. (2012). Histidine pairing at the metal transport site of mammalian ZnT transporters controls Zn^{2+} over Cd^{2+} selectivity. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7202–7207. doi: 10.1073/pnas.1200362109

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., et al. (2004). P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell* 16, 1327–1339. doi: 10.1105/tpc.020487

Kamiya, T., Tanaka, M., Mitani, N., Ma, J. F., Maeshima, M., and Fujiwara, T. (2009). NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of *Arabidopsis thaliana*. *J. Biol. Chem.* 284, 2114–2120. doi: 10.1074/jbc.M806881200

Kawachi, M., Kobae, Y., Kogawa, S., Mimura, T., Kramer, U., and Maeshima, M. (2012). Amino acid screening based on structural modeling identifies critical residues for the function, ion selectivity and structure of *Arabidopsis* MTP1. *FEBS J.* 279, 2339–2356. doi: 10.1111/j.1742-4658.2012.08613.x

Kopittke, P. M., De Jonge, M. D., Wang, P., Mckenna, B. A., Lombi, E., Paterson, D. J., et al. (2013). Laterally resolved speciation of arsenic in roots of wheat and rice using fluorescence-XANES imaging. *New Phytol.* 201, 1251–1262. doi: 10.1111/nph.12595

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., et al. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24, 4041–4051. doi: 10.1038/sj.emboj.7600864

Lee, S., Kim, Y. Y., Lee, Y., and An, G. (2007). Rice P1B-type heavy-metal ATPase, OsHMA9, is a metal efflux protein. *Plant Physiol.* 145, 831–842. doi: 10.1104/pp.107.102236

Ma, J. F., Yamaji, N., Mitani, N., Xu, X. Y., Su, Y. H., Mcgrath, S. P., et al. (2008). Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9931–9935. doi: 10.1073/pnas.0802361105

Meda, A. R., Scheuermann, E. B., Prechsl, U. E., Erenoglu, B., Schaaf, G., Hayen, H., et al. (2007). Iron acquisition by phytosiderophores contributes to cadmium tolerance. *Plant Physiol.* 143, 1761–1773. doi: 10.1104/pp.106.094474

Mendoza-Cozatl, D. G., Butko, E., Springer, F., Torpey, J. W., Komives, E. A., Kehr, J., et al. (2008). Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. *Plant J.* 54, 249–259. doi: 10.1111/j.1365-313X.2008.03410.x

Mendoza-Cozatl, D. G., Loza-Tavera, H., Hernandez-Navarro, A., and Moreno-Sanchez, R. (2005). Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants. *FEMS Microbiol. Rev.* 29, 653–671. doi: 10.1016/j.femsre.2004.09.004

Mendoza-Cozatl, D. G., Jobe, T. O., Hauser, F., and Schroeder, J. I. (2011). Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Curr. Opin. Plant Biol.* 14, 554–562. doi: 10.1016/j.pbi.2011.07.004

Mitani-Ueno, N., Yamaji, N., Zhao, F. J., and Ma, J. F. (2011). The aromatic/arginine selectivity filter of NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic. *J. Exp. Bot.* 62, 4391–4398. doi: 10.1093/jxb/err158

Molins, H., Michelet, L., Lanquar, V., Agorio, A., Giraudat, J., Roach, T., et al. (2013). Mutants impaired in vacuolar metal mobilization identify chloroplasts as a target for cadmium hypersensitivity in *Arabidopsis thaliana*. *Plant Cell Environ.* 36, 804–817. doi: 10.1111/pce.12016

Moore, K. L., Chen, Y., Van De Meene, A. M., Hughes, L., Liu, W., Geraki, T., et al. (2013). Combined NanoSIMS and synchrotron X-ray fluorescence reveal distinct cellular and subcellular distribution patterns of trace elements in rice tissues. *New Phytol.* 201, 104–115. doi: 10.1111/nph.12497.

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., et al. (2009). AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in *Arabidopsis*. *Plant Physiol.* 149, 894–904. doi: 10.1104/pp.108.130294

Murgia, I., Arosio, P., Tarantino, D., and Soave, C. (2012). Biofortification for combating "hidden hunger" for iron. *Trends Plant Sci.* 17, 47–55. doi: 10.1016/j.tplants.2011.10.003

Mustroph, A., Zanetti, M. E., Jang, C. J., Holtan, H. E., Repetti, P. P., Galbraith, D. W., et al. (2009). Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 18843–18848. doi: 10.1073/pnas.0906131106

Nordstrom, D. K. (2002). Public health. Worldwide occurrences of arsenic in ground water. *Science* 296, 2143–2145. doi: 10.1126/science.1072375

Nour-Eldin, H. H., and Halkier, B. A. (2013). The emerging field of transport engineering of plant specialized metabolites. *Curr. Opin. Biotechnol.* 24, 263–270. doi: 10.1016/j.copbio.2012.09.006

Olsson, I. M., Bensryd, I., Lundh, T., Ottosson, H., Skerfving, S., and Oskarsson, A. (2002). Cadmium in blood and urine-impact of sex, age, dietary intake, iron status, and former smoking-association of renal effects. *Environ. Health Perspect.* 110, 1185–1190. doi: 10.1289/ehp.021101185

Palmer, C. M., and Guerinot, M. L. (2009). Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nat. Chem. Biol.* 5, 333–340. doi: 10.1038/nchembio.166

Podar, D., Scherer, J., Noordally, Z., Herzyk, P., Nies, D., and Sanders, D. (2012). Metal selectivity determinants in a family of transition metal transporters. *J. Biol. Chem.* 287, 3185–3196. doi: 10.1074/jbc.M111.305649

Rellán-Álvarez, R., Abadía, J., and Álvarez-Fernández, A. (2008). Formation of metal-nicotianamine complexes as affected by pH, ligand exchange with citrate and metal exchange. A study by electrospray ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*22, 1553–1562. doi: 10.1002/rcm.3523

Rodriguez-Lado, L., Sun, G., Berg, M., Zhang, Q., Xue, H., Zheng, Q., et al. (2013). Groundwater arsenic contamination throughout China. *Science* 341, 866–868. doi: 10.1126/science.1237484

Rogers, E. E., Eide, D. J., and Guerinot, M. L. (2000). Altered selectivity in an *Arabidopsis* metal transporter. *Proc. Natl. Acad. Sci. U.S.A.* 97, 12356–12360. doi: 10.1073/pnas.210214197

Sasaki, A., Yamaji, N., Xia, J., and Ma, J. F. (2011). OsYSL6 is involved in the detoxification of excess manganese in rice. *Plant Physiol.* 157, 1832–1840. doi: 10.1104/pp.111.186031

Sasaki, A., Yamaji, N., Yokosho, K., and Ma, J. F. (2012). Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell* 24, 2155–2167. doi: 10.1105/tpc.112.096925

Satarug, S., Garrett, S. H., Sens, M. A., and Sens, D. A. (2010). Cadmium, environmental exposure, and health outcomes. *Environ. Health Perspect.* 118, 182–190. doi: 10.1289/ehp.0901234

Shin, H., Shin, H. S., Dewbre, G. R., and Harrison, M. J. (2004). Phosphate transport in *Arabidopsis*: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *Plant J.* 39, 629–642. doi: 10.1111/j.1365-313X.2004.02161.x

Sinclair, S. A., and Kramer, U. (2012). The zinc homeostasis network of land plants. *Biochim. Biophys. Acta* 1823, 1553–1567. doi: 10.1016/j.bbamcr.2012.05.016

Slewinski, T. L., Zhang, C., and Turgeon, R. (2013). Structural and functional heterogeneity in phloem loading and transport. *Front. Plant Sci.* 4:244. doi: 10.3389/fpls.2013.00244

Song, W. Y., Choi, K. S., Kim Do, Y., Geisler, M., Park, J., Vincenzetti, V., et al. (2010a). *Arabidopsis* PCR2 is a zinc exporter involved in both zinc extrusion and long-distance zinc transport. *Plant Cell* 22, 2237–2252. doi: 10.1105/tpc.109.070185

Song, W. Y., Park, J., Mendoza-Cozatl, D. G., Suter-Grotemeyer, M., Shim, D., Hortensteiner, S., et al. (2010b). Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21187–21192. doi: 10.1073/pnas.1013964107

Song, W. Y., Martinoia, E., Lee, J., Kim, D., Kim, D. Y., Vogt, E., et al. (2004). A novel family of cys-rich membrane proteins mediates cadmium resistance in *Arabidopsis*. *Plant Physiol.* 135, 1027–1039. doi: 10.1104/pp.103.037739

Song, W. Y., Mendoza-Cozatl, D., Lee, Y., Schroeder, J., Ahn, S. N., Lee, H. S., et al. (2013). Phytochelatin-metal(loid) transport into vacuoles shows different substrate preferences in barley and *Arabidopsis*. *Plant Cell Environ.* doi: 10.1111/pce.12227 [Epub ahead of print].

Sperotto, R. A., Ricachenevsky, F. K., Waldow Vde, A., and Fett, J. P. (2012). Iron biofortification in rice: it's a long way to the top. *Plant Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Takahashi, R., Ishimaru, Y., Senoura, T., Shimo, H., Ishikawa, S., Arao, T., et al. (2011). The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. *J. Exp. Bot.* 62, 4843–4850. doi: 10.1093/jxb/err136

Takahashi, R., Ishimaru, Y., Shimo, H., Ogo, Y., Senoura, T., Nishizawa, N. K., et al. (2012). The OsHMA2 transporter is involved in root-to-shoot translocation of Zn and Cd in rice. *Plant Cell Environ.* 35, 1948–1957. doi: 10.1111/j.1365-3040.2012.02527.x

Tennstedt, P., Peisker, D., Bottcher, C., Trampczynska, A., and Clemens, S. (2009). Phytochelatin synthesis is essential for the detoxification of excess zinc and contributes significantly to the accumulation of zinc. *Plant Physiol.* 149, 938–948. doi: 10.1104/pp.108.127472

Thomine, S., Lelièvre, F., Debarbieux, E., Schroeder, J. I., and Barbier-Brygoo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant J.* 34, 685–695. doi: 10.1046/j.1365-313X.2003.01760.x

Ueno, D., Yamaji, N., Kono, I., Huang, C. F., Ando, T., Yano, M., et al. (2010). Gene limiting cadmium accumulation in rice. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16500–16505. doi: 10.1073/pnas.1005396107

Uraguchi, S., Kamiya, T., Sakamoto, T., Kasai, K., Sato, Y., Nagamura, Y., et al. (2011). Low-affinity cation transporter (OsLCT1) regulates cadmium transport into rice grains. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20959–20964. doi: 10.1073/pnas.1116531109

Waters, B. M., and Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

White, P. J., and Broadley, M. R. (2005). Biofortifying crops with essential mineral elements. *Trends Plant Sci.* 10, 586–593. doi: 10.1016/j.tplants.2005.10.001

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

Yamaji, N., Xia, J., Mitani-Ueno, N., Yokosho, K., and Feng Ma, J. (2013). Preferential delivery of zinc to developing tissues in rice is mediated by P-type heavy metal ATPase OsHMA2. *Plant Physiol.* 162, 927–939. doi: 10.1104/pp.113.216564

Ye, W. L., Wood, B. A., Stroud, J. L., Andralojc, P. J., Raab, A., Mcgrath, S. P., et al. (2010). Arsenic speciation in phloem and xylem exudates of castor bean. *Plant Physiol.* 154, 1505–1513. doi: 10.1104/pp.110.163261

Zhao, F. J., Ma, J. F., Meharg, A. A., and Mcgrath, S. P. (2009). Arsenic uptake and metabolism in plants. *New Phytol.* 181, 777–794. doi: 10.1111/j.1469–8137.2008.02716.x

Zimmermann, M., Clarke, O., Gulbis, J. M., Keizer, D. W., Jarvis, R. S., Cobbett, C. S., et al. (2009). Metal binding affinities of Arabidopsis zinc and copper transporters: selectivities match the relative, but not the absolute, affinities of their amino-terminal domains. *Biochemistry* 48, 11640–11654. doi: 10.1021/bi901573b

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Biofortification of wheat grain with iron and zinc: integrating novel genomic resources and knowledge from model crops

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Wheat, like many other staple cereals, contains low levels of the essential micronutrients iron and zinc. Up to two billion people worldwide suffer from iron and zinc deficiencies, particularly in regions with predominantly cereal-based diets. Although wheat flour is commonly fortified during processing, an attractive and more sustainable solution is biofortification, which requires developing new varieties of wheat with inherently higher iron and zinc content in their grains. Until now most studies aimed at increasing iron and zinc content in wheat grains have focused on discovering natural variation in progenitor or related species. However, recent developments in genomics and transformation have led to a step change in targeted research on wheat at a molecular level. We discuss promising approaches to improve iron and zinc content in wheat using knowledge gained in model grasses. We explore how the latest resources developed in wheat, including sequenced genomes and mutant populations, can be exploited for biofortification. We also highlight the key research and practical challenges that remain in improving iron and zinc content in wheat.

Keywords: nutritional enhancement, cereals, transgenic, genomics, model to crop

INTRODUCTION

All living organisms require essential mineral micronutrients to maintain metabolism and humans obtain these from their diet (Welch and Graham, 2004). However staple grains such as wheat often contain suboptimal quantities of micronutrients, especially iron (Fe) and zinc (Zn), and most of this content is removed by milling. In regions where the human diet consists mainly of cereals this leads to deficiencies in micronutrients. The World Health Organization estimates that approximately 25% of the world's population suffers from anemia (WHO, 2008), and that Fe-deficiency anemia led to the loss of over 46,000 disability adjusted life years (DALYs) in 2010 alone (Murray and Lopez, 2013). An estimated 17.3% of people worldwide are at risk of inadequate Zn intake (Wessells and Brown, 2012) and Zn-deficiency leads to estimated annual deaths of 433,000 children under the age of five (WHO, 2009).

There are many possible strategies to improve micronutrient intake in the human diet including dietary diversification, mineral supplementation and post-harvest food fortification. However, these strategies depend on continued investment and infrastructure, and current levels of post-harvest fortification of Fe are often inadequate (White and Broadley, 2009; Gomez-Galera et al., 2010; Hurrell et al., 2010). Biofortification circumvents these problems by improving the micronutrient content of the crops themselves by increasing mineral levels and bioavailability in the edible parts. Improving crop varieties by either conventional breeding or transgenic methods has the advantage that once the initial research and development is completed, the benefits from these nutritionally-enhanced crops will be sustainable with little further investment (Gomez-Galera et al., 2010).

Many studies have shown that there is a wide variation in grain Fe and Zn concentrations in wild relatives of modern wheat and the concentrations found can significantly exceed those found in modern elite cultivars (Cakmak et al., 2000; Monasterio and Graham, 2000). This natural variation can be utilized to biofortify wheat for Fe and Zn, such as has been achieved using the transcription factor *NAM-B1* (Uauy et al., 2006) which was originally identified for increasing protein content in wild emmer (*Triticum turgidum* ssp *dicoccoides*). In near isogenic lines the presence of *NAM-B1* increased Fe and Zn grain concentrations by 18 and 12%, respectively, (Distelfeld et al., 2007). This gene is being widely used in breeding programes across several continents (Kumar et al., 2011; Randhawa et al., 2013; Tabbita et al., 2013).

Recent technological developments present new opportunities that can complement natural variation and genome-wide association studies, and lead to faster improvements in Fe and Zn grain content. Therefore here we focus on how the dramatic increase in wheat genomic sequence availability combined with functional genomic approaches can be used to their fullest potential to engineer new varieties of wheat with improved Fe and Zn content.

ADVANCES IN WHEAT RESOURCES APPLICABLE TO IMPROVING FE AND ZN GRAIN CONTENT

To date, molecular breeding in bread wheat has been hindered by its large genome size (16 Gb: five times that of humans), its polyploid nature (tetraploid pasta and hexaploid bread wheat), and the high nucleotide similarity between these genomes (>95% similar in genes). However, recent advances in technology will greatly increase the rate of discovery and functional characterization of wheat genes, and provide the tools with which to deploy this knowledge into improved varieties. Some of these advances are outlined below.

GENOME AND GENE SEQUENCE AVAILABILITY

In the last 5 years the amount of publicly available wheat genomic sequence has massively expanded. The International Wheat Genome Sequencing Consortium has coordinated the purification of individual chromosome arms using flow sorting (Safar et al., 2004) followed by shotgun sequencing and assembly into contigs of an average size of 2.5 kb. These genome-specific contigs have recently been released in *EnsemblPlants* allowing wheat researchers to separate and distinguish the homoeologous genomes for the first time. Physical maps of BAC libraries made from these purified chromosome arms are being constructed (Paux et al., 2008) to generate a high quality reference. A complementary strategy, whole genome shotgun (WGS) sequencing, has been used to generate a 5x coverage of the wheat genome, using orthologous sequences from multiple grasses to guide assembly (Brenchley et al., 2012). Draft sequences for the A and D genome progenitors, *T. urartu* (Ling et al., 2013) and *Aegilops tauschii* (Jia et al., 2013), were also created using a WGS approach followed by *de novo* assembly. Combining the WGS sequencing with the physical map strategy is leading to an unprecedented wealth of genomic information and will ultimately lead to a reliable reference sequence for polyploid wheat.

In parallel, the ability to access genic sequence through RNA-seq and exome capture (Saintenac et al., 2011; Trick et al., 2012; Winfield et al., 2012) is enabling the identification of single nucleotide polymorphisms and the development of publicly available genome-specific markers for genetic mapping in polyploid wheat (Wilkinson et al., 2012; Allen et al., 2013). Recently a comprehensive set of homoeolog-specific gene models for polyploid wheat has been published (Krasileva et al., 2013). In short, wheat researchers now have access to genome-specific contig assemblies (albeit partial and fragmented), draft reference genomes, gene models and large SNP datasets. Together, these tools should enable more precise mapping and deployment of grain Fe and Zn traits through marker assisted selection.

NOVEL EXPERIMENTS USING SEQUENCE DATA RESOURCES

The wealth of sequence data, together with the reduced cost of sequencing, allows new ways of investigating gene function related to grain Fe and Zn. For example, RNA-seq was applied to identify differentially expressed genes in lines with reduced expression of *NAM* genes (Cantu et al., 2011). Many classes of genes including transporters, hormone regulated genes and transcription factors were identified. This study generates leads for investigations into the early stages of senescence and nutrient remobilisation that relate directly to micronutrient content in wheat grains. The differentially expressed *NAM*-regulated genes can now be further pursued through the reverse genetic resources available in wheat (see below). The refinement of methods to analyze RNA-seq data (Duan et al., 2012) together with homoeolog-specific gene models will provide increased resolution to transcriptome studies as reads can be assigned more accurately to specific genomes (Krasileva et al., 2013).

REVERSE GENETIC MUTANT RESOURCES

The major advances and cost reduction in sequencing technology has also created the opportunity to characterize existing chemically mutagenised populations (Uauy et al., 2009; Sestili et al., 2010) for rapid discovery of mutants in specific genes. The newly developed gene models (Krasileva et al., 2013) are being combined with exome capture approaches to enrich for protein-coding genes in both tetraploid and hexaploid mutant populations. Over 3,000 individuals will be sequenced and mutations identified and organized for online access (Uauy and Dubcovsky, personal communication). Therefore in the very near future, researchers will be able to order mutants in their gene of interest through a simple *in silico* search as is standard in many model species. The availability of such resources will allow faster characterization of gene function in wheat and will provide valuable alleles for breeding.

TRANSGENIC METHOD IMPROVEMENTS

Producing transgenic wheat has previously been a major bottleneck in investigating gene function. The efficiency of wheat transformation still lags behind the efficiency of barley transformation but it is constantly improving and a wide range of promoters is available to target transgene expression to particular tissues or developmental stages (Harwood, 2012). In addition, high-throughput *Agrobacterium*-mediated transformation of wheat is now possible through a patented technology (PureIntro; WO 95/06722) from Japan Tobacco which has been licensed to several institutions and delivers transformation efficiencies above 30%. However challenges still remain. These relate primarily to costs and the ability to produce genotype-independent transformation protocols, since most reports utilize Bobwhite or Fielder which are not suitable for commercialisation of transgenic wheat (Li et al., 2012). The ability to transform any cultivar of wheat, at a reasonable price, would allow transformation into elite lines which would speed up breeding programes and also allow research to be carried out in a more appropriate background.

TRANSFERRING MODEL CROP KNOWLEDGE INTO WHEAT

THE PATHWAYS FROM THE ROOTS TO THE GRAIN AND THE IMPORTANCE OF BIOAVAILABILITY

Much work has been carried out to understand the distinct routes Fe and Zn take to reach the grain in diploid crop species such as rice, maize, and barley. Conservation of these pathways between species allows predictions about Fe and Zn transport in wheat where less is known. Recent reviews have covered the pathways in model crops extensively (Palmgren et al., 2008; Curie et al., 2009; Conte and Walker, 2011; Waters and Sankaran, 2011; White and Broadley, 2011; Borg et al., 2012; Kobayashi and Nishizawa,

2012; Lee et al., 2012; Sperotto et al., 2012; Schroeder et al., 2013) so here we will briefly outline the putative pathways in wheat and then discuss key steps to target for crop improvement (**Figure 1**).

The uptake of Fe and Zn from the soil occurs via two processes in plants: direct uptake of Fe^{2+} and Zn^{2+} by ZRT-, IRT-like proteins (ZIPs) or via secretion of phytosiderophores (PSs) which chelate Fe cations and are subsequent taken up by yellow stripe like (YSL) transporters (Sperotto et al., 2012). The chelation strategy is generally used for Fe uptake in monocots such as wheat. In many steps of Fe and Zn transport the same families of proteins are involved, however the two metals are treated separately by plants often by the involvement of different members of multigene families. Metal chelators such as nicotianamine (NA) are important for radial movement of Fe and Zn through the root (Rellán-Álvarez et al., 2010; Deinlein et al., 2012) and the transport of Zn into the vacuole affects overall Zn transport through the roots into the shoot (Morel et al., 2009; Haydon et al., 2012). Fe and Zn are loaded into the xylem where Zn can move as a cation or in a complex with organic acids such as citrate (Lu et al., 2013), and Fe is chelated by citrate (Rellán-Álvarez et al., 2010). Transfer from xylem to phloem can occur in the root or basal part of the shoot or during remobilisation from the leaves during grain filling and is facilitated by ZIP and YSL family proteins. In wheat all nutrients enter the grain from the phloem because the xylem is discontinuous (Zee and O'Brien, 1970). In the phloem Fe and Zn are transported as complexes with NA or small proteins. Transporters from the maternal tissue into the endosperm cavity and into the aleurone and embryo have been proposed; several are members of the ZIP, YSL, and metal tolerance protein (MTP) families (Borg et al., 2009; Tauris et al., 2009).

In wheat grain most Fe and Zn is located in the aleurone layer which is lost during milling. This problem is further compounded by the fact that Fe in these tissues is deposited mainly in protein storage vacuoles (PSVs; Regvar et al., 2011) where it is bound to phytate, which makes it poorly bioavailable to humans (Borg et al., 2009). Ferritin, which forms large Fe-rich nanoparticles, is generally regarded as a more bioavailable storage form and is present in the widely consumed endosperm amyloplasts (Balmer et al., 2006). Thus it is important to not only consider the total content of Fe and Zn in grain, but also the tissue localization and speciation (as chelates, protein particles or other), which affects their bioavailability.

Many of the steps described above have been modified by transgenic approaches in diploid crop species. We discuss below some promising studies and how this knowledge can be used to improve Fe and Zn grain content and bioavailability in wheat.

TRANSGENIC APPROACHES IN RICE

Several studies have over-expressed genes involved in the pathway for Fe and Zn transport in rice with promising results showing increased bioavailability of Fe and no negative impact on yield. Over-expression of NA synthase (NAS) led to 2–3 fold increases in Fe and Zn content in paddy grown grain and importantly feeding this grain to anemic mice led to the recovery of normal hemoglobin and haematocrit levels within 2 weeks, whereas wild-type grain did not (Lee et al., 2009). Multiplexing genes involved at several steps has enabled even larger increases in Fe content, although bioavailability was not tested. Expressing NAS, ferritin and phytase resulted in a 6-fold increase in Fe in polished rice grains (Wirth et al., 2009). The authors suggest that the combination of these three transgenes did not significantly affect overall Fe homeostasis, shown by expression analysis of 28 endogenous rice genes in Fe-deficient and sufficient conditions (Wang et al., 2013). This suggests that a mechanism combining both increased translocation (NAS) and expanded sink strength (ferritin) could be suitable to enhance rice (and wheat) endosperm Fe content.

APPLYING TRANSGENIC APPROACHES TO WHEAT

At present, studies in wheat are restricted to the endosperm-specific expression of wheat or soybean ferritin which led to increases in grain iron content of 1.5 to 1.9-fold and 1.1 to 1.6-fold respectively (Borg et al., 2012; Sui et al., 2012) and increasing phytase activity (Holm et al., 2002). These studies give proof of concept that grain Fe and Zn can be modified in wheat through transgenic approaches.

Using knowledge from model species, it is possible now to identify more rapidly and with higher confidence candidate genes that might play a role in Fe and Zn transport. Access to relatively complete genomic sequence for polyploid wheat will allow more comprehensive phylogenetic studies for putative wheat homologs of large gene families. For example we have used the sequences of the rice natural resistance-associated macrophage proteins (NRAMPs) to identify wheat homologs (**Table 1**). Wheat candidate genes with putative Fe and Zn transporter function inferred from phylogeny, can be taken forward and characterized in yeast mutants, with a view eventually to expressing these in transgenic wheat plants to increase vacuolar export and ultimately nutrient content in the grain.

Work in rice has shown that multiplexing genes can further increase gains and this strategy could also be suitable for wheat. An analogous approach which would act to regulate several steps of the Fe and Zn uptake pathways would be to engineer transcriptional regulators to enhance movement and uptake into grains. For example the *NAM-B1* transcription factor provides an entry point to increase Fe, Zn, and protein content: with greater understanding of its targets, and which transport steps are key control points, we could engineer expression patterns, downstream targets, or binding specificities to improve nutrient content in the grain. The lack of genomic resources in wheat prompted initial studies on *NAM-B1* to focus on rice, but these attempts failed since the orthologous rice *NAM* gene affects anther dehiscence rather than mineral remobilisation (Distelfeld et al., 2012). The advent of new technologies and genomic resources now allow these questions to be addressed directly in wheat. In addition to RNA-seq (Cantu et al., 2011), we have developed transgenic lines with epitope-tagged NAM proteins to perform chromatin immunoprecipitation followed by sequencing (ChIP-seq) to identify direct targets of NAM (Borrill and Uauy, unpublished data). The availability of gene models and genomic sequence (including promoter regions) now makes this a feasible undertaking in wheat.

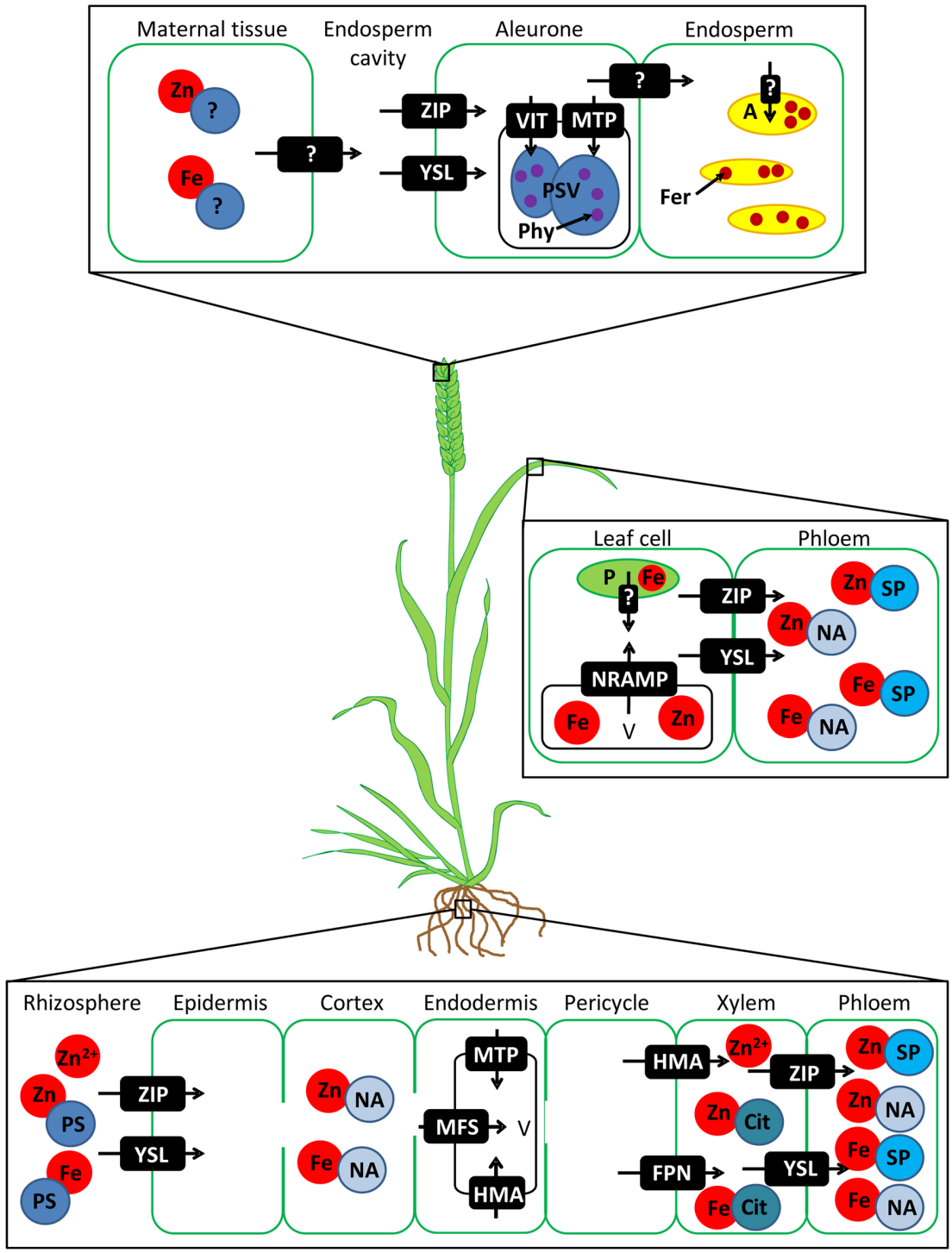

FIGURE 1 | Simplified proposed pathway for Fe and Zn uptake and translocation to the grain in wheat. Putative classes of transport proteins are shown in white text and are based on evidence from other species. Question marks show unidentified transporters. Free Zn^{2+} and phytosiderophore (PS) -bound Fe and Zn are absorbed from the soil into root epidermal cells. Fe and Zn move via the apoplast and symplast to the pericycle, but may be sequestered en-route in vacuoles. Fe and Zn are loaded into the xylem and transferred into the phloem in the root, basal shoot or leaf tissues (not shown). Fe and Zn are remobilised from leaf cell plastids (P) and vacuoles (V) and loaded into the phloem for transport to the ear. Fe and Zn are exported from the maternal tissue into the endosperm cavity. After uptake into the aleurone layer most Fe and Zn are sequestered in protein storage vacuoles (PSVs) bound to phytate (Phy). A small proportion of Fe and Zn may enter the endosperm and be stored bound to ferritin (Fer) in amyloplasts (A). ZIP = ZRT-, IRT-like protein, YSL = yellow stripe like transporter, MFS = major facilitator superfamily transporter, MTP = metal tolerance protein, HMA = heavy metal ATPase, FPN = ferroportin, NRAMP = natural resistance-associated macrophage protein, VIT = vacuolar iron transporter, NA = nicotianamine, Cit = citrate, SP = small proteins.

CHALLENGES IN TRANSFERRING KNOWLEDGE TO WHEAT

As illustrated by *NAM* in rice and wheat it is important to note that the precise mechanisms of transport and its regulation differ between species even within monocots, so not all knowledge can be directly translated. It has been proposed that Zn transport to the grain in wheat is constrained by two major bottlenecks; the root–shoot barrier and grain filling (Palmgren et al., 2008). However in rice these bottlenecks are reduced (Stomph et al., 2009) as shown by the storage of excess Zn in shoots as well as roots (Jiang et al., 2008), and the ability of rice to load Zn from the xylem into the grain without transfer to the phloem (Zee, 1971), which constitutes a limiting step in wheat. Fe transport in rice and other monocots also differs, for example rice uses both secretion of PSs and direct uptake of Fe from the soil (Bughio et al., 2002; Inoue et al., 2009; Nozoye et al., 2011), whereas barley and maize only absorb Fe via PSs (Römheld and Marschner, 1986; Zaharieva and Römheld, 2000; Murata et al., 2006; Walker and Connolly, 2008). These challenges are compounded by the fact that several fundamental questions remain unanswered even in model species. The specificity of many transporters is not fully characterized and the control of flux through pathways requires further investigation. Additionally, the relative contribution of sink/source strength and the possible effects of manipulating individual metals on total grain metal composition are not well understood.

CONCLUSIONS AND FUTURE DIRECTIONS

Our ability to carry out basic research in wheat will be extremely important to build upon and move beyond research in model species. This will be greatly advanced by recent developments in genomic resources, mutant catalogs and transgenic methods. Once genes to improve nutrient content have been identified, they will need to be transferred into agriculturally relevant wheat varieties and assessed for agronomical traits such as yield and disease resistance which are the main drivers for adoption of novel varieties by farmers.

Changes to agricultural conditions in the future will also impact upon the deployment of biofortified wheat varieties. Rising atmospheric carbon dioxide (CO_2) concentrations may lead to reduced

Table 1 | New genomic resources enable identification of NRAMP homologs in wheat.

Rice NRAMP (RAP locus ID)	Wheat homolog	Genome	Wheat sequence[a]
OsNRAMP1 (Os07g0258400)	*TaNRAMP1*	A	7AL_4537662 (URGI)[b]
		B	7BL_6744498 (Ensembl)
		D	7DL_3317468 (Ensembl)
OsNRAMP2 (Os03g0208500)	*TaNRAMP2*	A	4AS_5952279 (Ensembl)
		B	4BL_7000373 (Ensembl)
		D	4DL_14450878 (URGI)
OsNRAMP3 (Os06g0676000)	*TaNRAMP3*	A	7AL_4392690 (Ensembl)
		B	7BL_6748183 (Ensembl)
		D	7DL_3360602 (Ensembl)
OsNRAMP4 (Os02g0131800)	*TaNRAMP4*	A	6AS_4346871 (Ensembl)
		B	6BS_2318478 (Ensembl)[c]
		D	Absent
OsNRAMP5 (Os07g0257200)	*TaNRAMP5*	A	4AS_5926812 (Ensembl)
		B	Across multiple contigs
		D	4DL_14404139 (URGI)
OsNRAMP6 (Os01g0503400)	*TaNRAMP6*	A	Across multiple contigs
		B	Across multiple contigs
		D	Across multiple contigs
OsNRAMP7 (Os12g0581600)	*TaNRAMP7*	A	5AS_1501999 (Ensembl)[b]
		B	5BS_2288821 (Ensembl)
		D	5DS_2767814 (Ensembl)
—	*TaNRAMP8*	A	4AL_7173573 (URGI)
		B	4BS_3944622 (Ensembl)
		D	4DS_2292562 (Ensembl)

[a] *International wheat genome sequencing consortium chromosome-arm survey sequences are available at EnsemblPlants (Ensembl; http://plants.ensembl.org/index.html) and at Unité de Recherche Génomique Info (URGI; http://www.wheatgenome.org/Tools-and-Resources)*
[b] *partial sequence*
[c] *premature termination codon*

grain micronutrient content, especially Fe, as has been shown by free air CO_2 enrichment studies (Hogy et al., 2009; Fernando et al., 2012). Additionally lower levels of fertilizer may be used in the future due to budgetary and legal constraints. This may present a challenge to improve Fe and Zn grain concentrations because lower application of nitrogen fertilizer correlates to lower Fe and Zn grain concentrations (Cakmak et al., 2010). In addition, the drive for higher yields is usually accompanied by a dilution effect of minerals due to the additional grain starch accumulation. This scenario suggests that scientists and breeders will need to work ever more closely to achieve not just maintenance, but the required increased grain Fe and Zn contents.

Despite these challenges we believe that wheat researchers now have access to the tools and resources required to make significant improvements to Fe and Zn content in wheat grain and to bring these improved varieties to the field. These new varieties could make an important contribution to improving the health of millions of people worldwide to avoid Fe and Zn malnutrition which still affects over 25% of the global population.

AUTHOR CONTRIBUTIONS

Philippa Borrill and Cristobal Uauy wrote the manuscript; James M. Connorton, Janneke Balk, Anthony J. Miller, Dale Sanders contributed corrections and suggestions; Philippa Borrill, James M. Connorton, Cristobal Uauy analyzed the wheat genome sequence data for **Table 1**. Philippa Borrill, Janneke Balk, Anthony J. Miller, Dale Sanders, Cristobal Uauy conceived the perspective. All authors read and approved the final manuscript.

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REFERENCES

Allen, A. M., Barker, G. L. A., Wilkinson, P., Burridge, A., Winfield, M., Coghill, J., et al. (2013). Discovery and development of exome-based, co-dominant single nucleotide polymorphism markers in hexaploid wheat (*Triticum aestivum* L.). *Plant Biotechnol. J.* 11, 279–295. doi: 10.1111/pbi.12009

Balmer, Y., Vensel, W. H., Dupont, F. M., Buchanan, B. B., and Hurkman, W. J. (2006). Proteome of amyloplasts isolated from developing wheat endosperm presents evidence of broad metabolic capability. *J. Exp. Bot.* 57, 1591–1602. doi: 10.1093/jxb/erj156

Borg, S., Brinch-Pedersen, H., Tauris, B., and Holm, P. (2009). Iron transport, deposition and bioavailability in the wheat and barley grain. *Plant Soil* 325, 15–24. doi: 10.1007/s11104-009-0046-6

Borg, S., Brinch-Pedersen, H., Tauris, B., Madsen, L. H., Darbani, B., Noeparvar, S., et al. (2012). Wheat ferritins: improving the iron content of the wheat grain. *J. Cereal Sci.* 56, 204–213. doi: 10.1016/j.jcs.2012.03.005

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G. L. A., D'Amore, R., Allen, A. M., et al. (2012). Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature* 491, 705–710. doi: 10.1038/nature11650

Bughio, N., Yamaguchi, H., Nishizawa, N. K., Nakanishi, H., and Mori, S. (2002). Cloning an iron-regulated metal transporter from rice. *J. Exp. Bot.* 53, 1677–1682. doi: 10.1093/jxb/erf004

Cakmak, I., Ozkan, H., Braun, H. J., Welch, R. M., and Romheld, V. (2000). Zinc and iron concentrations in seeds of wild, primitive, and modern wheats. *Food Nutr. Bull.* 21, 401–403.

Cakmak, I., Pfeiffer, W. H., and Mcclafferty, B. (2010). Biofortification of durum wheat with zinc and iron. *Cereal Chem.* 87, 10–20. doi: 10.1094/CCHEM-87-1-0010

Cantu, D., Pearce, S. P., Distelfeld, A., Christiansen, M. W., Uauy, C., Akhunov, E., et al. (2011). Effect of the down-regulation of the high Grain Protein Content (GPC) genes on the wheat transcriptome during monocarpic senescence. *BMC Genomics* 12:492. doi: 10.1186/1471-2164-12–492

Conte, S. S., and Walker, E. L. (2011). Transporters contributing to iron trafficking in plants. *Mol. Plant* 4, 464–476. doi: 10.1093/mp/ssr015

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot. Lond.* 103, 1–11. doi: 10.1093/aob/mcn207

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T. H., et al. (2012). Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. *Plant Cell* 24, 708–723. doi: 10.1105/tpc.111.095000

Distelfeld, A., Cakmak, I., Peleg, Z., Ozturk, L., Yazici, A. M., Budak, H., et al. (2007). Multiple QTL-effects of wheat Gpc-B1 locus on grain protein and micronutrient concentrations. *Physiol. Plant* 129, 635–643. doi: 10.1111/j.1399-3054.2006.00841.x

Distelfeld, A., Pearce, S., Avni, R., Scherer, B., Uauy, C., Piston, F., et al. (2012). Divergent functions of orthologous NAC transcription factors in wheat and rice. *Plant Mol. Biol.* 78, 515–524. doi: 10.1007/s11103-012-9881-6

Duan, J., Xia, C., Zhao, G., Jia, J., and Kong, X. (2012). Optimizing de novo common wheat transcriptome assembly using short-read RNA-Seq data. *BMC Genomics* 13:392. doi: 10.1186/1471-2164-13-392

Fernando, N., Panozzo, J., Tausz, M., Norton, R., Fitzgerald, G., and Seneweera, S. (2012). Rising atmospheric CO2 concentration affects mineral nutrient and protein concentration of wheat grain. *Food Chem.* 133, 1307–1311. doi: 10.1016/j.foodchem.2012.01.105

Gomez-Galera, S., Rojas, E., Sudhakar, D., Zhu, C. F., Pelacho, A. M., Capell, T., et al. (2010). Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Res.* 19, 165–180. doi: 10.1007/s11248-009-9311-y

Harwood, W. A. (2012). Advances and remaining challenges in the transformation of barley and wheat. *J. Exp. Bot.* 63, 1791–1798. doi: 10.1093/jxb/err380

Haydon, M. J., Kawachi, M., Wirtz, M., Hillmer, S., Hell, R., and Krämer, U. (2012). Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in *Arabidopsis*. *Plant Cell* 24, 724–737. doi: 10.1105/tpc.111.095042

Hogy, P., Wieser, H., Kohler, P., Schwadorf, K., Breuer, J., Franzaring, J., et al. (2009). Effects of elevated CO2 on grain yield and quality of wheat: results from a 3-year free-air CO2 enrichment experiment. *Plant Biol.* 11, 60–69. doi: 10.1111/j.1438-8677.2009.00230.x

Holm, P. B., Kristiansen, K. N., and Pedersen, H. B. (2002). Transgenic approaches in commonly consumed cereals to improve iron and zinc content and bioavailability. *J. Nutr.* 132, 514S–516S.

Hurrell, R., Ranum, P., de Pee, S., Biebinger, R., Hulthen, L., Johnson, Q., et al. (2010). Revised recommendations for iron fortification of wheat flour and an evaluation of the expected impact of current national wheat flour fortification programs. *Food Nutr. Bull.* 31, S7–S21.

Inoue, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., et al. (2009). Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J. Biol. Chem.* 284, 3470–3479. doi: 10.1074/jbc.M806042200

Jia, J. Z., Zhao, S. C., Kong, X. Y., Li, Y. R., Zhao, G. Y., He, W. M., et al. (2013). *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. *Nature* 496, 91–95. doi: 10.1038/nature12028

Jiang, W., Struik, P. C., Van Keulen, H., Zhao, M., Jin, L. N., and Stomph, T. J. (2008). Does increased zinc uptake enhance grain zinc mass concentration in rice? *Ann. Appl. Biol.* 153, 135–147. doi: 10.1111/j.1744-7348.2008.00243.x

Kobayashi, T., and Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. *Annu. Rev. Plant Biol.* 63, 131–152. doi: 10.1146/annurev-arplant-042811-105522

Krasileva, K., Buffalo, V., Bailey, P., Pearce, S., Ayling, S., Tabbita, F., et al. (2013). Separating homeologs by phasing in the tetraploid wheat transcriptome. *Genome Biol.* 14:R66. doi: 10.1186/gb-2013-14-6-r66

Kumar, J., Jaiswal, V., Kumar, A., Kumar, N., Mir, R. R., Kumar, S., et al. (2011). Introgression of a major gene for high grain protein content in some Indian bread wheat cultivars. *Field Crops Res.* 123, 226–233. doi: 10.1016/j.fcr.2011.05.013

Lee, S., Jeon, J. S., and An, G. (2012). Iron homeostasis and fortification in rice. *J. Plant Biol.* 55, 261–267. doi: 10.1007/s12374-011-0386-7

Lee, S., Jeon, U. S., Lee, S. J., Kim, Y.-K., Persson, D. P., Husted, S., et al. (2009). Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22014–22019. doi: 10.1073/pnas.0910950106

Li, J. R., Ye, X. G., An, B. Y., Du, L. P., and Xu, H. J. (2012). Genetic transformation of wheat: current status and future prospects. *Plant Biotechnol. Rep.* 6, 183–193. doi: 10.1007/s11816-011-0213-0

Ling, H. Q., Zhao, S. C., Liu, D. C., Wang, J. Y., Sun, H., Zhang, C., et al. (2013). Draft genome of the wheat A-genome progenitor *Triticum urartu*. *Nature* 496, 87–90. doi: 10.1038/nature11997

Lu, L., Tian, S., Zhang, J., Yang, X., Labavitch, J. M., Webb, S. M., et al. (2013). Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant species *Sedum alfredii*. *New Phytol.* 198, 721–731. doi: 10.1111/nph.12168

Monasterio, I., and Graham, R. D. (2000). Breeding for trace minerals in wheat. *Food Nutr. Bull.* 21, 392–396.

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., et al. (2009). AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in *Arabidopsis*. *Plant Physiol.* 149, 894–904. doi: 10.1104/pp.108.130294

Murata, Y., Ma, J. F., Yamaji, N., Ueno, D., Nomoto, K., and Iwashita, T. (2006). A specific transporter for iron(III)-phytosiderophore in barley roots. *Plant J.* 46, 563–572. doi: 10.1111/j.1365-313X.2006.02714.x

Murray, C. J. L., and Lopez, A. D. (2013). Measuring the global burden of disease. *N. Engl. J. Med.* 369, 448–457. doi: 10.1056/NEJMra1201534

Nozoye, T., Nagasaka, S., Kobayashi, T., Takahashi, M., Sato, Y., Sato, Y., et al. (2011). Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. *J. Biol. Chem.* 286,5446–5454. doi: 10.1074/jbc.M110.180026

Palmgren, M. G., Clemens, S., Williams, L. E., Kraemer, U., Borg, S., Schjorring, J. K., et al. (2008). Zinc biofortification of cereals: problems and solutions. *Trends Plant Sci.* 13, 464–473. doi: 10.1016/j.tplants.2008.06.005

Paux, E., Sourdille, P., Salse, J., Saintenac, C., Choulet, F., Leroy, P., et al. (2008). A physical map of the 1-Gigabase bread wheat chromosome 3B. *Science* 322, 101–104. doi: 10.1126/science.1161847

Randhawa, H. S., Asif, M., Pozniak, C., Clarke, J. M., Graf, R. J., Fox, S. L., et al. (2013). Application of molecular markers to wheat breeding in Canada. *Plant Breed.* 132, 458–471. doi: 10.1111/pbr.12057

Regvar, M., Eichert, D., Kaulich, B., Gianoncelli, A., Pongrac, P., Vogel-Mikuš, K., et al. (2011). New insights into globoids of protein storage vacuoles in wheat aleurone using synchrotron soft X-ray microscopy. *J. Exp. Bot.* 62, 3929–3939. doi: 10.1093/jxb/err090

Rellán-Álvarez, R., Giner-Martínez-Sierra, J., Orduna, J., Orera, I., Rodríguez-Castrillón, J. Á., García-Alonso, J. I., et al. (2010). Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol.* 51, 91–102. doi: 10.1093/pcp/pcp170

Römheld, V., and Marschner H. (1986). Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. *Plant Physiol.* 80, 175–180. doi: 10.1104/pp.80.1.175

Safar, J., Bartos, J., Janda, J., Bellec, A., Kubalakova, M., Valarik, M., et al. (2004). Dissecting large and complex genomes: flow sorting and BAC cloning of individual chromosomes from bread wheat. *Plant J.* 39, 960–968. doi: 10.1111/j.1365-313X.2004.02179.x

Saintenac, C., Jiang, D., and Akhunov, E. (2011). Targeted analysis of nucleotide and copy number variation by exon capture in allotetraploid wheat genome. *Genome Biol.* 12, R88. doi: 10.1186/gb-2011-12-9-r88

Schroeder, J. I., Delhaize, E., Frommer, W. B., Guerinot, M. L., Harrison, M. J., Herrera-Estrella, L., et al. (2013). Using membrane transporters to improve crops for sustainable food production. *Nature* 497, 60–66. doi: 10.1038/nature11909

Sestili, F., Botticella, E., Bedo, Z., Phillips, A., and Lafiandra, D. (2010). Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis. *Mol. Breed.* 25, 145–154. doi: 10.1007/s11032-009-9314-7

Sperotto, R. A., Ricachenevsky, F. K., Waldow, V. D., and Fett, J. P. (2012). Iron biofortification in rice: it's a long way to the top. *Plant Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Stomph, T. J., Wen, J., and Struik, P. C. (2009). Zinc biofortification of cereals: rice differs from wheat and barley. *Trends Plant Sci.* 14, 123–124. doi: 10.1016/j.tplants.2009.01.001

Sui, X., Zhao, Y., Wang, S., Duan, X., Xu, L., Liang, R., et al. (2012). Improvement Fe content of wheat (*Triticum aestivum*) grain by soybean ferritin expression cassette without vector backbone sequence. *J. Agric. Biotechnol.* 20, 766–773.

Tabbita, F., Lewis, S., Vouilloz, J. P., Ortega, M. A., Kade, M., Abbate, P. E., et al. (2013). Effects of the Gpc-B1 locus on high grain protein content introgressed into Argentinean wheat germplasm. *Plant Breed.* 132, 48–52. doi: 10.1111/pbr.12011

Tauris, B., Borg, S., Gregersen, P. L., and Holm, P. B. (2009). A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. *J. Exp. Bot.* 60, 1333–1347. doi: 10.1093/jxb/erp023

Trick, M., Adamski, N., Mugford, S., Jiang, C.-C., Febrer, M., and Uauy, C. (2012). Combining SNP discovery from next-generation sequencing data with bulked segregant analysis (BSA) to fine-map genes in polyploid wheat. *BMC Plant Biol.* 12:14. doi: 10.1186/1471-2229-12-14

Uauy, C., Distelfeld, A., Fahima, T., Blechl, A., and Dubcovsky, J. (2006). A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* 314, 1298–1301. doi: 10.1126/science.1133649

Uauy, C., Paraiso, F., Colasuonno, P., Tran, R. K., Tsai, H., Berardi, S., et al. (2009). A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biol.* 9:115. doi: 10.1186/1471-2229-9-115

Walker, E. L., and Connolly, E. L. (2008). Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. *Curr. Opin. Plant Biol.* 11, 530–535. doi: 10.1016/j.pbi.2008.06.013

Wang, M., Gruissem, W., and Bhullar, N. K. (2013). Nicotianamine synthase overexpression positively modulates iron homeostasis-related genes in high iron rice. *Front. Plant Sci.* 4:156. doi: 10.3389/fpls.2013.00156

Waters, B. M., and Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: Genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

Welch, R. M., and Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* 55, 353–364. doi: 10.1093/jxb/erh064

Wessells, K. R., and Brown, K. H. (2012). Estimating the global prevalence of zinc deficiency: results based on zinc availability in national food supplies and the prevalence of stunting. *PLoS ONE* 7:e50568. doi: 10.1371/journal.pone.0050568

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets - iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

White, P. J., and Broadley, M. R. (2011). Physiological limits to zinc biofortification of edible crops. *Front. Plant Sci.* 2:80. doi: 10.3389/fpls.2011.00080

WHO. (2008). *Worldwide Prevalence of Anaemia 1993–2005: WHO Global Database on Anaemia*, eds B. de Benoist, E. McLean, I. Egli, and M. Cogswell (Geneva: World Health Organization Press).

WHO. (2009). *Global Health Risks. Mortality and Burden of Disease Attributable to Selected Major Risks*. Available at: http://www.who.int/healthinfo/global_burden_disease/GlobalHealthRisks_report_annex.pdf (accessed October 30, 2013).

Wilkinson, P., Winfield, M., Barker, G., Allen, A., Burridge, A., Coghill, J., et al. (2012). CerealsDB 2.0*:* an integrated resource for plant breeders and scientists. *BMC Bioinformatics* 13:219. doi: 10.1186/1471-2105-13-219

Winfield, M. O., Wilkinson, P. A., Allen, A. M., Barker, G. L. A., Coghill, J. A., Burridge, A., et al. (2012). Targeted re-sequencing of the allohexaploid wheat exome. *Plant Biotechnol. J.* 10, 733–742. doi: 10.1111/j.1467-7652.2012.00713.x

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., et al. (2009). Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnol. J.* 7, 631–644. doi: 10.1111/j.1467-7652.2009.00430.x

Zaharieva, T., and Römheld, V. (2000). Specific Fe uptake system in strategy I plants inducible under Fe deficiency. *J. Plant Nutr.* 23, 1733–1744. doi: 10.1080/01904160009382137

Zee, S. Y., and O'Brien, T. P. (1970). A special type of tracheary element associated with xylem-discontinuity in floral axis of wheat. *Aust. J. Biol. Sci.* 23, 783–791. doi: 10.1071/BI9700783

Zee, S. Y. (1971). Vascular tissue and transfer cell distribution in the rice spikelet. *Aust. J. Biol. Sci.* 25, 411–414.

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The diverse roles of FRO family metalloreductases in iron and copper homeostasis

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Iron and copper are essential for plants and are important for the function of a number of protein complexes involved in photosynthesis and respiration. As the molecular mechanisms that control uptake, trafficking and storage of these nutrients emerge, the importance of metalloreductase-catalyzed reactions in iron and copper metabolism has become clear. This review focuses on the ferric reductase oxidase (FRO) family of metalloreductases in plants and highlights new insights into the roles of FRO family members in metal homeostasis. *Arabidopsis* FRO2 was first identified as the ferric chelate reductase that reduces ferric iron-chelates at the root surface-rhizosphere interface. The resulting ferrous iron is subsequently transported across the plasma membrane of root epidermal cells by the ferrous iron transporter, IRT1. Recent work has shown that two other members of the FRO family (FRO4 and FRO5) function redundantly to reduce copper to facilitate its uptake from the soil. In addition, FROs appear to play important roles in subcellular compartmentalization of iron as FRO7 is known to contribute to delivery of iron to chloroplasts while mitochondrial family members FRO3 and FRO8 are hypothesized to influence mitochondrial metal ion homeostasis. Finally, recent studies have underscored the importance of plasma membrane-localized ferric reductase activity in leaves for photosynthetic efficiency. Taken together, these studies highlight a number of diverse roles for FROs in both iron and copper metabolism in plants.

Keywords: ferric reductase oxidase, metalloreductase, iron, copper, plant

INTRODUCTION

Iron (Fe) is essential for plants and is required for the function of a large number of enzymes involved in photosynthesis, respiration and a number of other processes. Iron's utility in myriad biochemical processes stems from its ability to readily accept and donate electrons. It is most often associated with protein complexes either as a component of heme or Fe-S clusters. The ability of Fe to participate in electron transfer reactions is nevertheless problematic as well, since Fe^{3+} and Fe^{2+} are able to participate in the generation of the highly reactive hydroxyl radical (Halliwell and Gutteridge, 1992). As a result, it is critical that cells carefully control cellular Fe metabolism.

Iron limits plant growth in many soil types despite the fact that it is usually quite abundant. This is due to the fact that ferric iron is very poorly soluble in aerobic soils at neutral to basic pH. In the presence of oxygen, iron precipitates into insoluble Fe(III)-oxyhydroxide complexes. Thus, the molecular mechanisms utilized by plants for iron acquisition often include a first step that solubilizes ferric iron followed by a second step in which iron is transported from the soil and into root cells. Plants have evolved two types of strategies to combat iron deficiency. Strategy I is a reduction-based method used by all dicots and non-grass monocots while strategy II is used by grass species and involves chelation of ferric iron followed by uptake (Guerinot and Yi, 1994).

In response to iron deficiency, strategy I plants engage in a three stage process to acquire iron. First, the surrounding rhizosphere is acidified via proton extrusion by a root plasma membrane-localized proton ATPase, AHA2 (*Arabidopsis* H^+ATPase 2; Santi and Schmidt, 2009). This serves to increase solubility of ferric iron complexes. Ferric iron chelates are then reduced to ferrous iron by FRO2 (ferric reductase oxidase 2) and Fe^{2+} ions are subsequently taken up into root cells by the divalent metal transporter, IRT1 (iron regulated transporter 1; Eide et al., 1996; Yi and Guerinot, 1996; Robinson et al., 1999; Vert et al., 2002). In contrast, strategy II plants secrete phytosiderophores (PSs), such as mugineic acid, which bind to ferric iron with high affinity (Walker and Connolly, 2008). The resulting Fe(III)-PS complexes are transported across the root plasma membrane via the yellow stripe1 (YS1) iron transporter (Curie et al., 2001).

In this review, we focus on the roles of the FRO family of metalloreductases in reduction of iron and copper in plants. To this end, we briefly review what is known about reduction of iron at the root surface and highlight new work that has demonstrated a role for FRO family members in reduction of copper for uptake by plants. In addition, we focus on the emerging roles of FROs in trafficking of iron to subcellular compartments.

THE FRO FAMILY OF METALLOREDUCTASES

The reduction of ferric iron to ferrous iron at the root surface is a process that has been well documented and characterized across several plant species including *Arabidopsis* (Yi and Guerinot, 1996), pea (Waters et al., 2002), and tomato (Li et al., 2004), as well as the green alga *Chlamydomonas reinhardtii* (Eckhardt and Buckhout,

1998). The first plant metalloreductase gene was cloned from *Arabidopsis* (Robinson et al., 1999). FRO2 was identified based on its sequence similarity to the yeast ferric reductase, FRE1, as well as to a subunit of the human NADPH oxidase, gp91phox, which is involved in the production of reactive oxygen species to protect against invading pathogens (Robinson et al., 1999; Vignais, 2002). FRO2 was shown to complement the phenotype of an *Arabidopsis* ferric reductase defective-1 mutant (*frd1*), thus proving that *FRO2* encodes the root surface ferric chelate reductase. As expected for an enzyme involved in iron acquisition from the soil, *FRO2* is expressed in the root epidermis and is strongly induced by iron limitation (Connolly et al., 2003). Constitutive high-level expression of *FRO2* in soybean confers enhanced tolerance to iron deficiency-induced chlorosis (Vasconcelos et al., 2006).

FRO2 belongs to a superfamily of flavocytochromes and is involved in transfer of electrons from the cytosol across the plasma membrane to reduce extracellular ferric iron to ferrous iron. Studies of the topology of FRO2 show that the protein contains eight transmembrane (TM) helices, four of which comprise the highly conserved core of the protein (Schagerlof et al., 2006). This core is conserved throughout the flavocytochrome b family. The large water-soluble domain of FRO2, which contains NADPH, flavin adenine dinucleotide (FAD), and oxidoreductase sequence motifs, is located in the cytosol. FRO2 also contains four highly conserved histidine residues that likely coordinate two intramembranous heme groups that are instrumental in the electron transfer process (Robinson et al., 1999; Schagerlof et al., 2006). Although FRO2 appears to be solely responsible for reduction of ferric iron chelates in the rhizosphere, the *Arabidopsis* genome encodes a total of eight FRO family members. The seven additional FRO proteins are believed to function as metalloreductases primarily involved in the reduction of iron and possibly copper; here, we highlight new insight into the roles of FRO family members in copper reduction and intracellular metal trafficking.

PLASMA MEMBRANE-LOCALIZED ROOT COPPER REDUCTASES

Studies of the yeast FRE family have uncovered roles for these proteins in reduction of both iron and copper (Hassett and Kosman, 1995; Georgatsou et al., 1997; Martins et al., 1998). Consistent with their roles in the high-affinity iron and copper uptake systems, their expression is regulated by both iron and copper status. Like their FRE counterparts, *Arabidopsis FRO* genes are differentially regulated by deficiencies of iron and/or copper (Mukherjee et al., 2006). Studies of FRO2 have suggested that it may have a role in the reduction of Cu^{2+} to Cu^{+} at the root surface, in addition to its role in iron reduction (Yi and Guerinot, 1996; Robinson et al., 1999). *Arabidopsis* plants show an increase in root copper reductase activity under iron limitation and *frd1* mutants fail to induce this activity in response to iron limitation (Robinson et al., 1999). However, copper concentrations are not reduced in *frd1* mutants, suggesting that reduction of copper by FRO2 is not physiologically relevant; this result opens up the possibility that other FROs function to reduce copper at the root surface. It is possible that copper uptake may proceed without prior reduction of Cu^{2+} to Cu^{+}, perhaps via a ZRT, IRT-like protein (ZIP)-type transporter. Interestingly, expression of *ZIP2* and *ZIP4* is upregulated under copper limitation (Wintz et al., 2003). However, stable isotope studies support a reduction-based pathway for copper uptake (Jouvin et al., 2012). Indeed, recent studies have shown that FRO4 and FRO5 act redundantly to reduce copper at the root surface (Bernal et al., 2012)

The SPL7 (SQUAMOSA promoter binding-like7) transcription factor functions as a master regulator of the copper deficiency response in *Arabidopsis* (Yamasaki et al., 2009). Recently, RNA-Seq revealed that *FRO4* and *FRO5* are strongly upregulated in roots under copper limitation. In addition, induction of *FRO4* and *FRO5* in roots under copper limitation depends on SPL7 (Bernal et al., 2012). *FRO4* and *FRO5* lie in tandem on chromosome 5 and share high sequence similarity at the amino acid level (Mukherjee et al., 2006). SPL7 has been shown to bind to a CuRE (Cu responsive element) in promoters of copper regulated genes (Yamasaki et al., 2004; Yamasaki et al., 2009) similar to its homolog in *C. reinhardtii*, CCR1 (COPPER RESPONSIVE REGULATOR1; Quinn and Merchant, 1995; Kropat et al., 2005; Sommer et al., 2010). *FRO4* and *FRO5* each contain GTAC motifs in their upstream promoter regions, suggesting that they may be direct targets of SPL7 (Bernal et al., 2012). *fro4*, *fro5*, and *fro4fro5* double mutant lines display significant decreases in copper deficiency-inducible copper reductase activity. In addition, use of a fluorescent dye [coppersensor-1 (CS1)] that binds Cu^{+} showed that uptake of Cu^{+} in the *fro4* and *fro5* single mutants was markedly lower than in wild type plants and *fro4fro5* double mutant plants show hardly any detectable Cu^{+}, demonstrating that FRO4 and FRO5 function redundantly as copper reductases in the high affinity copper uptake pathway (Bernal et al., 2012). In addition, although *spl7* plants lack expression of *FRO4* and *FRO5* and corresponding Cu-deficiency inducible root Cu reductase activity, *spl7* does display elevated *FRO2* transcript abundance and root ferric chelate reductase activity. These results clearly establish that FRO4 and FRO5 (rather than FRO2) are responsible for reduction of Cu at the root surface (Bernal et al., 2012). It remains unclear whether FRO4 and FRO5 are involved in Fe homeostasis, however, expression of *FRO5* is induced under iron deficiency (Wu et al., 2005; Mukherjee et al., 2006).

PUTATIVE PLASMA MEMBRANE-LOCALIZED LEAF FERRIC REDUCTASE

Following uptake from the soil, iron must be loaded into the xylem, where it is found as a ferric-citrate complex (Rellan-Alvarez et al., 2010). How iron is transported into leaf cells remains unknown, but it is thought that Fe(III)-chelates may need to be reduced prior to transport into leaf cells. *FRO6* is expressed at high levels in leaves (Mukherjee et al., 2006), and overexpression of *FRO6* in tobacco showed that *FRO6* can facilitate the reduction of iron in leaves (Li et al., 2011). *FRO6* expression is not affected by iron status (Mukherjee et al., 2006). Instead, analysis of *FRO6-GUS* lines has shown that *FRO6* expression is controlled in a light-dependent manner. Indeed, the *FRO6* promoter contains several light-responsive elements and etiolated *FRO6-GUS* seedlings exhibit no *FRO6* promoter activity (Feng et al., 2006). Together, these data suggest that FRO6 may function to reduce iron

in leaves when light is available, perhaps to enable the assembly of new photosynthetic complexes.

INTRACELLULAR METALLOREDUCTASES

Chloroplasts and mitochondria represent significant sinks for Fe. Indeed, the vast majority of Fe found within leaves is located within chloroplasts. Essential cofactors such as heme and Fe–S clusters are synthesized in chloroplasts and mitochondria. Despite this, the molecular mechanisms by which iron is trafficked to these two organelles are not well understood. Recent studies implicate FRO family members in iron delivery to chloroplasts and mitochondria. Intriguingly, although work in yeast has shown that metalloreductases are important in vacuolar metal homeostasis, to date there is no evidence to support an analogous role in plants.

CHLOROPLASTIC FERRIC REDUCTASE

Although the precise mechanisms involved in chloroplast iron acquisition are still somewhat murky (Landsberg, 1984; Terry and Abadia, 1986; Bughio et al., 1997; Shikanai et al., 2003), it seems likely that chloroplasts take up both Fe(II) and Fe(III) via multiple pathways as observed in modern day cyanobacteria. Free living cyanobacteria have been shown to acquire iron through Fe^{2+} iron transporters from a pool of Fe(III)-dicitrate complexes (Katoh et al., 2001) and it is thus clear that some species of cyanobacteria are able to use a reduction-based mechanism for iron uptake (Kranzler et al., 2014). Plant chloroplasts, which are thought to have originated from ancient cyanobacteria, appear to utilize a similar strategy for iron uptake as studies of *Arabidopsis* FRO7 demonstrate that chloroplasts employ a reduction-based strategy for iron acquisition. FRO7 localizes to chloroplasts and loss of FRO7 function results in a significant reduction in chloroplast surface ferric reductase activity. In addition, *fro7* chloroplasts show a ∼30% reduction in chloroplast Fe content. *fro7* grows poorly on medium lacking sucrose and shows reduced photosynthetic efficiency, consistent with the idea that FRO7 is critical for delivery of Fe for proper assembly of photosynthetic complexes. When sown on alkaline soil, *fro7* seeds germinate but the resulting seedlings are severely chlorotic and the plants fail to set seed unless supplemented with excess iron (Jeong et al., 2008). Recent work in sugar beet further supports the existence of a reduction-based mechanism for iron uptake by chloroplasts, as well (Solti et al., 2012).

A presumptive Fe transporter, PIC1, has been identified that localizes to the chloroplast envelope (Duy et al., 2007). Whether FRO7 and PIC1 work together in chloroplast iron uptake currently remains unknown and it is not yet clear whether PIC1 transports ferric or ferrous iron. Other proteins that are presumed to function in chloroplast Fe transport are MAR1 (a homolog of ferroportin 1 and 2), which may transport an iron chelator (Conte et al., 2009), MFL1/2 [which resemble mitoferrins but function in chloroplasts; (Tarantino et al., 2011) and NAP14 (Shimoni-Shor et al., 2010)]. In addition, a chloroplast-and mitochondria-localized NEET-type protein was recently identified which may be involved in Fe–S cluster transfer to apoproteins (Nechushtai et al., 2012).

PUTATIVE MITOCHONDRIAL FERRIC REDUCTASES

Studies in *Arabidopsis* have identified a putative iron-chaperone (Busi et al., 2006; Vazzola et al., 2007) and putative mitochondrial effluxer proteins involved in iron metabolism (Kushnir et al., 2001; Chen et al., 2007). In addition, a recent report described the identification of a mitochondrial Fe transporter in rice (MIT1) which is essential for plant growth (Bashir et al., 2011). Despite this, we are far from a comprehensive understanding of mitochondrial Fe homeostasis (Nouet et al., 2011; Vigani et al., 2013). Although two *Arabidopsis* metalloreductases (FRO3 and FRO8) have been predicted to localize to mitochondrial membranes, neither one has been functionally characterized. A mitochondrial proteomics study has placed FRO8 at the mitochondrial membrane (Heazlewood et al., 2004). The expression patterns of *FRO3* and *FRO8* are largely non-overlapping, suggesting that they do not function redundantly (Jain and Connolly, 2013). Little information is available for *FRO8* but its expression is concentrated in the vasculature of senescing leaves (Wu et al., 2005). *FRO3* is expressed most highly in the vasculature of young seedlings and its expression is strongly induced under iron deficiency; for this reason, *FRO3* has been widely used as an iron deficiency marker (Mukherjee et al., 2006; Tarantino et al., 2010). Interestingly, *FRO3* expression is negatively regulated by the basic helix loop helix (bHLH) transcription factor PYE (POPEYE); PYE appears to control a pericycle-specific Fe deficiency response in roots (Dinneny et al., 2008; Long et al., 2010). *FRO3* expression also is responsive to copper status (Mukherjee et al., 2006; Yamasaki et al., 2009). Despite this, the roles of FRO3 and FRO8 remain unclear. It is interesting to note that although the yeast metalloreductase FRE5 localizes to mitochondria (Sickmann et al., 2003), there are no reports to date that demonstrate a role for a metalloreductase in mitochondria in any organism.

VACUOLAR IRON TRAFFICKING

Acidic compartments like vacuoles have a relatively oxidizing atmosphere as compared to the cytosol. In yeast, iron in vacuoles is largely present as ferric polyphosphate complexes (Raguzzi et al., 1988). The remobilization of iron from the yeast vacuolar compartment is mediated by the FRE6 ferric chelate reductase (Singh et al., 2007). FRE6 also plays a role in copper remobilization from vacuoles; reduced copper is subsequently exported to the cytosol via CTR2 (copper transporter 2; Rees and Thiele, 2007). Vacuolar iron transporters have been reported in plants; *Arabidopsis* vacuolar iron transporter (VIT1), transports iron into the organelle while NRAMP3 (natural resistance against microbial pathogens3) and NRAMP4 mediate the export of iron (Lanquar et al., 2005; Kim et al., 2006). However, no vacuolar metalloreductases have been reported in plants, to date.

CONCLUSION

Plants require iron and copper for vital processes such as photosynthesis, respiration, and nitrogen fixation. While it has been known for some time that ferric chelate reductases play a vital role in iron uptake from the soil by all plant species except for grasses, other roles for FROs in metal homeostasis have only recently emerged. Indeed, new studies have shown that FROs are important for copper acquisition from the soil (**Figure 1**) and for intracellular

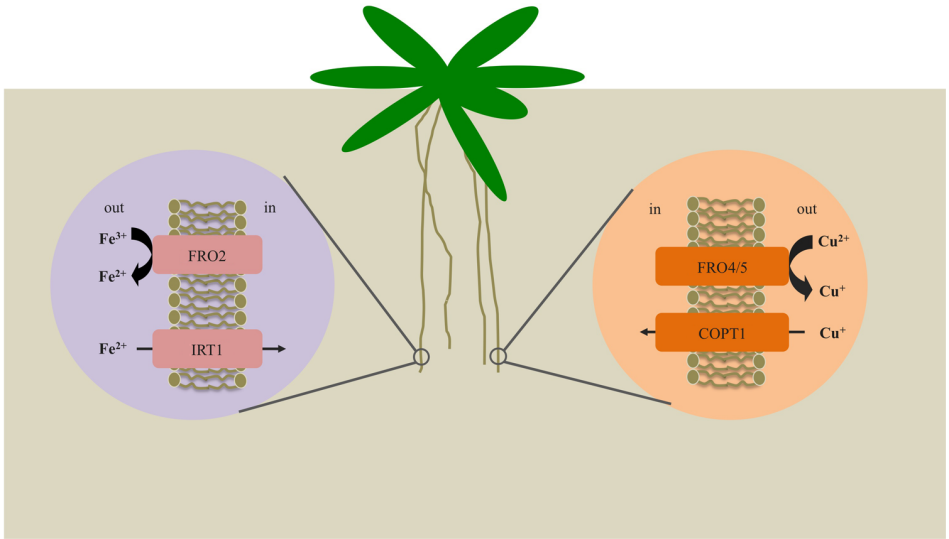

FIGURE 1 | Mechanisms for iron and copper uptake by *Arabidopsis* roots. Under iron-deficient conditions, expression of *FRO2* and *IRT1* is enhanced. FRO2 serves to reduce solubilized Fe^{3+} to Fe^{2+}, which is then transported across the root plasma membrane via IRT1. Under copper-deficient conditions, FRO4 and FRO5 are highly expressed in the roots and function to reduce Cu^{2+} to Cu^{+} prior to uptake by COPT1.

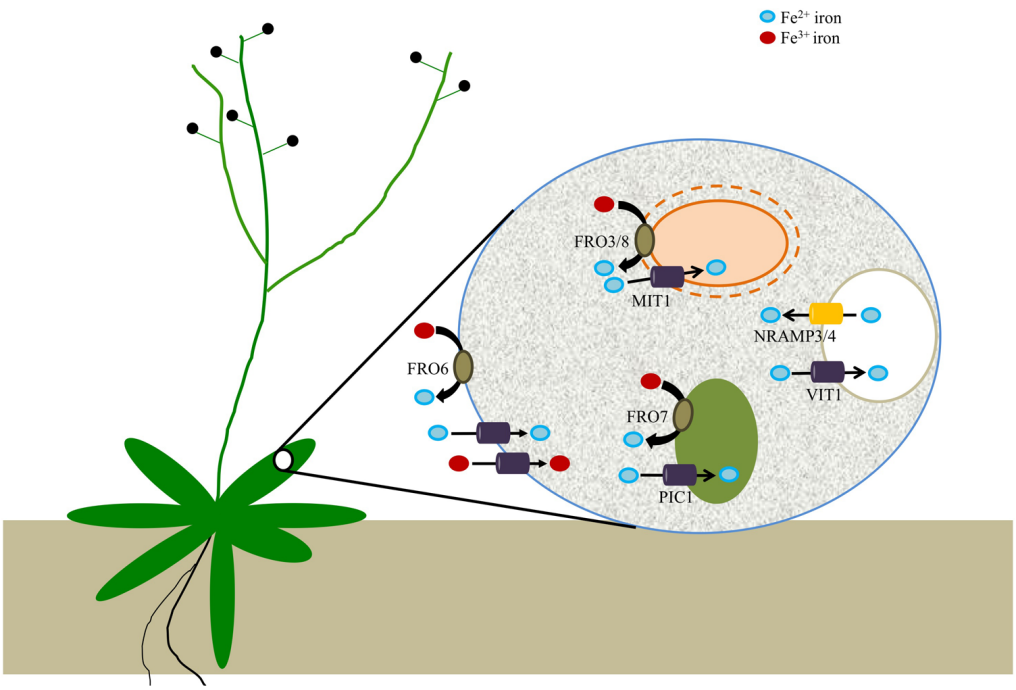

FIGURE 2 | *Arabidopsis* ferric reductases and transporters that contribute to cellular iron homeostasis. Evidence suggests that FRO6 functions to reduce Fe^{3+} to Fe^{2+} at the cell surface of leaf cells; Fe^{2+} is subsequently transported across the membrane via an unknown transporter(s), while other unknown transporters may be involved in the uptake of Fe^{3+}. Iron is then trafficked to a set of intracellular organelles. Chloroplasts utilize a reduction-based mechanism for iron acquisition via FRO7, whereas FRO3 and FRO8 may serve an analogous function in mitochondria. PIC1 serves as a chloroplast iron transporter while rice MIT mediates iron uptake by mitochondria. Although there is not yet any evidence for vacuolar metalloreductases in plants, it is known that VIT1 is important for iron uptake by vacuoles while *NRAMP3/4* function in vacuolar Fe efflux.

distribution of Fe (**Figure 2**). Together, these studies have shed considerable light on the molecular mechanisms employed by plants to maintain Fe and Cu homeostasis. In addition, this new knowledge should facilitate novel strategies aimed at improving crop yields on nutrient-poor soils and biofortification of plant foods to help ameliorate nutrient deficiencies in humans. Future studies will likely focus on the precise roles of mitochondrial FROs in mitochondrial metal metabolism. Furthermore, our understanding of iron trafficking within cells is severely hampered by our limited understanding of the various subcellular iron pools. New tools that provide insight into the redox status and types of iron species found in each of the various cellular compartments will go a long way toward the development of a comprehensive understanding of iron metabolism in plants.

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REFERENCES

Bashir, K., Ishimaru, Y., Shimo, H., Nagasaka, S., Fujimoto, M., Takanashi, H., et al. (2011). The rice mitochondrial iron transporter is essential for plant growth. *Nat. Commun.* 2, 1–7. doi: 10.1038/ncomms1326

Bernal, M., Casero, D., Singh, V., Wilson, G. T., Grande, A., Yang, H., et al. (2012). Transcriptome sequencing identifies SPL7-regulated Cu acquisition genes FRO4/FRO5 and the Cu dependence of Fe homeostasis in *Arabidopsis*. *Plant Cell* 24, 738–761. doi: 10.1105/tpc.111.090431

Bughio, N., Takahashi, M., Yoshimura, E., Nishizawa, N. K., and Mori, S. (1997). Light-dependent iron transport into isolated barley chloroplasts. *Plant Cell Physiol.* 38, 101–105. doi: 10.1093/oxfordjournals.pcp.a029079

Busi, M. V., Maliandi, M. V., Valdez, H., Clemente, M., Zabaleta, E. J., Araya, A., et al. (2006). Deficiency of *Arabidopsis thaliana* frataxin alters activity of mitochondrial Fe-S proteins and induces oxidative stress. *Plant J.* 48, 873–882. doi: 10.1111/j.1365-313X.2006.02923.x

Chen, S., Sanchez-Fernandez, R., Lyver, E. R., Dancis, A., and Rea, P. A. (2007). Functional characterization of AtATM1, AtATM2 and AtATM3, a subfamily of *Arabidopsis* half-molecule ABC transporters implicated in iron homeostasis. *J. Biol. Chem.* 282, 21561–21571. doi: 10.1074/jbc.M702383200

Connolly, E. L., Campbell, N., Grotz, N., Prichard, C. L., and Guerinot, M. L. (2003). Overexpression of the FRO2 iron reductase confers tolerance to growth on low iron and uncovers post-transcriptional control. *Plant Physiol.* 133, 1102–1110. doi: 10.1104/pp.103.025122

Conte, S., Stevenson, D., Furner, I., and Lloyd, A. (2009). Multiple antibiotic resistance in *Arabidopsis* is conferred by mutations in a chloroplast-localized transport protein. *Plant Physiol.* 151, 559–573. doi: 10.1104/pp.109.143487

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S. L., Briat, J. F., and Walker, E. L. (2001). Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409, 346–349. doi: 10.1038/35053080

Dinneny, J. R., Long, T. A., Wang, J. Y., Jung, J. W., Mace, D., Pointer, S., et al. (2008). Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. *Science* 320, 942–945. doi: 10.1126/science.1153795

Duy, D., Wanner, G., Meda, A. R., Von Wiren, N., Soll, J., and Philippar, K. (2007). PIC1, an ancient permease in *Arabidopsis* chloroplasts, mediates iron transport. *Plant Cell* 19, 986–1006. doi: 10.1105/tpc.106.047407

Eckhardt, U., and Buckhout, T. J. (1998). Iron assimilation in *Chlamydomonas reinhardtii* involves ferric reduction and is similar to strategy I higher plants. *J. Exp. Bot.* 9, 1219–1226.

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5624–5628. doi: 10.1073/pnas.93.11.5624

Feng, H., An, F., Zhang, S., Ji, Z., Ling, H. Q., and Zuo, J. (2006). Light-regulated, tissue-specific, and cell differentiation-specific expression of the *Arabidopsis* Fe(III)-chelate reductase gene AtFRO6. *Plant Physiol.* 140, 1345–1354. doi: 10.1104/pp.105.074138I

Georgatsou, E., Mavrogiannis, L. A., Fragiadakis, G. S., and Alexandraki, D. (1997). The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J. Biol. Chem.* 272, 13786–13792. doi: 10.1074/jbc.272.21.13786

Guerinot, M. L., and Yi, Y. (1994). Iron: nutritious, noxious, and not readily available. *Plant Physiol.* 104, 815–820. doi:10.1104/pp.104.3.815

Halliwell, B., and Gutteridge, J. M. C. (1992). Biologically relevant metal ion-dependent hydroxyl radical generation. *FEBS Lett.* 307, 108–112. doi: 10.1016/0014-5793(92)80911-Y

Hassett, R., and Kosman, D. J. (1995). Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J. Biol. Chem.* 270, 128–134. doi: 10.1074/jbc.270.1.128

Heazlewood, J. L., Tonti-Filippini, J. S., Gout, A. M., Day, D. A., Whelan, J., and Millar, A. H. (2004). Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. *Plant Cell* 16, 241–256. doi: 10.1105/tpc.016055

Jain, A., and Connolly, E. L. (2013). Mitochondrial iron transport and homeostasis in plants. *Front. Plant Sci.* 4:348. doi: 10.3389/fpls.2013.00348

Jeong, J., Cohu, C., Kerkeb, L., Pilon, M., Connolly, E. L., and Guerinot, M. L. (2008). Chloroplast Fe(III) chelate reductase activity is essential for seedling viability under iron limiting conditions. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10619–10624. doi: 10.1073/pnas.0708367105

Jouvin, D., Weiss, D. J., Mason, T. F., Bravin, M. N., Louvat, P., Zhao, F., et al. (2012). Stable isotopes of Cu and Zn in higher plants: evidence for Cu reduction at the root surface and two conceptual models for isotopic fractionation processes. *Environ. Sci. Technol.* 46, 2652–2660. doi: 10.1021/es202587m

Katoh, H., Hagino, N., Grossman, A. R., and Ogawa, T. (2001). Genes essential to iron transport in the cyanobacterium *Synechocystis* sp. *strain PCC* 6803. *J. Bacteriol.* 183, 2779–2784. doi: 10.1128/JB.183.9.2779-2784.2001

Kim, S. A., Punshon, T., Lanzirotti, A., Li, L., Alonso, J. M., Ecker, J. R., et al. (2006). Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science* 314, 1295–1298. doi: 10.1126/science.1132563

Kranzler, C., Lis, H., Finkel, O. M., Schmetterer, G., Shaked, Y., and Keren, N. (2014). Coordinated transporter activity shapes high-affinity iron acquisition in cyanobacteria. *ISME J.* 8, 409–417. doi: 10.1038/ismej.2013.161

Kropat, J., Tottey, S., Birkenbihl, R. P., Depege, N., Huijser, P., and Merchant, S. (2005). A regulator of nutritional copper signaling in Chlamydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18730–18735. doi: 10.1073/pnas.0507693102

Kushnir, S., Babiychuk, E., Storozhenko, S., Davey, M. W., Papenbrock, J., De Rucke, R., et al. (2001). A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the *Arabidopsis* mutant starik. *Plant Cell* 13, 89–100.

Landsberg, E. (1984). Regulation of iron-stress-response by whole-plant activity. *J. Plant Nutr.* 7, 609–621. doi: 10.1080/01904168409363226

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., et al. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24, 4041–4051. doi: 10.1038/sj.emboj.7600864

Li, L., Cheng, X., and Ling, H. Q. (2004). Isolation and characterization of Fe(III)-chelate reductase gene LeFRO1 in tomato. *Plant Mol. Biol.* 54, 125–136. doi: 10.1023/B:PLAN.0000028774.82782.16

Li, L. Y., Cai, Q. Y., Yu, D. S., and Guo, C. H. (2011). Overexpression of AtFRO6 in transgenic tobacco enhances ferric chelate reductase activity in leaves and increases tolerance to iron-deficiency chlorosis. *Mol. Biol. Rep.* 38, 3605–3613. doi: 10.1007/s11033-010-0472-9I

Long, T. A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D. E., and Benfey, P. N. (2010). The bHLH transcription factor POPEYE regulates response to iron deficiency in *Arabidopsis* roots. *Plant Cell* 22, 2219–2236. doi: 10.1105/tpc.110.074096

Martins, L. J., Jensen, L. T., Simon, J.R., Keller, G. L., and Winge, D. R. (1998). Metalloregulation of FRE1 and FRE2 homologs in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 23716–23721. doi: 10.1074/jbc.273.37.23716

Mukherjee, I., Campbell, N. H., Ash, J. S., and Connolly, E. L. (2006). Expression profiling of the *Arabidopsis* ferric chelate reductase (*FRO*) gene family reveals differential regulation by iron and copper. *Planta* 223, 1178–1190. doi: 10.1007/s00425-005-0165-0

Nechushtai, R., Conlan, A. R., Harir, Y., Song, L., Yogev, O., Eisenberg-Domovich, Y., et al. (2012). Characterization of *Arabidopsis* NEET reveals an ancient role for NEET proteins in iron metabolism. *Plant Cell* 24, 2139–2154. doi: 10.1105/tpc.112.097634

Nouet, C., Motte, P., and Hanikenne, M. (2011). Chloroplastic and mitochondrial metal homeostasis. *Trends Plant Sci.* 16, 395–404. doi: 10.1016/j.tplants.2011.03.005

Quinn, J. M., and Merchant, S. (1995). Two copper-responsive elements associated with the Chlamydomonas Cyc6 gene function as targets for transcriptional activators. *Plant Cell* 7, 623–628. doi: 10.1105/tpc.7.5.623

Raguzzi, F., Lesuisse, E., and Crichton, R. R. (1988). Iron storage in *Saccharomyces cerevisiae*. *FEBS Lett.* 231, 253–258. doi: 10.1016/0014-5793(88)80742-7

Rees, E. M., and Thiele, D. J. (2007). Identification of a vacuole-associated metalloreductase and its role in Ctr2-mediated intracellular copper mobilization. *J. Biol. Chem.* 282, 21629–21638. doi: 10.1074/jbc.M703397200

Rellan-Alvarez, R., Giner-Martinez-Sierra, J., Orduna, J., Orera, I., Rodriguez-Castrillon, J. A., Garcia-Alonso, J. I., et al. (2010). Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol.* 51, 91–102. doi: 10.1093/pcp/pcp170

Robinson, N. J., Procter, C. M., Connolly, E. L., and Guerinot, M. L. (1999). A ferric-chelate reductase for iron uptake from soils. *Nature* 397, 694–697. doi: 10.1038/17800

Santi, S., and Schmidt, W. (2009). Dissecting iron deficiency-induced proton extrusion in *Arabidopsis* roots. *New Phytol.* 183, 1072–1084. doi: 10.1111/j.1469-8137.2009.02908.x

Schagerlof, U., Wilson, G., Hebert, H., Al-Karadaghi, S., and Hagerhall, C. (2006). Transmembrane topology of FRO2, a ferric chelate reductase from *Arabidopsis thaliana*. *Plant Mol. Biol.* 62, 215–221. doi: 10.1007/s11103-006-9015-0

Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K. K., and Pilon, M. (2003). PAA1, a P-type ATPase of *Arabidopsis*, functions in copper transport in chloroplasts. *Plant Cell* 15, 1333–1346. doi: 10.1105/tpc.011817

Shimoni-Shor, E., Hassidim, M., Yuval-Naeh, N., and Keren, N. (2010). Disruption of Nap14, a plastid-localized non-intrinsic ABC protein in *Arabidopsis thaliana* results in the over-accumulation of transition metals and in aberrant chloroplast structures. *Plant Cell Environ.* 33, 1029–1038. doi: 10.1111/j.1365-3040.2010.02124.x

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H. E., et al. (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13207–13212. doi: 10.1073/pnas.2135385100

Singh, A., Kaur, N., and Kosman, D. J. (2007). The metalloreductase Fre6p in Fe-efflux from the yeast vacuole. *J. Biol. Chem.* 282, 28619–28626. doi: 10.1074/jbc.M703398200

Solti, Á., Kovács, K., Basa, B., Vértes, A., Sárvári, É., and Fodor, F. (2012). Uptake and incorporation of iron in sugar beet chloroplasts. *Plant Physiol. Biochem.* 52, 91–97. doi: 10.1016/j.plaphy.2011.11.010

Sommer, F., Kropat, J., Malasarn, D., Grossoehme, N. E., Chen, X., Giedroc, D. P., et al. (2010). The CRR1 nutritional copper sensor in Chlamydomonas contains two distinct metal-responsive domains. *Plant Cell* 22, 4098–4113. doi: 10.1105/tpc.110.080069

Tarantino, D., Morandini, P., Ramirez, L., Soave, C., and Murgia, I. (2011). Identification of an *Arabidopsis* mitoferrinlike carrier protein involved in Fe metabolism. *Plant Physiol. Biochem.* 49, 520–529. doi: 10.1016/j.plaphy.2011.02.003

Tarantino, D., Santo, N., Morandini, P., Casagrande, F., Braun, H. P., Heinemeyer, J., et al. (2010). AtFer4 ferritin is a determinant of iron homeostasis in *Arabidopsis thaliana* heterotrophic cells. *J. Plant Physiol.* 167, 1598–1605. doi: 10.1016/j.jplph.2010.06.020

Terry, N., and Abadia, J. (1986). Function of iron in chloroplasts. *J. Plant Nutr.* 9, 609–646. doi: 10.1080/01904168609363470

Vasconcelos, M., Eckert, H., Arahana, V., Graef, G., Grusak, M. A., and Clemente, T. (2006). Molecular and phenotypic characterization of transgenic soybean expressing the *Arabidopsis* ferric chelate reductase gene, FRO2. *Planta* 224, 1116–1128. doi: 10.1007/s00425-006-0293-1

Vazzola, V., Losa, A., Soave, C., and Murgia, I. (2007). Knockout of frataxin gene causes embryo lethality in *Arabidopsis*. *FEBS Lett.* 581, 667–672. doi: 10.1016/j.febslet.2007.01.030

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., et al. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

Vigani, G., Zocchi, G., Bashir, K., Philippar, K., and Briat, J. F. (2013). Signals from chloroplasts and mitochondria for iron homeostasis regulation. *Trends Plant Sci.* 18, 305–311. doi: 10.1016/j.tplants.2013.01.006

Vignais, P. V. (2002). The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol. Life Sci.* 59, 1428–1459. doi: 10.1007/s00018-002-8520-9

Walker, E. L., and Connolly, E. L. (2008). Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. *Curr. Opin. Plant Biol.* 11, 530–535.

Waters, B. M., Blevins, D. G., and Eide, D. J. (2002). Characterization of FRO1, a pea ferric-chelate reductase involved in root iron acquisition. *Plant Physiol.* 129, 85–94. doi: 10.1104/pp.010829

Wintz, H., Fox, T., Wu, Y. Y., Feng, V., Chen, W., Chang, H. S., et al. (2003). Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *J. Biol. Chem.* 278, 47644–47653. doi: 10.1074/jbc.M309338200

Wu, H., Li, L., Du, J., Yuan, Y., Cheng, X., and Ling, H. Q. (2005). Molecular and biochemical characterization of the Fe(III) chelate reductase gene family in *Arabidopsis thaliana*. *Plant Cell Physiol.* 46, 1505–1514. doi: 10.1093/pcp/pci163

Yamasaki, H., Hayashi, M., Fukazawa, M., Kobayashi, Y., and Shikanai, T. (2009). SQUAMOSA promoter binding protein-like7 is a central regulator for copper homeostasis in *Arabidopsis*. *Plant Cell* 21, 347–361. doi: 10.1105/tpc.108.060137

Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., et al. (2004). A novel zinc-binding motif revealed by solution structures of DNA-binding domains of *Arabidopsis* SBP-family transcription factors. *J. Mol. Biol.* 337, 49–63. doi: 10.1016/j.jmb.2004.01.015

Yi, Y., and Guerinot, M. L. (1996). Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. *Plant J.* 10, 835–844. doi: 10.1046/j.1365-313X.1996.10050835.x

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Metal species involved in long distance metal transport in plants

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The mechanisms plants use to transport metals from roots to shoots are not completely understood. It has long been proposed that organic molecules participate in metal translocation within the plant. However, until recently the identity of the complexes involved in the long-distance transport of metals could only be inferred by using indirect methods, such as analyzing separately the concentrations of metals and putative ligands and then using *in silico* chemical speciation software to predict metal species. Molecular biology approaches also have provided a breadth of information about putative metal ligands and metal complexes occurring in plant fluids. The new advances in analytical techniques based on mass spectrometry and the increased use of synchrotron X-ray spectroscopy have allowed for the identification of some metal-ligand species in plant fluids such as the xylem and phloem saps. Also, some proteins present in plant fluids can bind metals and a few studies have explored this possibility. This study reviews the analytical challenges researchers have to face to understand long-distance metal transport in plants as well as the recent advances in the identification of the ligand and metal-ligand complexes in plant fluids.

Keywords: metals, metal complexes, phloem, transport, xylem

INTRODUCTION

To reach their final destination within the plant (e.g., organelles such as chloroplast or mitochondria) micronutrients taken up from the growth medium, including metals such as Fe, Mn, Zn, and Cu, must follow a complex path through a number of different plant compartments and membrane systems (Clemens et al., 2002; Colangelo and Guerinot, 2006; Briat et al., 2007; Haydon and Cobbett, 2007; Curie et al., 2009; Puig and Peñarrubia, 2009; Conte and Walker, 2011; Sinclair and Krämer, 2012). The vascular system, including the xylem and phloem conduits, is an essential segment for long distance translocation of micronutrients within this path. It has long been proposed that a significant fraction of metals would be present in plant fluids not as free ions but in less reactive chemical forms, e.g., non-covalently bound to organic compounds, to prevent uncontrolled binding and also because free metals often exert some degree of toxicity. The formation of metal complexes provides both solubility and shielding during long-distance transport, since the metallic atom is enveloped by an array of bound molecules or anions (the so-called ligands; in this review only ligands consisting in organic molecules are considered), which donate one or more electron pairs to the metal to form the complexes.

Indirect evidence for long distance, organic ligand-assisted transport of metals has been extensively reported. Possible ligand candidates are a range of small molecules, including organic acids -carboxylates- such as citrate (Cit) and malate (Mal), amino acids [including nicotianamine (NA), histidine (His), cysteine (Cys) and high-affinity Fe(III) chelating compounds derived from NA called phytosiderophores (PSs), such as mugineic (MA) and 2′-deoxymugineic (DMA) acids], as well as peptides and proteins (e.g., metallothioneins). In the case of toxic metals such as Cd, Hg, and others, plants also respond by synthesizing compounds such as the poly-glutathione peptides phytochelatins (PCs) that decrease the amount of free metal ions in plant fluids. Recent papers have reviewed the roles of NA (Curie et al., 2009; Clemens et al., 2013) and PCs (Pal and Rai, 2010) in plant metal homeostasis, the intra- and extracellular excretion of carboxylates (Meyer et al., 2010) and the plant metallo-chaperones (Tehseen et al., 2010). During the last decade, the identification of many genes involved in plant long-distance metal transport has also been achieved, and many reviews have covered this issue in relation to either several metals (Colangelo and Guerinot, 2006; Haydon and Cobbett, 2007; Krämer et al., 2007; Curie et al., 2009; Palmer and Guerinot, 2009; Puig and Peñarrubia, 2009; Krämer, 2010; Waters and Sankaran, 2011) or to specific ones such as Fe (Briat et al., 2007; Kim and Guerinot, 2007; Morrissey and Guerinot, 2009; Conte and Walker, 2011; Sperotto et al., 2012; Thomine and Vert, 2013), Zn (Sinclair and Krämer, 2012), Cu (Yruela, 2009), Mn (Pittman, 2005), and Cd (Mendoza-Cózatl et al., 2011).

To date, analytical data on the actual metal complexes existing in plant fluids are still scarce. This is relevant, because the chemical forms in which a metal occurs in solution (metal speciation) affect solubility, precipitation, acid/base equilibria, electron-transfer reactions, diffusivity, ability to undergo photolysis and others. The metal species existing in any given compartment determine biological activity, including the capability to be a substrate for membrane transport proteins involved in loading and unloading to xylem and phloem, as well as the possibility

that metal toxicity can occur. This review summarizes the current knowledge on metal species occurring in plant fluids [xylem sap, phloem sap and other fluids such as apoplastic fluid and embryo sac liquid (ESL)], and discusses general problems relevant to these studies as well as the methodological approaches currently used.

METHODS FOR PLANT FLUID SAMPLING AND INHERENT PROBLEMS

Metal translocation within plants is dynamic, with the composition of plant fluids often changing with time. This mandates to establish an adequate standard sampling protocol, including sampling time and others, which must be applied to all samples so that results are fully comparable. A major limitation in the analysis of plant fluids is the need for samples of adequate purity and in sufficient volume to carry out measurements. The purity of the fluid samples must always be assessed by measuring cytosolic and/or vacuolar components associated to cell rupture. The activities of cytosolic marker enzymes, such as Mal dehydrogenase (*mdh*) or others (López-Millán et al., 2000), or the concentrations of K as a vacuolar marker (Lohaus et al., 2001; Barabasz et al., 2012) are generally used with that purpose.

Xylem sap, the fluid contained in xylem vessels -composed of tracheary elements separated by rather large perforation plates-, is relatively easy to sample (reviewed by Alexou and Peuke, 2013). The most frequently used technique is de-topping plants and letting xylem to bleed after discarding the first drops (López-Millán et al., 2012). Other techniques involve centrifugation of excised stems (López-Millán et al., 2000) or the use of a Schölander pressure chamber (Larbi et al., 2003). With any of these extraction techniques the xylem sap volume sampled could reach several hundreds of μL.

The phloem sap, the fluid contained in sieve cells—separated by sieve plates—, is a special case, because there is still controversy about changes in composition induced by wounding (Atkins et al., 2011) and the presence of different types of phloem saps (Zhang et al., 2012). The purity of the phloem sap is usually assessed by measuring sugar concentrations and/or evaluating the presence of Rubisco proteins or transcripts (Giavalisco et al., 2006; Rodríguez-Medina et al., 2011; Lattanzio et al., 2013). Other authors consider pH values around 8.0 indicative of phloem purity (Ando et al., 2013). Some methods in use for phloem sap sampling (reviewed by Dinant and Kehr, 2013) are those involving an incision near the inflorescence (in *Cucurbitaceae*, *Brassicaceae* and some *Lupinus* species; Lattanzio et al., 2013), those using aphid or leaf-hopper stylectomy (with the disadvantage of resulting in minute phloem sap volumes, $\leq$1 μL per cut stylet; Ando et al., 2013), or by exudation of excised shoots maintained at >80% relative humidity in a closed chamber (the latter being only a qualitative approach; Marentes and Grusak, 1998). When using insect stylectomy, evaporation during sampling is a relevant issue; accurate determination of the phloem volume has been recently achieved by measuring the droplet diameter as it forms on the tip of the severed aphid stylet (Palmer et al., 2013).

The fluid in the apoplastic space in the leaf (free space outside the plasma membrane) is an interface between the xylem and the symplast. Some methods for leaf apoplastic fluid sampling involve direct centrifugation of leaves without petiole (previously centrifuged at low speed to remove xylem sap from the mid vein; López-Millán et al., 2000) or by using a Schölander pressure chamber (Larbi et al., 2003). Other authors infiltrate the leaves with a solution under vacuum and then obtain by centrifugation a leaf apoplastic wash fluid (Lohaus et al., 2001). Since the latter procedure leads to a dilution of the apoplastic fluid, concentrations should be corrected considering estimations of apoplastic volumes occupied by water and air, using vacuum infiltration with a ^{14}C-sorbitol labeled solution and silicone oil, respectively (Lohaus et al., 2001).

Recently, the ESL has been successfully used to study the processes of metal transport in pea seeds (Grillet et al., 2014). This liquid is enriched by the bulk flow of nutrients delivered by the seed coat and feeds the embryo.

METHODOLOGICAL APPROACHES TO UNRAVEL METAL SPECIES OCCURRING IN PLANT FLUIDS

Until recently, researchers had to rely on indirect methods to putatively identify the chemical forms of metals occurring in plant fluids. These methods were based on separate measurements of the concentrations of metals and possible ligands and the prediction of the existent chemical species by means of titration in "artificial" saps (Liao et al., 2000; Irtelli et al., 2009; Alves et al., 2011) or *in silico* by using a set of known, experimentally determined stability constants of metal-containing complexes and chemical speciation software; this was always done assuming that chemical equilibrium was achieved (White et al., 1981; López-Millán et al., 2000; Callahan et al., 2006; Harris et al., 2012). However, chemical equilibrium is unexpected in plant fluids, since continuous changes in composition usually occur in these dynamic plant compartments.

Molecular biology approaches have provided key information on putative metal ligands and metal complexes that could play a crucial role in inter- and intra-organ metal transport (see reviews by Briat et al., 2007; Haydon and Cobbett, 2007; Kim and Guerinot, 2007; Milner and Kochian, 2008; Palmer and Guerinot, 2009; Pal and Rai, 2010; Waters and Sankaran, 2011; Leitenmaier and Küpper, 2013; Thomine and Vert, 2013). Genotypes with loss-of-function of genes involved in the trafficking of metals, ligands and/or their metal complexes, as well as metal hyperaccumulator (e.g., *T. caerulescens*) plant species have been studied. Other approaches include mutant and transgenic lines with impaired or enhanced metal ligand synthesis; a classical example of impaired synthesis is the tomato mutant "*chloronerva*," which lacks NA (Pich and Scholz, 1996). Transgenic genotypes have also been constructed where the synthesis of ligands such as PCs is restricted to a specific tissue (Gong et al., 2003).

Another approach is searching for molecules with affinity for metals in plant fluids, using their immobilization by metal affinity chromatography (IMAC) followed by identification of the isolated molecules using some of the mass spectrometry (MS) techniques described below (Lattanzio et al., 2013). In this approach, the occurrence of the corresponding metal-ligand complex in plant fluids is only inferred from the presence of suitable ligands.

A further approach to identify metal complexes in plant tissues -including fluids- relies on the identification of the chemical

environment surrounding the metal by means of synchrotron X-ray absorption spectroscopy (XAS) techniques such as X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure spectroscopy (EXAFS) (Lombi et al., 2011; Donner et al., 2012). This has been used for different metals such as Fe (Yoshimura et al., 2000; Terzano et al., 2013), Mn (Yun et al., 1998), Cu (Küpper et al., 2009; Song et al., 2013), Zn (Salt et al., 1999; Küpper et al., 2004; Trampczynska et al., 2010; Lu et al., 2013), Ni (McNear et al., 2010), Cd (Salt et al., 1995; Vogel-Mikus et al., 2010; Huguet et al., 2012; Yamaguchi et al., 2012), Hg (Carrasco-Gil et al., 2011, 2013; McNear et al., 2012), and Pb (Tian et al., 2010; Chu et al., 2012). This technique allows direct *in situ* metal speciation in plants, without any preliminary extraction or preparation (see reviews by Lombi et al., 2011; Donner et al., 2012; Sarret et al., 2013). However, spectra are difficult to interpret when more than two or three species are dominant, and these techniques are generally used to confirm the presence of known species rather than to find new ones (Monicou et al., 2009). Furthermore, the applications of these techniques have been usually restricted to tissues from metal hyper-accumulators, due to the relatively low sensitivity (metal concentration should be higher than $10\,\mu g\ g^{-1}$ dry weight). In spite of that, some data on metal speciation have been reported for sap and vasculature tissues, although mainly in metal hyper-accumulator species. This has been done for Zn in *T. caerulescens* (Salt et al., 1999; Küpper et al., 2004; Trampczynska et al., 2010) and *Sedum alfredii* (Lu et al., 2013), for Cd in *Brassica juncea* (Salt et al., 1995) and *Arabidopsis halleri* (Huguet et al., 2012), and for Ni in *Alyssum murale* (McNear et al., 2010). Recently, the use of highly brilliant synchrotrons as X-ray sources has increased one order of magnitude the sensitivity of EXAFS for trace elements (e.g., below $1\,\mu g\ g^{-1}$ dry weight for Fe), thus allowing speciation of Fe in tomato xylem sap (Terzano et al., 2013) and Cd around the vascular bundles in node I -the one beneath the panicle- in *Oryza sativa* (Yamaguchi et al., 2012). Unfortunately, artifacts can arise from high intensity radiation damage to the sample (i.e., degradation of Fe(III) complexes with carboxylates and NA, as reported in Terzano et al., 2013).

Recent advances in analytical techniques, and specifically in MS, have enabled a new insight on metal speciation. The use of highly selective and sensitive molecular and metal-specific MS techniques has allowed the identification and quantification of individual, well-defined metal species in plant tissues (including metabolites and proteins), even at sub-nM levels (Meija et al., 2006; Monicou et al., 2009). These approaches generally use hyphenated techniques based on the separation of compounds by high-resolution techniques [e.g., liquid chromatography (HPLC) and capillary electrophoresis (CE)] and the determination of the metals and/or metal complexes by MS techniques [including inductively coupled plasma MS (ICP-MS), electrospray MS (ESI-MS), and others]. These new methodologies (reviewed by Monicou et al., 2009; Khouzam et al., 2012) offer unique advantages for the *de novo* identification of metallo-metabolites occurring at low concentrations, in particular in plant materials such as plant fluids that do not require multi-step extraction from the plant tissues. Metabolite structures can be elucidated using the empirical formulas of the parent compound and fragment ions (data provided by high-resolution and high-accuracy MS) and the lineage of fragment ions observed in tandem MS or multistage MS (MSn). Furthermore, complexes with metals having more than one stable isotope, such as Fe, Zn, Cu, Cd, and Hg, provide metal-specific isotopic signatures that allow for identification of MS signals corresponding to metal containing molecules (see an example for metal-NA complexes in Rellán-Álvarez et al., 2008). In the five past years, methods based on these analytical techniques have achieved the direct identification of several metal complexes with possible relevance in long distance transport, including carboxylate complexes with Fe(III) and Ni(II), NA and PS complexes with Fe(III), Fe(II), Ni(II), Co(II), Mn(II), Cu(II) and Zn(II), and His complexes with Cu(II) (Weber et al., 2006; Xuan et al., 2006, 2007; Rellán-Álvarez et al., 2008, 2010; Dell'mour et al., 2010; Köster et al., 2011a,b; Tsednee et al., 2012). However, until now only a few of these metal-ligand complexes have been really found in plant fluids (see below).

Another MS technique, high-precision multi-collection-ICP-MS (developed 20 years ago), which yields high-precision measurements of stable isotopes of transition elements (e.g., Fe, Cu, and Zn), has allowed the study of their fractionation during plant uptake and translocation (reviewed by von Blanckenburg et al., 2009). This technique provides information on the mechanisms involved in metal partitioning within the plant, but not on metal speciation or specific binding environments. Recent studies on fractionation of Fe and Cu stable isotopes, using plants relying on Strategy I or II for Fe uptake, have revealed that both metals undergo redox cycling during root-to-shoot translocation in Fe-deficient Strategy I plant species, but not in Strategy II ones (Guelke-Stelling and von Blanckenburg, 2007, 2012; Kiczka et al., 2010; Ryan et al., 2013). A recent *in silico* study predicted that redox Fe changes affect Fe isotopic fractionation, with Δ^{56}Fe (^{56}Fe/^{54}Fe) being 3‰ heavier in Fe(III)-PS than in Fe(II)-NA (Moynier et al., 2013). Even in the absence of redox Fe changes, changes in speciation alone would create up to 1.5‰ differences in Δ^{56}Fe: Fe(III)-PS is up to 1.5‰ heavier than Fe(III)-Cit and Fe(II)-NA is up to 1‰ heavier than Fe(II)-Cit (Moynier et al., 2013). These estimations are in agreement with the fact that roots of Strategy-II plants, which rely on Fe(III)-PS uptake, are isotopically heavier (by about 1‰) than the shoots, where Fe had presumably been transported as Fe(III)-Cit in the xylem or Fe(II)-NA in the phloem. Isotopic variations observed between younger and older leaves could also be explained by the occurrence of Fe acquisition *via* xylem and phloem (Moynier et al., 2013). Zinc sequestration in roots is mediated by a number of mass dependent processes that favor the heavier isotopes, including binding to cell walls, precipitation in intercellular spaces, binding to high affinity ligands in the root cell and sequestration in the vacuole (Aucour et al., 2011; Caldelas et al., 2011). Translocation processes of Zn within the plant also lead to significant fractionation of Zn stable isotopes, since shoots of several plant species were enriched in light isotopes, with the tallest species showing the largest fractionation in the youngest leaves (Moynier et al., 2009). This effect can be modified by the Zn status in those Zn hyper-accumulators that accumulate Zn mainly in the roots (e.g., *A. halleri*), due to the large fractionation that occurs before shoot Zn's re-translocation.

METALS IN PLANT FLUIDS

The study of long distance metal transport has traditionally used differences in metal accumulation ratios (root to shoot metal content ratios) within the plant, considering different scenarios (i.e., growth conditions, genotypes, etc.). Only recently, high-throughput elemental analysis technologies have allowed the characterization of the full ionome in a large number of lines of several plant species (www.ionomicshub.org) (Baxter et al., 2008; Singh et al., 2013). This information, in combination with bioinformatics and genetic tools, has yielded candidate genes coding for transporters as well as gene networks involved in long distance metal transport.

Direct analyses of metals in plant fluids have been carried out in a number of studies, and their concentrations are generally in the μM range (**Table 1**). Therefore, the use of sensitive analytical techniques such as graphite furnace atomic absorption spectroscopy (GAAS) or inductively coupled plasma-mass spectrometry (ICP-MS) is mandatory for their determination. Prior to the analyses and immediately after the sampling of plant fluids, metals are usually stabilized in the solution by acidifying it. To avoid metal cross-contamination, high purity acids (ultra trace analysis quality grade) should be used for sample acidification, and also for sample digestion and cleaning of all materials (Husted et al., 2011; Ando et al., 2013). Concentrations found in the xylem sap, phloem sap and leaf apoplastic fluid of different plant species are in the ranges 4–168 μM for Fe, 0.5–245 μM for Zn, 0.3–30 μM for Cu, 4–400 μM for Mn and nd–0.1 μM for Cd and Ni (**Table 1**). These concentrations usually increase when either the metals are in excess in the plant growth medium or when plants (specially those genotypes known as metal stress tolerant) are treated with control metal concentrations after a period of metal deficiency. In these cases concentrations may reach 120–177 μM Fe in tomato xylem sap (Orera et al., 2010; Rellán-Álvarez et al., 2010, 2011a), 148 μM Zn in sugar beet xylem sap (Sagardoy, 2012), 43 μM Cu in *O. sativa* phloem sap (Ando et al., 2013), 2300 μM Mn in *O. sativa* leaf apoplastic fluid (Führs et al., 2010) and 1–100 μM Cd in *O. sativa*, *B. vulgaris*, and *S. lycopersicum* (Mori et al., 2009; Kato et al., 2010; Sagardoy, 2012). Higher concentrations of metals in plant fluids are also found in hyper-accumulator species: up to 450 μM Ni (Mari et al., 2006) and 524 μM Zn (Lasat et al., 1998) in *T. caerulense*, up to 1750 μM Ni in *Alyssum lesbiacum* (Kerkeb and Krämer, 2003) and up to 100 μM Cd and 486 μM Zn in *A. halleri* (Ueno et al., 2008) (**Table 1**). Altered levels of metals in plant fluids are also found in mutant genotypes as well as in transgenic plants overexpressing or losing the function of either one or several gene components of the uptake, sequestration or transport mechanisms (Nakamura et al., 2008; Ishimaru et al., 2011; Sasaki et al., 2011). The addition of protein synthesis inhibitors or ligands to the growth media has also a large effect on the metal concentrations in plant fluids. For instance, the addition of cycloheximide to the hyper-accumulator *A. lesbiacum* led to a 70% reduction of the xylem sap Ni concentration, whereas the addition of His to the non-accumulator *B. juncea* increased Ni in xylem sap (Kerkeb and Krämer, 2003).

Transport metal studies have also been approached using radioactive or stable isotopes. Radioactive metal isotopes supplied to plants can be detected by autoradiography, both at the whole plant and/or organ level (Cakmak et al., 2000a,b; Erenoglu et al., 2002). The application of ^{59}Fe-citrate to source tissues of pea plants showed that Fe is complexed prior to phloem loading (Grusak, 1994). The high remobilization of ^{109}Cd from the leaves and stem to the maturing grain was associated with high accumulation of Cd in durum wheat grain (Harris and Taylor, 2001). A more recent example is the use of ^{109}Cd autoradiography in *A. halleri* (a Zn and Cd hyper-accumulator species) showing that after 3 weeks an enrichment of the leaf petiole, central vein and trichomes occurs, whereas after 9 weeks leaf edges were the most Cd-enriched tissues and Cd concentrations were lower in regions along leaf vascular bundles (Huguet et al., 2012). A visualization of metals at the microscopic level can be obtained using thin sections. For instance, ^{109}Cd radioisotope imaging using 30-μm thickness sections has demonstrated Cd xylem-phloem transfer immediately after root uptake in *O. sativa* (Kobayashi et al., 2013).

The use of stable metal isotopes and highly selective and sensitive ICP-MS detection has also allowed tracing long-distance transport of metals within plants. Metal sources labeled with stable isotopes and applied to the roots or to leaves were used to evaluate the translocation within the plant of ^{67}Zn (Watmough et al., 1999; Benedicto et al., 2011), ^{57}Fe (Rodríguez-Castrillón et al., 2008; Rojas et al., 2008; Orera et al., 2010), ^{54}Fe (Orera et al., 2010), and ^{207}Pb (Watmough et al., 1999). Recently, the application of stable isotopes combined with laser ablation ICP-MS has allowed to localize and quantify the metal tracer together with other metals in plant tissue thin sections. This has been applied for the quantitative imaging of Cu and other essential elements (such as K, Mg, Mn, P, S, and B) in the leaves of the Cu-tolerant plant *Elsholtzia splendens* treated with ^{65}Cu (Wu et al., 2009). Whereas these are useful approaches for analyzing the spatial distribution or temporal changes of metals within the plant, only a snapshot of the distribution of metal at a given moment can be obtained. In contrast, the Positron-Emitting Tracer Imaging System technique, PETIS, allows for real-time, image quantification studies of the movement of elements in intact plants. The uptake and translocation of metals has been investigated in several graminaceous species using PET tracers ^{52}Fe, ^{52}Mn, ^{62}Zn, and ^{107}Cd (Tsukamoto et al., 2006, 2009; Suzuki et al., 2008; Fujimaki et al., 2010; Ishikawa et al., 2011). For instance, this tool demonstrated the direct translocation of Fe from roots to young leaves *via* phloem in *H. vulgare* (Tsukamoto et al., 2009).

LIGANDS IN PLANT FLUIDS

Organic ligands involved in xylem and phloem metal translocation have been revealed mainly by physiological, genetic, and molecular studies, with the exception of some recent studies performing metabolite profiling using a combination of powerful analytical techniques (e.g., the LC-MS, GC-MS, and NMR analyses of latex of the Ni hyper-accumulator tree *Sebertia acuminata* by Callahan et al., 2008). Metal transport is associated with the occurrence in plant fluids of different classes of metal ligands, including: (i) compounds with just only O atoms as electron donors, such as different carboxylates (mainly Cit and Mal but also some less known ones such as methylated aldaric acid Callahan et al., 2008) and some *ortho*-dihydroxy phenolic compounds (e.g., protocatechuic acid; Ishimaru et al.,

Table 1 | Metal concentrations (in μM) in xylem sap, leaf apoplastic fluid and phloem sap in different plant species.

Species	Stress	Fe	Mn	Cu	Zn	Ni	Cd	References
XYLEM SAP								
A. lesbiacum[H]	Ni					nd–1750[S]		Kerkeb and Krämer, 2003
A. serpyllifolium[H]	Ni					1000[S]		Alves et al., 2011
A. halleri[H]	Cd				486[S]		nd–100[S]	Ueno et al., 2008
A. thaliana		5[G]–10						Durrett et al., 2007
B. vulgaris	Fe	2[S]–6						López-Millán et al., 2000
	Fe	2[S]–4–11[SA]						Larbi et al., 2010
	Fe	4[S]–15–30[SA]						Orera et al., 2010
	Zn	5–9[S]			20–148[S]			Sagardoy, 2012
	Cd	5–11[S]			20–26[S]		<0.1–10[S]	Sagardoy, 2012
B. carinata	Cu			0.3–1[S]				Irtelli et al., 2009
B. juncea[H]	Ni					175[S]–500[SA]		Kerkeb and Krämer, 2003
	Cd						nd–50[S]	Salt et al., 1995
	Cd						nd–61[S]	Wei et al., 2007a
B. napus	Cd	10–15[S]	23–26[S]	0.6–4[S]	0.5–0.6[S]		nd–12[S]	Nakamura et al., 2008
	Cd	0.7[S]–7					nd–11[S]	Mendoza-Cózatl et al., 2008
B. oleracea			11–80		9–70			Shelp, 1987
H. vulgare	Fe	2[S]–30	8–58[S]	2–5[S]	5–20[S]			Alam et al., 2001
	Fe	nd–100[SA]						Kawai et al., 2001
G. leucopteris		20	4	0.6	14			Hocking, 1983
G. max	Zn	1[S]–6	7[S]–10	1[S]–6[S]	3–28[S]			White et al., 1981
N. glauca		11	4	2	22			Hocking, 1980
O. sativa		8[G]–14						Yokosho et al., 2009
	Fe	4[G,S]–6[S]						Yokosho et al., 2009
	Fe	2[S]–11						Kakei et al., 2009
	Cu	36[S]–40	107[S]–134–175[S]	5–9[S]	8–16[S]			Ando et al., 2013
	Cu			0.2[G]–1[G]–5				Deng et al., 2013
		30[G]–100	8–9[G]				nd	Ishimaru et al., 2011
	Cd	15[GS]–50[S]	8[GS]–8[S]				5[GS]–8[S]	Ishimaru et al., 2011
	Cd	40[S]–122[S]			12[S]–32[S]		0.1[S]–0.6[S]	Yoneyama et al., 2010
	Cd						3[S]–12[S]	Kato et al., 2010
	Cd						<0.1–2[S]	Uraguchi et al., 2009
P. communis	Fe	0.5[S]–4						Larbi et al., 2003
P. persica	Fe	1[S]–6						Larbi et al., 2003
S. lycopersicum	Fe	10[S]–150[SA]						Orera et al., 2010
	Fe	5[S]–20–121[SA]						Rellán-Álvarez et al., 2010
	Fe	5[S]–15–177[SA]						Rellán-Álvarez et al., 2011a
	Fe	4[S]–6[S]						Terzano et al., 2013
	Cu	13[GSA]–43	15–56[GS]	1[G]–6	2[GSA]–4[G]			Pich and Scholz, 1996
	Zn	3[S]–9	5[S]–7	1[S]–7	2–84[S]			White et al., 1981
	Zn	32–89[GS]			12–214[GS]			Barabasz et al., 2012
	Cd	2[S]–10			3–12[S]		nd–95[S]	Sagardoy, 2012
T. arvense	Zn				15–100[S]			Lasat et al., 1998
T. caerulense[H]	Zn				54–524[S]			Lasat et al., 1998
	Ni					nd–450[S]		Mari et al., 2006
LEAF APOPLASTIC FLUID								
B. vulgaris	Fe	3[S]–6						López-Millán et al., 2000
	Fe	7[S]–19–65[SA]						Larbi et al., 2010
H. vulgare	Mn		11–22[S]					Führs et al., 2010
O. sativa	Mn		400–2300[S]					Führs et al., 2010
	Mn		150[S]–600[GS]					Sasaki et al., 2011

(Continued)

Table 1 | Continued

Species	Stress	Fe	Mn	Cu	Zn	Ni	Cd	References
P. communis	Fe	2^{S}–5						López-Millán et al., 2001
S. lycopersicum	Zn	2^{S}–4^{GS}			1^{S}–8^{GS}			Barabasz et al., 2012
PHLOEM SAP								
B. oleracea			4–76		78–245			Shelp, 1987
G. leucopteris		83	24	8	66			Hocking, 1983
N. glauca		168	16	19	243			Hocking, 1980
O. sativa	Cu	54^{S}–67–74^{S}	7^{S}–10	20^{S}–30–43^{S}	14^{S}–22–24^{S}			Ando et al., 2013
	Cd						nd–18^{S}	Tanaka et al., 2007
	Cd	50^{S}–63^{S}			34^{S}–115^{S}		0.1^{S}–0.5^{S}	Yoneyama et al., 2010
	Cd						1^{S}–3^{S}	Kato et al., 2010
R. communis		40–64	8–12	16–28	40–74			Schmidke and Stephan, 1995
		37						Schmidke et al., 1999

Specific cases are marked as follows: [S] High or low metal supply; [A] High or low metal supply in combination with a chemical (e.g., histidine, ABA, metal resupply); [G] Mutant or transgenic genotypes; and [H] Hyper-accumulator plant species. nd: not detected.

2011); (ii) compounds with O and N atoms as electron donors, such as amino acids (proteinogenic ones such as His and non-proteinogenic ones such as NA) and PSs (e.g., MA and DMA); and (iii) compounds with S atoms in which at least one of them acts as an electron donor, such as Cys, S-containing peptides (e.g., glutathione and their derivatives, PCs) and Cys-containing proteins (e.g., metallothioneins).

Carboxylates are usually found in the μM-mM range in plant fluids, whereas NA, DMA, His, PCs and others are generally in the μM range (examples are given in **Table 2**). Difficulties in determining ligands in plant fluids are inherent to their ability to form metal complexes. The total concentration of ligand (often very low) is distributed in different chemical forms: uncomplexed, complexed to different metals and even complexed to specific metals in different stoichiometries. Therefore, to prevent errors in ligand quantification, the conditions used during sample extraction, treatment and analytical determination seek either the complete dissociation of the existing metal-complexes (while preventing the formation of new ones) or the quantitative formation of the metal complexes [e.g., Fe(III)-PSs]. Also, derivatization of the ligands may be used. An example of the latter is the use of compounds such as fluorenylmethyloxycarbonyl to protect the amino groups of NA by blocking ligand atoms involved in metal complexation (these compounds also tag the ligand with a moiety that favors its detection) (Wada et al., 2007).

Classical analytical methodologies for organic ligands were based on direct HPLC-UV/VIS analyses for carboxylates (López-Millán et al., 2000), NA (Pich et al., 1994), PSs (Alam et al., 2001) and also for PCs (in the latter case, HPLC-UV/VIS is used in combination with pre- or post-column derivatization with UV/VIS-active tags, such as Ellman's reagent). New methodologies, based generally on HPLC-ESI-MS, have been developed using high resolution MS such as time-of-flight (TOF) MS for carboxylates (Rellán-Álvarez et al., 2011b and references therein) and NA and PSs (Wada et al., 2007; Kakei et al., 2009; Schmidt et al., 2011), Fourier transform ion cyclotron resonance-MS for NA and PSs (Weber et al., 2006), and quadrupole-TOF MS for NA and PSs (Tsednee et al., 2012). Other techniques have also been used, including CE coupled to UV/VIS (Xuan et al., 2007) or MS detection (Dell'mour et al., 2010), and GC-MS (for NA and/or PSs; Wirth et al., 2009; Rellán-Álvarez et al., 2011a). Nicotianamine and PSs have been analyzed, either using previous derivatization (Wada et al., 2007; Kakei et al., 2009; Schmidt et al., 2011) or by direct analysis (Xuan et al., 2007; Tsednee et al., 2012). In the case of PCs, analytical methods are also based on HPLC-MS techniques using either molecular detection (HPLC-ESI-MS and HPLC-ESI-MS/MS) or elemental detection (ICP-MS), and are often combined with derivatization (reviewed by Wood and Feldmann, 2012). For instance, N-(2-ferrocene-ethyl)maleimide is an electroactive pre-column tag that yields thiol-PCs conjugates, which can be separated and quantified by ESI-MS or ICP-MS detection, with detection limits for S at the nM level.

Quantification of ligands in plant fluids has been always done using external calibration with or without internal standardization. The latter is required when using HPLC-ESI-MS-based technologies, because the degree of ionization of a given analyte in different matrices can vary significantly and signal suppression (or enhancement) commonly occurs. Ideally, an isotope-labeled compound should be used as internal standard (IS) per each analyte to achieve accurate quantification; however, since isotope labeled compounds have a limited commercial offer and are quite expensive, either one or two isotope-labeled compounds or structural analogues are generally used. For instance, ^{13}C-labelled Mal and succinate have been used for quantification of carboxylates (Rellán-Álvarez et al., 2011b). For quantification of NA and PSs, different ISs, including structural NA analogs such as nicotyl-lysine (Wada et al., 2007) and nicotine (Tsednee et al., 2012), or ^{15}N-labelled NA produced using a recombinant *Schizosaccharomyces pombe* strain expressing NAS (Schmidt et al., 2011) have been used.

Since many carboxylates have more than one carboxylate group and some also have a α-hydroxycarboxylate binding center (i.e.,

Table 2 | Metal ligand concentrations (malate and citrate in mM and the rest in μM) in xylem sap, leaf apoplastic fluid and phloem sap.

Species	Stress	Malate	Citrate	NA	DMA	His	PCs	References
XYLEM SAP								
A. lesbiacum[H]	Ni					40–500[S]		Kerkeb and Krämer, 2003
A. serpyllifolium[H]	Ni	2[S]	0.5[S]			nd		Alves et al., 2011
A. deliciosa	Fe	0.2–0.4[S]						Rombolà et al., 2002
A. halleri[H]	Cd	0.02[S]	0.3[S]			12[S]		Ueno et al., 2008
A. thaliana			0.06[G]–0.09					Durrett et al., 2007
			0.2–0.9[G]					Schuler et al., 2012
B. vulgaris	Fe	2–30[S]	0.2–5[S]			nd–4[S]		López-Millán et al., 2000
	Fe	2–14[S]	1–3[S]					Larbi et al., 2010
	Zn	0.5[C]–2[S]	0.06–0.3[S]					Sagardoy et al., 2011
B. carinata	Cu			64–271[S]		17–140[S]		Irtelli et al., 2009
B. juncea[H]	Ni					40[S]–50		Kerkeb and Krämer, 2003
	Cd	0.02–0.23[S]	0.01[S]–0.7					Wei et al., 2007a
B. napus	Cd	0.7–0.9[S]	0.5–0.6[S]			66[S]–76		Nakamura et al., 2008
H. vulgare	Fe	0.01–0.03[S]	<0.01–0.02[S]		30–450[S]			Alam et al., 2001
	Fe				90[S]			Kawai et al., 2001
	Fe	0.8–5[S]	0.3–1.3[S]					López-Millán et al., 2012
G. max	Zn	0.04[S]–0.9	0.08[S]–1.5[S]			26[S]–62[S]		White et al., 1981
N. glauca						10		Hocking, 1980
O. sativa		0.15–0.18[G]	0.05[G]–0.1					Yokosho et al., 2009
	Fe	0.3[S]–0.4[GS]	0.1[GS]–0.2[S]					Yokosho et al., 2009
	Fe			10–18[S]	10–48[S]			Kakei et al., 2009
	Cu		0.08	28	34			Ando et al., 2013
P. communis	Fe	0.1–3	0.03–0.5[S]					Larbi et al., 2003
P. persica	Fe	0.7–2[S]	0.05–0.8[S]					Larbi et al., 2003
Q. suber	Cd	1–2[S]	0.5–1.2[S]					Gogorcena et al., 2011
S. lycopersicum	Fe	0.6–4[S]	0.04–0.6[S]					López-Millán et al., 2009
	Fe		0.01–0.17[S]					Rellán-Álvarez et al., 2010
	Cu			nd[GS]–20[S]				Pich and Scholz, 1996
	Zn	0.06[S]–0.8[S]	<0.04[S]–0.3[S]			6[S]–18		White et al., 1981
	Cd	0.1–0.3[S]	0.01[S]–0.03–0.06[S]					Sagardoy, 2012
T. arvense	Zn	0.1–0.3[S]	nd			110–140[S]		Lasat et al., 1998
	Ni					nd[S]–57		Persans et al., 1999
T. caerulense[H]	Zn	0.1–0.2[S]	nd		nd			Lasat et al., 1998
T. goesingense[H]	Ni					8–18[S]		Persans et al., 1999
LEAF APOPLASTIC FLUID								
B. vulgaris	Fe	0.7–2[S]	0.7–4[S]			<1–4[S]		López-Millán et al., 2000
	Fe		0.2–4[S]					Larbi et al., 2010
P. communis	Fe	1.6[S]–2	0.8–1.8[S]					López-Millán et al., 2001
PHLOEM SAP								
C. maxima		1.6	2.1					Fiehn, 2003
L. texensis		5.5	1.1					Lattanzio et al., 2013
N. glauca						370		Hocking, 1980
O. sativa	Cu		>0.08	66	152			Ando et al., 2013
	Cd		>0.001–>0.002[S]	66–83[S]	152–176[S]		nd–63[S]	Kato et al., 2010

[S] High or low metal supply; [G] Mutant or transgenic genotypes; and [H] Hyper-accumulator plant species. Nd, not detected; NA, nicotianamine; DMA, 2′-deoxymugineic acid; His, histidine; PCs, phytochelatins.

α-hydroxy acids such as Cit and Mal), they can act as mono- or poly-dentate (bidentate and so on) ligands, and form complexes with several metals (e.g., Fe, Mn, Cu, Ni, Zn, Pb, etc.). Carboxylates with shorter chains or closely packed carboxyl groups with adjacent alcohol groups (α-hydroxy acids) form stronger complexes. These characteristics, as well as the increases in carboxylate levels found in some metal-stressed plants (**Table 2**), have supported that carboxylates could be associated to long-distance metal transport. For instance, Fe-deficiency causes a well-known increase in carboxylate concentrations (mainly Cit) in xylem sap (Abadía

et al., 2002; Rellán-Álvarez et al., 2010) and leaf apoplastic fluid (López-Millán et al., 2000, 2001; Larbi et al., 2010). This occurs not only in several Strategy I plant species (**Table 2**; Abadía et al., 2002 and references therein; Rellán-Álvarez et al., 2010 and references therein) but also in Strategy II plant species (Alam et al., 2001; Yokosho et al., 2009; López-Millán et al., 2012), likely as a result of an increased anaplerotic C fixation mediated by the root phospoenolpyruvate carboxylase (López-Millán et al., 2000; Andaluz et al., 2002; López-Millán et al., 2012). This increased carboxylate flux in xylem sap would supply C to the Fe-deficient foliage that is deprived of C skeletons (López-Millán et al., 2000, 2012) and could also increase Fe supply via formation of Cit complexes with extracellular Fe pools (see below). This is in line with the use of citric acid in industry for cleaning and prevention of the clogging of pipes with colloidal and particulate Fe. An increased activity of citrate synthase (CS) and/or an overexpression of CS genes has been reported in plants grown with low Fe supply (e.g., *Beta vulgaris* López-Millán et al., 2000, *Pyrus communis* López-Millán et al., 2001, *A. thaliana* Thimm et al., 2001, *Actinidia deliciosa* Rombolà et al., 2002, *S. lycopersicum* López-Millán et al., 2009, *Pisum sativum* Jelali et al., 2010, *Malus xiaojinensis* Han et al., 2012 and citrus Martínez-Cuenca et al., 2013) and more recently in *M. xiaojinensis* plants grown with excessive Fe supply (Han et al., 2012). In fact, overexpression of an apple CS gene increased tolerance to Fe stress (low and excessive Fe supply) in transgenic *A. thaliana* and *Nicotiana tabacum* plants (Han et al., 2012, 2013). Increases in xylem carboxylate concentrations have also been described with other metal stresses, including excess of metals in crop (e.g., Zn in sugar beet Sagardoy et al., 2011 and Cd in tomato Sagardoy, 2012), and forest species (Cd in *Quercus suber* Gogorcena et al., 2011), as well as in metal hyper-accumulators (e.g., the Zn hyper-accumulator *T. caerulescens* Lasat et al., 1998, the Cd hyper-accumulator genotype of *B. juncea* L. Wei et al., 2007a and the Ni hyper-accumulator tree *S. acuminata* Callahan et al., 2008). It has been hypothesized that the increases in xylem carboxylates may constitute a general mechanism to cope with situations causing reduced photosynthetic activity (Sagardoy et al., 2011).

Catechols such as caffeic and protocatechuic acids are phenolic compounds with two adjacent hydroxyl groups in the aromatic ring, which have very high affinity for Fe(III). These compounds are involved in long-distance transport of Fe in *O. sativa*, since the mutant *phenolics efflux transporter* (*pez1*) had reduced concentrations of Fe, protocatechuic and caffeic acids in the xylem, along with increased root apoplasmic Fe (Ishimaru et al., 2011).

Histidine (His) is one of the strongest metal-coordinating ligands among the proteinogenic amino acids, and has three metal binding sites: carboxylate, α-amino and imidazole groups. The coordination to metals through the latter group forms rigid bonds and strong complexes, especially with Ni and Cu. However, evidence for a role of His in long-distance metal transport in plants is mostly related to Ni in the xylem of hyper-accumulators of the genus *Allysum*. Histidine (in the μM-mM range) and Ni xylem sap concentrations are significantly and linearly correlated in several *Alyssum* Ni hyper-accumulators (such as *A. lesbiacum*) in response to increased metal concentrations in the growth media (Krämer et al., 1996). This increased xylem loading of Ni is associated with constitutively higher root concentrations of His. Moreover, exogenous applications of His to either the roots or shoots of the non-accumulator plant species *Alyssum montanum* and *Brassica juncea* greatly increases the root-to-shoot mobility of Ni (Kerkeb and Krämer, 2003). In *A. lesbiacum* shoot His concentrations only increased when plants were exposed to Ni, and the levels of transcripts of the enzymes of the His biosynthesis pathway were constitutively higher in *A. lesbiacum* than in the non-accumulator *A. montanum*, especially for the first enzyme in the biosynthetic pathway, ATP-phosphoribosyltransferase (ATP-PRT) (Ingle et al., 2005). Moreover, the overexpression of an ATP–PRT cDNA in *A. thaliana* resulted in increases in shoot His and Ni tolerance (Wycisk et al., 2004). However, it has been recently reported that His does not play a role in Ni translocation in the xylem sap of *Alyssum* ssp. under field conditions (Alves et al., 2011; Centofanti et al., 2013). The Ni-His complex could occur in xylem sap under N-sufficient conditions, whereas under N-limited conditions, such as those usually found in the field, Ni translocation would occur as a free ion or complexed with carboxylates (Alves et al., 2011).

Nicotianamine (NA) and related molecules such as PSs are multi-dentate aminoacid chelators having more than one α-aminocarboxylate binding centers, which confer high affinity not only for Fe but also for other metals such as Zn, Cu, Mn, Ni, and Cd. Nicotianamine has affinity for Fe(III) and Fe(II), whereas PSs have an α-hydroxycarboxylate binding center that confers selectivity for Fe(III). Nicotianamine is ubiquitous in higher plants and present in all tissues, and is involved in plant metal trafficking (Curie et al., 2009; Clemens et al., 2013). In the NA-free tomato mutant *chloronerva*, which displays a strong interveinal chlorosis in young leaves, the long-distance transport of Cu but not that of Fe is impaired, indicating the importance of NA in Cu trafficking (Pich and Scholz, 1996). Unlike NA, PSs are restricted to grasses and secreted to the rhizosphere, and they are responsible for Fe and Zn acquisition (Suzuki et al., 2006). Both NA and PSs form metal stable complexes at the pH values occurring in plant fluids (von Wirén et al., 1999, 2000; Rellán-Álvarez et al., 2008). Furthermore, hydroxylated PSs such as MA and epi-MA have a higher affinity for Fe(III) than the non-hydroxylated DMA at the pH values from 5 to 7 typical of xylem sap, and this represents a competitive advantage when moving through slightly acid environments (von Wirén et al., 2000).

In xylem sap of *O. sativa*, NA and DMA concentrations are in the ranges 10–20 μM and 10–50 μM, respectively (Kakei et al., 2009), whereas in phloem sap higher concentrations were found, in the range of 66–83 for NA and 152–176 μM for DMA (Kato et al., 2010). Metal stresses caused increases in xylem sap NA or PSs concentrations in several species, including MA and DMA in Fe-deficient *H. vulgare* (Alam et al., 2001; Kawai et al., 2001), DMA in Fe-deficient *O. sativa* (Kakei et al., 2009), and NA in Cu-deficient Brassica (Irtelli et al., 2009) and Fe-deficient *Prunus persica* (Rellán-Álvarez et al., 2011a). Nickel-induced NA root-accumulation occurred in *T. caerulescens*, a Cd/Zn/Ni hyper-accumulator, but not in *T. arvense*, and this suggests that NA could be involved in Ni translocation *via* xylem in *T. caerulescens*, resulting in a higher capacity to transport Ni to shoots (Mari et al., 2006).

Phytochelatins are oligomers of the tri-peptide glutathione (-GluCysGly) and act as metal (Cd, Hg, Zn, and others) chelators through the thiol (-SH) group of Cys. Phytochelatins form a family of structures with increasing repetitions of the -Glu-Cys dipeptide units, followed by a terminal Gly, (-Glu-Cys)n-Gly or (-EC)n-Gly, where *n* generally ranges from 2 to 5 but can be as high as 11. A number of structural variants have been identified in a wide variety of plant species, and different metals, including Cd, Pb, Zn, and Hg, have been found to induce PCs production (reviewed by Pal and Rai, 2010). The occurrence of long-distance transport (either from shoot-to-root or from root-to-shoot) of PCs and some intermediates of their biosynthesis (e.g., **γ**-glutamylcysteine) during heavy-metal detoxification was first shown with transgenic and grafted Arabidopsis plants where PCs synthesis was restricted to specific tissues (Gong et al., 2003; Chen et al., 2006; Li et al., 2006). However, the direct determination of PCs in plant fluids has only been achieved more recently: glutathione and PCs were found in the phloem of Cd-treated *B. napus* (by HPLC coupled to both fluorescence and ESI-MS; Mendoza-Cózatl et al., 2008) and *O. sativa* (by CE-MS; Kato et al., 2010), and As-treated *R. communis* (by HPLC-ESI-MS; Ye et al., 2010). Lower concentrations of PCs (or trace levels) were found in the xylem of *B. napus* (Mendoza-Cózatl et al., 2008) and *R. communis* (Ye et al., 2010), suggesting that phloem is the major vascular system for PC-facilitated long-distance metal transport.

Proteins can also be involved in metal transport in fluids. A significant fraction of metals has been associated with the high molecular weight fraction in the phloem sap of *R. communis* (Fe Krüger et al., 2002) and *O. sativa* (Cu Ando et al., 2013 and Cd Kato et al., 2010). Among metal-binding proteins, metallothioneins (MTs) are low molecular weight (5–10 kDa), Cys-rich proteins, which are able to bind a variety of metals by the formation of mercaptide bonds with the numerous Cys residues present in the proteins (see review by Freisinger, 2011). Metallothioneins are implicated in several processes related to metal homeostasis, detoxification, distribution, and redox regulation, in particular under normal (non-stressed) physiological conditions. Evidence supports its role as metal carriers, mainly in the phloem sap. An up-regulation of MTs in *H. vulgare* was found during senescence (when metal remobilization occurs from senescing leaves), heavy metal treatments and Cu deficiency (Heise et al., 2007). Metallothioneins have been reported to occur in the phloem of *Apium graveolens* (Vilaine et al., 2003), *R. communis* (Barnes et al., 2004), *O. sativa* (Aki et al., 2008) and *L. texensis* (Lattanzio et al., 2013), when grown under non-stressed conditions. The induction of MTs (MT1) expression in leaf veins (and to a lesser extent in mesophyll cells) in response to Cu stress in *A. thaliana* suggest that this MT could be important for scavenging Cu in leaf veins (García-Hernández et al., 1998). Also, in hyper-accumulator plants, MTs could help detoxify the excess Cu accumulated by the high expression of the Cd/Zn ATPase HMA4 (Leitenmaier and Küpper, 2011, 2013).

CHALLENGES ANALYZING METAL COMPLEXES IN PLANT FLUIDS

Several challenges are faced when studying metal speciation in plant fluids, because of the changes in metal speciation that may occur at sampling and/or during storage, and especially during sample preparation, separation and determination (Husted et al., 2011). Challenges when attempting the analysis of the metal chemical form(s) existing in a plant fluid occur because: (i) dynamic metal–ligand systems such as those in plant fluids inevitably include labile or transient metal species; (ii) biochemical processes such as enzymatic activities may cause degradation of metal complexes, (iii) metal species occur in plant fluids at very low concentrations (in the μM range; see below; **Table 1**), (iv) the metal complex distribution strongly depends not only on pH, but also on the metal-to-ligand ratios (Weber et al., 2006; Xuan et al., 2006, 2007; Rellán-Álvarez et al., 2008). The latter is specially important in plant fluids, since unlike stable metal chelates with the hexadentate ligands NA and PSs, which always occur with 1:1 stoichiometry, many of the known metal ligands existing in xylem and phloem saps (e.g., amino acids and carboxylates) may act as bi- and tri-dentate ligands, resulting in numerous metal-ligand species with different stoichiometries and charge states (see examples for Fe-Cit complexes in Silva et al., 2009; Rellán-Álvarez et al., 2010). For instance, in a solution with a 1:2 Fe:Cit ratio and pH 4, up to thirteen different Fe-Cit species were detected in aqueous solution by ESI-MS, whereas only two species occurred in a solution prepared at 1:100 Fe:Cit ratio at the same pH (Silva et al., 2009). Also, even for stable metal species, ligand exchange reactions may occur (altering the actual composition of the sample) at any step previous to detection, due to the presence of competing ligands and/or redox mediators. Ligand exchanges have already been reported in the cases of Fe(III)-NA (with Cit; Rellán-Álvarez et al., 2008) and Fe(III)-DMA (with NA; Weber et al., 2006). Some of these challenges are especially critical in separation-based methods (e.g., HPLC, CE), because the separation of the free ligand does change the metal-to-ligand ratio, and also because the pH may change considerably when organic solvent modifiers are used (Rellán-Álvarez et al., 2008, 2010; Köster et al., 2011a).

Sampling and storage procedures (temperature, light, etc.), can be considered as key aspects to preserve the metal species occurring in samples during the whole analytical process (reviewed by Mesko et al., 2011). Temperature needs to be as low as possible to reduce metal species transformation. For this purpose, lyophilization and shock-freezing in liquid N are the most common procedures used to preserve metal species in fluids. The latter is considered the safest technique to prevent metal species changes because it can be performed immediately at the sampling site and also because sample is stored in an inert gas atmosphere. Light can cause changes in metal speciation because it can induce electron transfer reactions affecting the stability of the metal complexes and also the structural integrity of the ligands. For instance, photochemical reduction of Fe(III) complexes with ligands such as di- and tri-carboxylic acids is well known (Bennett et al., 1982), and are accompanied by oxidative decarboxylation of the ligand. This issue could limit the use of irradiation with high intensity synchrotron X-rays for metal speciation (Terzano et al., 2013).

Finally, accurate quantification of metal species, generally performed either on-line or off-line after separation, is currently carried out using metal (ICP-MS) or molecular (ESI-MS) detection, in combination with isotope dilution analysis (IDA) that requires the use of an isotopolog of the analyte. When ICP-MS

is used, a stable isotope of the metal is added after the separation of the metal complexes. For instance, Rellán-Álvarez et al. (2010) used ^{57}Fe post-column addition to quantify Fe-Cit species in tomato xylem sap. When molecular detection such as ESI-MS is used, the isotopolog should be either a metal complex with a stable isotope-labeled ligand, or alternatively a structural analogue of the ligand. As mentioned above, the limited supply of stable isotope labeled ligands is an additional constraint for metal speciation.

METAL SPECIES IN XYLEM SAP

Most of the studies exploring the chemical forms of metal complexes in plant fluids have been conducted using xylem sap. Metals occurring in the xylem sap may be preferentially complexed by the more acidic carboxylic acids (existing at concentrations from 2 to 9 mM in the xylem) rather than the much more basic amino acids (existing at concentrations from 1 to 3 mM in the xylem) due to the relatively acidic pH of this fluid, which is generally in the pH range from 5 to 6.5 (Harris et al., 2012).

A Fe(III)-Cit complex [tri-Fe(III), tri-Cit complex (Fe_3Cit_3)] was found for the first time in the xylem sap of tomato, using an integrated HPLC-MS approach, consisting in hydrophilic interaction liquid chromatography (HILIC) coupled to both ICP-MS and ESI-MS(TOF), combined with the use of stable isotope (^{54}Fe) labeling; the identification was based on exact molecular mass, isotopic signature, Fe determination and retention time (Rellán-Álvarez et al., 2010). Citrate had been considered for many years a likely candidate for Fe xylem transport, but the possible Fe-Cit species in the xylem sap were only predicted from the co-migration of Fe and Cit during paper electrophoresis of xylem sap (Tiffin, 1966) or from *in silico* calculations (von Wirén et al., 1999; López-Millán et al., 2000, 2001; Rellán-Álvarez et al., 2008). The Fe_3Cit_3complex was only found in xylem samples with Fe concentrations above 20 μM (the limit of detection for the complex), such as those in Fe-deficient plants after Fe-resupply. The complex could not be detected in Fe-deficient and control plants, which have lower xylem sap Fe concentrations. The existence of other Fe-Cit complexes is likely, and the complex Fe_2Cit_2 was also detected in Fe-Cit standards along with Fe_3Cit_3, with the allocation of Fe between the two complexes depending on the Fe:Cit ratio. Since in plant xylem sap a wide range of Fe to citrate ratios could exist, it is likely that both Fe(III)-Cit species could occur in different conditions (Rellán-Álvarez et al., 2010). Later, other Fe-Cit species were found along with Fe_3Cit_3 in *H. vulgare* leaf extracts using HILIC coupled to high-resolution Fourier transform ion cyclotron resonance (FT-ICR) MS (Köster et al., 2011a). More recently, the Fe speciation in tomato xylem sap was assessed for the first time using XANES on a highly brilliant synchrotron (PETRA III, beamline P06; Terzano et al., 2013). Although this study confirmed the occurrence of Fe(III)-Cit and also found Fe(III)-acetate complexes in xylem sap, the authors indicated that complexes found could be artifacts as a result of the high intensity radiation used. Studies with *FRD* mutants (i.e., *Atfrd3* and *Osfrdl1*), which lack a protein responsible for efflux of Cit in cells of the xylem vasculature, also support the role of Cit as a major ligand responsible for Fe complexation (Durrett et al., 2007; Yokosho et al., 2009). These mutants showed leaf chlorosis and low levels of Fe in xylem and leaves, as well as decreased Cit levels of in the xylem sap. Taken together, all these studies support that Fe-Cit is responsible for the translocation of an important fraction of Fe to the shoot, and that FRD mediated-Cit efflux is required to sustain normal rates of root–shoot Fe delivery. More recently, it was shown that FRD mediated-Cit efflux is also a major player in mobility of Fe in inter-cellular spaces lacking symplastic connections (Roschzttardtz et al., 2011).

The possible role of NA in long-distance Fe transport in the xylem is still being explored (Curie et al., 2009). However, strong evidence supports that this amino acid is not essential for xylem Fe transport: the NA-deficient tomato mutant *chloronerva* does accumulate Fe in old leaves (Pich et al., 1994) and the *A. thaliana* NA synthase (NAS) quadruple mutant (with low levels of NA) also accumulates Fe in leaves (Klatte et al., 2009). Until now, Fe-NA chelates have not been detected in xylem sap (Rellán-Álvarez et al., 2010), and *in silico* and/or *in vitro* speciation studies tend to exclude NA as a possible xylem Fe carrier at the slightly acidic pH values typical of xylem (von Wirén et al., 1999; Rellán-Álvarez et al., 2008). However, it has been recently suggested that NA may play a role in long distance transport of Fe when carboxylates are in short supply, as it occurs in *FRD* mutants (Schuler et al., 2012) or in plant species with less acidic xylem such in field-grown *Prunus persica* trees (where the xylem sap pH is in the range from 6.5 to 7.5 Larbi et al., 2003; Rellán-Álvarez et al., 2011a). The most accepted role of NA is in intra-organ Fe distribution, where this ligand could be crucial for xylem Fe unloading. Iron distribution within leaves is impeded in the tomato mutant *chloronerva* (Pich et al., 1994) that also showed a lower presence of Fe(II) ions in the veins when leaves were analyzed by XANES (Yoshimura et al., 2000). This suggests that the occurrence of Fe as Fe(II)-NA complex in leaf veins is crucial for the intra-organ Fe allocation (Yoshimura et al., 2000). No transporter responsible for moving Fe-complexes into the xylem sap has been conclusively identified so far, but it has been suggested that Fe-NA could be effluxed into the xylem by a yellow-stripe-like (YSL) transporter (Colangelo and Guerinot, 2006). Regarding PSs, only a minor peak assigned to Fe(III)-DMA was found in press sap from the roots of Fe-deficient wheat plants by HILIC-ESI-MS (Xuan et al., 2006).

The Zn species present in the xylem are still an open question (Clemens et al., 2013). Three studies tackling Zn speciation in xylem sap were carried out using EXAFS or XANES with the hyper-accumulators *T. caerulescens* (Salt et al., 1999; Monsant et al., 2011) and *S. alfredii* (Lu et al., 2013). In all cases, although the major fraction of Zn consisted in free hydrated Zn^{2+} ions, the remaining fraction was coordinated with carboxylates such as Cit and Mal. The occurrence of Zn-Cit in xylem sap was also predicted by *in silico* studies in non hyper-accumulator species (White et al., 1981; Mullins et al., 1986). However, other EXAFS study indicated that most of the Zn in petioles and stems of *T. caerulescens* is present as Zn-His (Küpper et al., 2004) and a recent study (including the re-evaluation of previous EXAFS spectra from this species) proposes His as a Zn ligand within cells and NA as Zn chelator involved in long distance transport (Trampczynska et al., 2010). In a recent room temperature XANES study with *T. caerulescens* intact plants, Zn-His and

Zn-phytate complexes were found in roots, whereas Zn(II)-Mal and Zn(II)-Cit were the major species in shoots (Monsant et al., 2011). *In vitro* metal exchange experiments also support the existence of the complex Zn(II)-NA in the xylem sap (Rellán-Álvarez et al., 2008). It has also been speculated that Zn would be associated with S ligands in Cys, GSH or PCs in hyper-accumulators (Milner and Kochian, 2008). In the Zn hyper-accumulator *A. halleri*, suppression of NA synthase (NAS2) resulted in strongly reduced NA root accumulation, and a concomitant decrease in root-to-shoot translocation of Zn (Deinlein et al., 2012). This study found NA and thiols as the dominant Zn ligands in the low molecular weight fraction of root extracts by using size-exclusion chromatography (SEC)-ICP-MS combined with off-line ESI-MS ligand detection in the Zn-containing LC fractions. The overexpression of *A. thaliana* ZINC-INDUCED-FACILITATOR1 (ZIF1) altered the subcellular partitioning of NA, which was accumulated in roots, and led to a Zn accumulation in roots at the expense of shoots (Haydon et al., 2012). This indicates that the formation of Zn(II)-NA could be responsible for Zn hyper-accumulation. However, the complex Zn(II)-NA has never been found in the xylem sap yet. In grasses, a Zn(II)-DMA complex was detected in press sap from roots of Fe-deficient wheat plants using HILIC-ESI-MS (Xuan et al., 2006).

The Cu(II)-DMA complex has been recently been found in xylem sap of *O. sativa* (Ando et al., 2013). The Cu(II)-DMA complex was identified by SEC combined with both off-line Cu determination (using GFAAS) and off-line molecular detection of the complex by ESI-MS, based on exact molecular mass and isotopic signature. In this study, the Cu, NA, and DMA xylem sap concentrations were 5, 28, and 34 μM, respectively (the molar ratio DMA:Cu was *ca.* 7). The same complex was already found in press sap from roots of Fe-deficient wheat plants, both using HPLC-MS (Xuan et al., 2006) and CE and UV-VIS detection (Xuan et al., 2007). The presence of Cu(II)-DMA is not unexpected, since it has a quite high stability constant (18.7; Murakami et al., 1989). Nicotianamine and DMA are present in comparable concentrations in the xylem, but Cu(II)-NA has not been found in xylem sap so far, in spite of having a similar stability constant to that of Cu(II)-DMA (18.6; Callahan et al., 2006). A preferential Cu complexation by DMA vs. NA was already predicted by *in silico* speciation (von Wirén et al., 1999). In plant species other than grasses, the low leaf Cu concentration found in the NA-free tomato mutant *chloronerva* is in strong support that Cu(II)-NA is involved in xylem Cu transport (Pich and Scholz, 1996). The EXAFS spectra of *T. caerulescens* shoots also suggested the presence of Cu(II)-NA (Mijovilovich et al., 2009). Further support for the role of NA in xylem Cu transport was obtained when the NA aminotransferase gene from *H. vulgare* was introduced into the non-graminaceous plant *N. tabacum*. When compared to wild type, these transgenic plants showed decreased Cu concentration in young leaves and flowers (Takahashi et al., 2003), attributable to the depletion of endogenous NA. The occurrence of a Cu-His complex has been reported in the xylem sap from the Ni hyper-accumulator *A. lesbiacum* by EXAFS (Krämer et al., 1996) and in *H. vulgare* leaf aqueous extracts by HILIC-FT-ICR/MS (Köster et al., 2011a).

Long-distance Ni transport has been demonstrated in hyper-accumulators (*Allysum* and *Thlaspi* species) coordinated with several ligands, including different carboxylates (e.g., Cit, Mal, etc.) and amino acids (e.g., NA and His). An in depth study of Ni ligands using a combination of advanced MS and NMR techniques in the latex of the Ni hyper-accumulator tree *S. acuminata* revealed the presence of Ni in complexes with methylated aldaric acid and Cit (Callahan et al., 2008). In this latex, containing 26% dry matter of Ni, Ni(II) was forming complexes of 1:2 stoichiometry [Ni(II):carboxylate] with these two carboxylates as well as with Mal, aconitate, erythronate, galacturonate, tartarate, aconitate, and saccharate. A $Ni(II)Cit_2$ complex (accounting for 99.4% of the Ni) was previously identified in an aqueous extract of *S. acuminata* using SEC monitorized on-line with ICP-MS, followed by off-line ESI-MS/MS analyses of the Ni-containing SEC-fractions (Schaumlöffel et al., 2003). In leaf extracts of several New Caledonia Ni hyper-accumulator plant species, Ni-Cit complexes were also found as the most prominent Ni containing ions detected by SEC-ESI-MS/MS (Callahan et al., 2012). In another Ni hyper-accumulator, *A. murale*, EXFAS studies indicated that Ni was found coordinated with Mal, His and other low molecular weight compounds in the plant sap and vasculature (McNear et al., 2010), and an *in silico* speciation study of the xylem sap of the hyper-accumulator *A. serpyllifolium* predicted approximately 18% of Ni bound to organic acids (Alves et al., 2011). However, a detailed study of the metal and ligand concentration in the xylem sap of Alyssum species treated with Ni for long periods indicated that most of the Ni in xylem sap of this species is present as the hydrated cation, and that the increases in His and other chelators may constitute only a short term response (Centofanti et al., 2013). The Ni(II)-NA complex is quite stable ($\log K = 16.1$) and accordingly Ni has also been found complexed by NA in hyper-accumulators as well as model plant species. Nickel(II)-NA was found in the xylem sap (Mari et al., 2006) and plant extracts (Vacchina et al., 2003; Ouerdane et al., 2006) of *T. caerulescens*, in a water extract of the latex in *S. acuminata* (Schaumlöffel et al., 2003), and in leaf extracts of New Caledonia Ni hyper-accumulator plant species (Callahan et al., 2012), using SEC-ESI-MS/MS or SEC in combination with ICP-MS detection and off-line ESI-MS/MS. In Arabidopsis xylem sap, the Ni(II)-NA complex was also detected using both HPLC-MS (Xuan et al., 2006) and CE coupled to UV-VIS (Xuan et al., 2007). On the other hand, studies on natural variation among *Arabidopsis* accessions indicated that a Ni(II)-Mal complex may also be involved in translocation of Ni from roots to shoots (Agraval et al., 2013).

In the case of toxic metals such as Cd, complexation with organic ligands in xylem vessels may not be necessary, because toxicity exerted in this apoplastic, extracellular system is low and may not require a metal detoxification strategy. In fact, using ^{113}Cd NMR analysis combined with a stable isotope (^{113}Cd) labeling technique, Cd was found as an ionic form in *A. halleri* xylem sap (Ueno et al., 2008). However, several EXAFS spectroscopy studies indicate that Cd is coordinated with O or N ligands in *B. juncea* xylem sap (Salt et al., 1995) and with O in aerial parts of *T. caerulescens* (Küpper et al., 2004), *A. halleri*

(Huguet et al., 2012) and *T. praecox* (Vogel-Mikus et al., 2010). In roots of *B. juncea*, a possible coordination of Cd with S ligands was also reported (Salt et al., 1995), with the bond length being similar to that of a purified Cd(II)-PC complex, supporting the occurrence of Cd(II)-PC complexes in plants (Salt et al., 1995). The occurrence of Cd association with PCs in the xylem sap of *B. juncea* has been proposed using SEC and off-line metal GFAAS, and using the retention times of several Cu-complexes with low molecular weight ligands (including PCs, GSH, Cys, organic acids, and inorganic anions) as a mean for identification (Wei et al., 2007b).

The chemical form of Al in xylem sap has been identified as the Al-Cit complex using ^{27}Al-NMR analysis in several Al hyper-accumulators, including *Fagopyrum esculentum* (Ma and Hiradate, 2000), *Melastoma malabathricum* (Watanabe and Osaki, 2001) and *Camellia sinensis* (Morita et al., 2004). Since Al in roots was as an Al-oxalate (1:3) complex, ligand exchange from oxalate to Cit should occur in these plant species (Ma and Hiradate, 2000; Watanabe and Osaki, 2001).

METAL SPECIES IN PHLOEM SAP

The distribution of micronutrients to developing organs of plants depends to a great extent on phloem transport. Unlike xylem, phloem consists of columns of living cells. Metals are sparingly soluble at the alkaline pH values typical of the phloem sap (pH range from 7 to 8), and they are also highly reactive species, with some of them such as Fe undergoing easily changes of valence that favor the production of highly reactive oxygen species *via* Fenton reactions. Therefore, metal complexation with appropriate ligands can provide solubility and shielding during phloem transport of metals to the nutrient sinks.

In the phloem sap of *O. sativa*, Fe has been found predominantly (77%) associated with high molecular weight molecules (using a 3 kDa membrane filter; Nishiyama et al., 2012). In this study, Fe-containing compounds or complexes of 10–30 kDa and the Fe(III)-DMA complex were detected in the phloem sap using anion exchange HPLC separation followed by an identification based on Fe determination and the comparison of the retention time with those of standards, in combination with exact molecular mass and Fe isotopic signature obtained using ESI-MS(TOF). A protein capable to bind Fe was described in the phloem sap of *R. communis* (ITP; Iron Transport Protein; Krüger et al., 2002) using two-dimensional gel electrophoresis protein separation (2-DE SDS-PAGE) followed by electro-blotting to PVDF membranes and staining of Fe-containing proteins with Ferene. Recently, two more low molecular weight Fe-binding proteins were also identified in *L. texensis* phloem sap using a similar approach combined with Fe affinity chromatography, although none of them are considered candidates for Fe transport (Lattanzio et al., 2013). The Fe(II)-NA complex has not been found so far in the phloem sap, although *in silico* and/or *in vitro* studies support that the Fe-NA complex is likely to occur at the neutral to basic pH values of the phloem sap (von Wirén et al., 1999; Rellán-Álvarez et al., 2008), and YSL transporters able to transport Fe-NA complexes have been described in *Arabidopsis* and *O. sativa* phloem vascular tissues (Curie et al., 2009). Perhaps NA is only important in Fe phloem loading (Schuler et al., 2012), and once in that compartment Fe may be transported in another form such as bound to proteins.

Almost all Zn in the *O. sativa* phloem sap was found associated with low molecular weight molecules when a 3 kDa membrane filter was used as a cut-off (Nishiyama et al., 2012). In this study, Zn was identified as the Zn(II)-NA complex, using SEC and off-line Zn determination and off-line ESI-MS, based on exact molecular mass, isotopic signature, and retention time. In *L. texensis* phloem sap, four low molecular weight Zn-binding proteins were identified using 2-DE SDS-PAGE, nanoLC-MS/MS and Zn affinity chromatography, one of them being a metallothionein-like protein type 2B, but they were not considered as good candidates for Zn transport (Lattanzio et al., 2013).

Regarding Cu, the phloem sap of *O. sativa* has been shown to contain the complexes Cu(II)-NA, Cu(II)-His and high-molecular-weight compounds >3 kDa (the latter being at least 30% of the total Cu) (Ando et al., 2013). Copper(II)-NA and Cu(II)-His were identified using SEC combined with both off-line Cu determination by GFAAS and molecular detection of the complex in the major Cu-containing fractions. Copper-containing proteins detected in phloem sap so far include Cu/Zn-superoxide dismutase, a Cu-chaperone (CCH homolog) and several MTs in *O. sativa* (Aki et al., 2008) and *L. texensis* (Lattanzio et al., 2013).

A Cd-containing complex has not been directly identified in the phloem sap so far. However, 90% of the Cd in the phloem sap from Cd-treated *O. sativa* plants was found in a complexed form using SEC-ICP-MS (Kato et al., 2010). Based on the elution times of *in vitro* prepared Cd-complexes with glutathione and several PCs and on the changes caused on Cd elution by sap digestion with proteinase K, it was proposed that Cd was found associated with a 13 kDa protein and SH-containing compounds. Cadmium has also been associated with S in the phloem and companion cells of *A. thaliana* using energy-dispersive X-ray microanalysis (van Belleghem et al., 2007). This, along with the occurrence of PCs in phloem of Cd-treated plants (see above) suggest the occurrence of Cd-PC complexes in the phloem sap.

Much less information is available on the chemical forms of other metals in the phloem sap. In *R. communis*, Mn was detected in association with low molecular peptides (van Goor and Wiersma, 1976), while Ni was shown to be complexed with negatively charged organic compounds with a molecular weight in the range of 1000–5000 Da (Wiersma and van Goor, 1979).

METAL SPECIES IN OTHER FLUIDS

The ESL is a fluid that facilitates metal transport from the seed coat to the embryo. Iron speciation in isolated *P. sativum* ESL was achieved using an integrated analytical approach, combining XANES, HILIC-ICP-MS, and HILIC-ESI-MS (Grillet et al., 2014). The application of the XANES technique indicated that most of the Fe was present as Fe(III), probably associated to carboxylates—although the XANES spectra of Fe-Cit and Fe-Mal could not be distinguished—with only a minor amount of Fe(II) species being present. Most (88%) of the Fe occurring in the ESL was found as the species $Fe(III)_3Cit_2Mal_2$, $Fe(III)_3Cit_3Mal$ (both are mixed ligand species) and $Fe(III)Cit_2$; only a minor amount of Fe(II)-NA was found using HILIC separation coupled to the two cited MS detectors. Metal species were identified

based on elution time, Fe determination, exact mass determination, isotopic signature, and MS^2 fragmentation pattern of the Fe species as identification tools. Pea embryos are capable of reducing Fe(III) in these complexes by effluxing to the ESL high amounts of ascorbate that chemically reduce Fe(III) from the Fe-Cit and Fe-Mal complexes. Ascorbate efflux also occurs in *A. thaliana* embryos and is significantly decreased, along with Fe concentrations, in ascorbate deficient mutants (Grillet et al., 2014). These data provide support for a new local Fe transport system that may play a major role to control Fe loading in seeds in dicotyledonous plants.

Apoplastic fluid composition is determined by the balance of import via xylem, absorption by cells, and export by phloem, and plays important roles in the transport and storage of mineral nutrients (Sattelmacher, 2001). However, as far as we know, there are no reports tackling direct metal speciation on apoplastic fluids. *In silico* calculations have been carried out to speciate Fe in leaf apoplastic fluid of *B. vulgaris* (López-Millán et al., 2000; Larbi et al., 2010) and field-grown *P. communis* (López-Millán et al., 2001) using experimental concentrations of Fe, inorganic ions, carboxylates, sugars and amino acids measured from fluids isolated from Fe-sufficient and Fe-deficient plants. In both plant species, Fe was predicted to occur in the leaf apoplastic fluid as Fe-Cit complexes, with FeCitOH and $Fe(III)Cit_2$ being the major species. The effect of Fe deficiency altered the balance between these two Fe-Cit species and the contribution of $Fe(III)Cit_2$ increased with Fe deficiency in *B. vulgaris*, whereas in *P. communis* $Fe(III)Cit_2$ was lower in Fe-deficient trees.

SUMMARY AND CONCLUDING REMARKS

The new developments in MS techniques and the increased use of X-ray spectroscopy methods at synchrotron facilities have permitted the discovery of a number of natural metal species (*ca.* 19) in xylem and phloem saps (**Figure 1**; clear symbols). Moreover, evidence supporting the occurrence in such fluids of further putative metal species (*ca.* 15) has been provided by means of physiological studies, many of them applying molecular biology tools (**Figure 1**; faded symbols). Approximately 50% of the confirmed metal species and 27% of the putative ones have been reported in the xylem sap of plants (mostly hyper-accumulators) treated with high metal supply including Zn, Ni, and Cd. The second most abundant group of metal species has been described in the phloem sap of plants (including grasses) grown at low and adequate metal supply, and accounts for 37% of the confirmed metal species and 33% of those considered putative. This second (phloem) group includes mainly species containing Fe and Cu. Metal carboxylate complexes have always been found in xylem, whereas metals associated with proteins or high molecular weight compounds have been reported in phloem. Complexes with high affinity metal ligands (e.g., NA for Cu and Zn, PSs for Fe and PCs for Cd) have been commonly found in phloem sap, where complexation is needed to ensure metal solubility at the existing high pH as well as to avoid uncontrolled binding of metals in living cells, ensuring metal transport and delivery to sinks. Also, high affinity metal ligands have been found as metal carriers in the xylem sap when plants are grown with low or high metal supply. When metal availability is scarce, organic ligand-assisted xylem transport (e.g., Fe complexed by carboxylates or DMA) can increase transport efficiency, because complexation ensures metal delivery, while avoiding unwanted reactions and scavenging any metal pools occurring in the apoplast. Under conditions of high metal supply, organic ligand-assisted xylem transport (e.g., Ni-NA or Cd-PCs) may attenuate the toxicity derived of the exceptionally high metal concentrations as well as to ensure a correct delivery of other metals.

Most of the knowledge on metal complexes in xylem and phloem has been gained using plants (mostly hyper-accumulators) grown on hydroponics with either a short-time excess metal supply treatment or with a established metal deficiency followed by a short-time metal re-supply treatment (the latter in the case of Fe or Zn). Since these conditions are far from the natural ones, some of the reported metal species can differ from the actual complexes existing in plants in natural conditions (Centofanti et al., 2013). The reason to use such growth conditions is to force upwards the metal concentrations usually found in plant fluid samples (which rarely are $>50\,\mu M$; **Table 1**). These low concentrations make particularly challenging the speciation of metals in plant fluids in natural conditions. Also, plant fluids contain ions, sugars, proteins and other biomolecules along with the metal of interest and the rest of metals. This leads to a delicate balance among several metal species, which can include free metal ions and metal complexes having a great diversity of size, charge, and stability. Therefore, the direct analysis of the metal species in plant fluids requires of highly conservative (avoiding any alteration of sample that can damage metal species), sensitive and selective analytical techniques. Unfortunately, none of different techniques available comply with all of these characteristics.

X-ray spectroscopy methods are non-invasive and apparently conservative, although damage of the sample can occur, but they are much less sensitive than ICP-MS and much less selective than ESI-MS/MS. In contrast, MS-based techniques need to be combined with separation methodologies that are inherently invasive since they carry out the separation of the sample in fractions of different characteristics (e.g., molecular size in SEC), and this can significantly affect the distribution of metal chemical forms, particularly in the case of weak metal complexes specially sensitive to changes in pH and ligand-to-metal ratios. For these reasons, metal species found using MS techniques usually require confirmation using alternative separation methods to validate the actual existence of such metal-complexes in the samples, and sometimes alternative detection methods (e.g., NMR of isolated fractions) are needed to elucidate identity. Ultimately, the occurrence of the metal complexes identified *via* MS should be also confirmed in intact samples with XAS methods. On the other hand, the use of X-ray spectroscopy methods requires having pure standards of putative metal species, because data interpretation relies on linear combination fitting procedures where the sample spectra is fitted using those of standard compounds. Also, XAS techniques can hardly give any information on well-defined metal species when the sample contains several species with similar XANES spectra (e.g., metal complexes with carboxylates). Therefore, XAS also needs complementary techniques (e.g., ESI-MS/MS) to provide putative metal species as well as to confirm the correct identity of the metal species occurring in the samples.

The path of any given metal to its final destination (e.g., the chloroplast) involves transport across multiple membranes

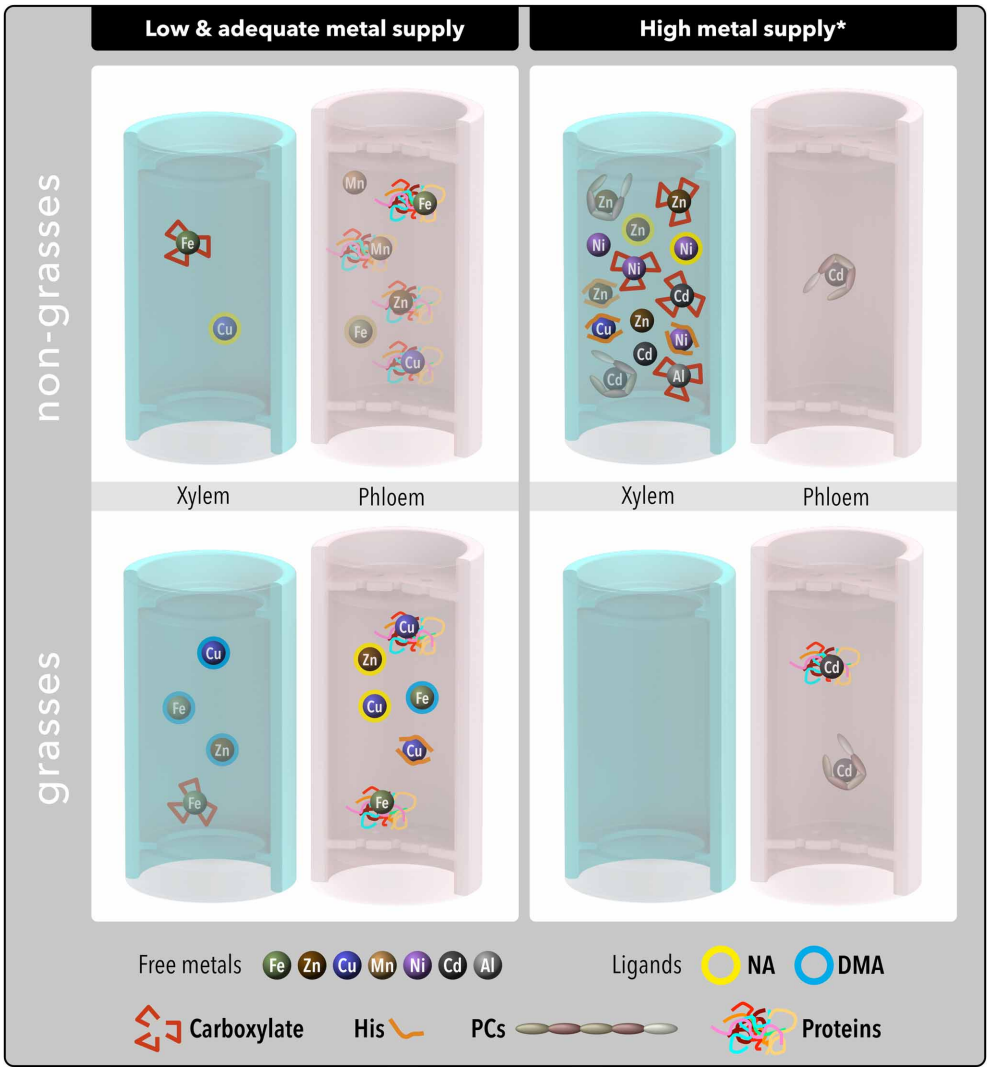

FIGURE 1 | Schematic representation showing the metal species found in the xylem and phloem saps of non-grass (upper panels) and grass plant species (lower panels) grown at low and adequate metal supply (left panels) or at high metal supply (right panels). Metals occurred in free ionic forms and in complexes with different ligands such as nicotianamine (NA), 2′-deoxymugineic acid (DMA), carboxylates (e.g., citrate), histidine (His), phytochelatins (PCs) and proteins. Putative metal species proposed to occur in plant fluids as supported by strong evidence from indirect approaches (e.g., molecular biology and others) are shown with faded symbols. *Most of the data with high metal supply have been described in metal hyper-accumulator plant species.

mediated by different transporter proteins and most likely metal complexation by different ligands in each compartment within the path. Although a complete picture of this complex process is still lacking even for one metal, an increasingly more complete and accurate knowledge of the metal species in plant fluids would be achieved performing studies that integrate molecular biology approaches, untargeted analyses of plant fluids using complementary MS-based and NMR techniques, and targeted XAS methods. The limits of detection and quantification of the techniques actually used are still far from ideal to analyze fluids coming from plants grown in natural conditions, and therefore more analytical efforts are required to completely decipher the metal species transported in plant fluids.

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REFERENCES

Abadía, J., López-Millán, A. F., Rombolà, A. D., and Abadía, A. (2002). Organic acids and Fe deficiency: a review. *Plant Soil* 241, 75–86. doi: 10.1023/A:1016093317898

Agraval, B., Czymmek, K. J., Sparks, D. L., and Bais, H. P. (2013). Transient influx of nickel in root mitochondria modulates organic acid and reactive oxygen species production in nickel hyper-accumulator *Alysuum murale*. *J. Biol. Chem.* 288, 7351–7362. doi: 10.1074/jbc.M112.406645

Aki, T., Shigyo, M., Nakano, R., Yoneyama, T., and Yanagisawa, S. (2008). Nano scale proteomics revealed the presence of regulatory proteins including three FT-like proteins in phloem and xylem saps from rice. *Plant Cell Physiol.* 49, 767–790. doi: 10.1093/pcp/pcn049

Alam, S., Kamei, S., and Kawai, S. (2001). Effect of iron deficiency on the chemical composition of the xylem xap of barley. *Soil Sci. Plant Nutr.* 47, 643–649. doi: 10.1080/00380768.2001.10408428

Alexou, M., and Peuke, A. D. (2013). Methods for xylem sap collection. *Methods Mol Biol.* 953, 195–207. doi: 10.1007/978-1-62703-152-3_13

Alves, S., Navais, C., Simões Gonçalves, M. de L., and Correia Dos Santos, M. M. (2011). Nickel speciation in the xylem sap of the hiperaccumulator *Alyssum serpyllifolium* ssp. Lusitanicum growing on serpentine soils of northeast Portugal. *J. Plant Physiol.* 168, 1715–1722. doi: 10.1016/j.jplph.2011.04.004

Andaluz, S., López-Millán, A. F., Peleato, M. L., Abadía, J., and Abadía, A. (2002). Increases in phospho*enol*pyruvate carboxylase activity in iron-deficient sugar beet roots: analysis of spatial localization and post-translational modification. *Plant Soil* 241, 43–48. doi: 10.1023/A:1016000216252

Ando, Y., Nagata, S., Yanagisawa, S., and Yoneyama, T. (2013). Copper in xylem and phloem saps from rice (*Oryza sativa*): the effect of moderate copper concentrations in the growth medium on the accumulation of five essential metals and a speciation analysis of copper-containing compounds. *Funct. Plant Biol.* 40, 89–100. doi: 10.1071/FP12158

Atkins, C. A., Smith, P. M. C., and Rodríguez-Medina, C. (2011). Macro-molecules in phloem exudates-a review. *Protoplasma* 248, 165–172. doi: 10.1007/s00709-010-0236-3

Aucour, A. M., Pichat, S., Macnair, M. R., and Oger, P. (2011). Fractionation of stable zinc isotopes in the zinc hyperaccumulator *Arabidopsis halleri* and nonaccumulator *Arabidopsis petraea*. *Environ. Sci. Technol.* 45, 9212–9217. doi: 10.1021/es200874x

Barabasz, A., Wilkowska, A., Ruszczyñsk, A., Bulska, E., Hanikenne, M., Czarny, M., et al. (2012). Metal response of transgenic tomato plants expressing P1B-ATPase. *Physiol. Plant.* 145, 315–331. doi: 10.1111/j.1399-3054.2012.01584.x

Barnes, A., Bale, J., Constantinidou, C., Ashton, P., Jones, A., and Pritchard, J. (2004). Determining protein identity from sieve element sap in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. *J. Exp. Bot.* 55, 1473–1481. doi: 10.1093/jxb/erh161

Baxter, I. R., Vitek, O., Lahner, B., Muthukumar, B., Borghi, M., Morrissey, J., et al. (2008). The leaf ionome as a multivariable system to detect a plant's physiological status. *Proc. Nat. Acad. Sci. U.S.A.* 105, 12081–12086. doi: 10.1073/pnas.0804175105

Benedicto, A., Hernández-Apaolaza, L., Rivas, I., and Lucena, J. J. (2011). Determination of ^{67}Zn distribution in navy bean (*Phaseolus vulgaris* L.) after foliar application of ^{67}Zn-lignosulfonates using isotope pattern deconvolution. *J. Agric. Food Chem.* 59, 8829–8838. doi: 10.1021/jf2002574

Bennett, J. H., Lee, E. H., Krizek, D. T., Olsen, R. A., and Brown, J. C. (1982). Photochemical reduction of iron. *II. Plant related factors. J. Plant Nutr.* 5, 335–344. doi: 10.1080/01904168209362962

Briat, J. F., Curie, C., and Gaymard, F. (2007). Iron utilization and metabolism in plants. *Curr. Opin. Plant Biol.* 10, 276–282. doi: 10.1016/j.pbi.2007.04.003

Cakmak, I., Welch, R. M., Erenoglu, B., Römheld, V., Norvell, W. A., and Kochian, L. V. (2000a). Influence of varied zinc supply on retranslocation of cadmium (^{109}Cd) and rubidium (^{86}Rb) applied on mature leaf of durum wheat seedlings. *Plant Soil* 219, 279–284. doi: 10.1023/A:1004777631452

Cakmak, I., Welch, R. M., Hart, J., Norvell, W. A., Oztürk, L., and Kochian, L. V. (2000b). Uptake and retranslocation of leaf−applied cadmium (^{109}Cd) in diploid, tetraploid and hexaploid wheats. *J. Exp. Bot.* 51, 221–226. doi: 10.1093/jexbot/51.343.221

Caldelas, C., Dong, S., Araus, J. L., and Weiss, D. J. (2011). Zinc isotopic fractionation in *Phragmites australis* in response to toxic levels of zinc. *J. Exp. Bot.* 62, 2169–2178. doi: 10.1093/jxb/erq414

Callahan, D. L., Baker, A. J. M., Kolev, S. D., and Wedd, A. G. (2006). Metal ion ligands in hyperaccumulating plants. *J. Biol. Inorg. Chem.* 11, 2–12. doi: 10.1007/s00775-005-0056-7

Callahan, D. L., Roessner, U., Dumontet, V., De Livera, A. M., Doronila, A., Baker, A. J. M., et al. (2012). Elemental and metabolite profiling of nickel hyperaccumulators from New Caledonia. *Phytochemistry* 81, 80–89. doi: 10.1016/j.phytochem.2012.06.010

Callahan, D. L., Roessner, U., Dumontet, V., Perrier, N., Wedd, A. G., O'Hair, R. A., et al. (2008). LC-MS and GC-MS metabolite profiling of nickel(II) complexes in the latex of the nickel-hyperaccumulating tree *Sebertia acuminata* and identification of methylated aldaric acid as new nickel(II) ligand. *Phytochemistry* 69, 240–251. doi: 10.1016/j.phytochem.2007.07.001

Carrasco-Gil, S., Álvarez-Fernández, A., Sobrino-Plata, J., Millán, R., Carpena-Ruiz, R. O., Leduc, D. L., et al. (2011). Complexation of Hg with phytochelatins is important for plant Hg tolerance. *Plant Cell Environ.* 34, 778–791. doi: 10.1111/j.1365-3040.2011.02281.x

Carrasco-Gil, S., Siebner, H., LeDuc, D. L., Webb, S. M., Millán, R., Andrews, J. C., et al. (2013). Mercury localization and speciation in plants grown hydroponically or in a natural environment. *Environ. Sci. Technol.* 47, 3082–3090. doi: 10.1021/es303310t

Centofanti, T., Sayers, Z., Cabello-Conejo, M. I., Kidd, P., Nishizawa, N., Kakei, Y., et al. (2013). Xylem exudate composition and root-to-shoot nickel translocation in *Alyssum* species. *Plant Soil* 373, 59–75. doi: 10.1007/s11104-013-1782-1

Chen, A., Komives, E. A., and Schroeder, J. I. (2006). An improved grafting technique for mature *Arabidopsis* plants demonstrates long-distance shoot-to-root transport of phytochelatins in *Arabidopsis*. *Plant Physiol.* 141, 108–120. doi: 10.1104/pp.105.072637

Chu, B. B., Luo, L. Q., Xu, T., Yuan, J., Sun, J. L., Zeng, Y., et al. (2012). XANES study of lead speciation in duckweed. *Spectrosc. Spect. Anal.* 32, 1975–1978. doi: 10.3964/j.issn.1000-0593(2012)07-1975-04

Clemens, S., Deinlein, U., Ahmadi, H., Höreth, S., and Uraguchi, S. (2013). Nicotianamine is a major player in plant Zn homeostasis. *Biometals* 26, 623–632. doi: 10.1007/s10534-013-9643-1

Clemens, S., Palmgren, M. G., and Krämer, U. (2002). A long way ahead: understanding and engineering plant metal accumulation. *Trends Plant Sci.* 7, 309–315. doi: 10.1016/S1360-1385(02)02295-1

Colangelo, E. P., and Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Curr. Opin. Plant Biol.* 9, 322–330. doi: 10.1016/j.pbi.2006.03.015

Conte, S. S., and Walker, E. L. (2011). Transporters contributing to iron trafficking in plants. *Mol. Plant* 4, 464–476. doi: 10.1093/mp/ssr015

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T. H., et al. (2012). Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. *Plant Cell* 24, 708–723. doi: 10.1105/tpc.111.095000

Dell'mour, M., Koellensperger, G., Quirino, J. P., Haddadm P. R., Stanetty, C., Oburger, E., et al. (2010). Complexation of metals by phytosiderophores revealed by CE-ESI-MS and CE-ICP-MS. *Electrophoresis* 31, 1201–1207. doi: 10.1002/elps.200900635

Deng, F., Yamaji, N., Xia, J., and Ma, J. F. (2013). A member of the heavy metal P-Type ATPase OsHMA5 is involved in xylem loading of copper in rice. *Plant Physiol.* 163, 1353–1362. doi: 10.1104/pp.113.226225

Dinant, S., and Kehr, J. (2013). Sampling and analysis of phloem sap. *Methods Mol Biol.* 953, 185–194. doi: 10.1007/978-1-62703-152-3_12

Donner, E., Punshon, T., Guerinot, M. L., and Lombi, E. (2012). Functional characterisation of metal(loid) processes in planta through the integration of synchrotron techniques and plant molecular biology. *Anal Bioanal. Chem.* 402, 3287–3298. doi: 10.1007/s00216-011-5624-9

Durrett, T. P., Gassmann, W. and Rogers, E. E. (2007). The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiol.* 144, 197–205. doi: 10.1104/pp.107.097162

Erenoglu, B., Nikolic, M., Römheld, V., and Cakmak, I. (2002). Uptake and transport of foliar applied zinc (65Zn) in bread and durum wheat cultivars differing in zinc efficiency. *Plant Soil* 241, 251–257. doi: 10.1023/A:1016148925918

Fiehn, O. (2003). Metabolic networks of *Cucurbita maxima* phloem. *Phytochemistry* 62, 875–886. doi: 10.1016/S0031-9422(02)00715-X

Freisinger, E. (2011). Structural features specific to plant metallothioneins. *J. Biol. Inorg. Chem.* 16, 1035–1045. doi: 10.1007/s00775-011-0801-z

Führs, H., Behrens, C., Gallien, S., Heintz, D., Dorsselaer, A. V., Braun, H. P., et al. (2010). Physiological and proteomic characterization of manganese sensitivity

and tolerance in rice (*Oryza sativa*) in comparison with barley (*Hordeum vulgare*). *Ann. Bot.* 105, 1129–1140. doi: 10.1093/aob/mcq046

Fujimaki, S., Suzui, N., Ishioka, N. S., Kawachi, N., Ito, S., Chino, M., et al. (2010). Tracing cadmium from culture to spikelet: non-invasive imaging and quantitative characterization of absorption, transport, and accumulation of cadmium in an intact rice plant. *Plant Physiol.* 152, 1796–1806. doi: 10.1104/pp.109.151035

García-Hernández, M., Murphy, A. and Taiz, L. (1998). Metallothioneins 1 and 2 have distinct but overlapping expression patterns in *Arabidopsis*. *Plant Physiol.* 118, 387–397. doi: 10.1104/pp.118.2.387

Giavalisco, P., Kapitza, K., Kolasa, A., Buhtz, A., and Kehr J. (2006). Towards the proteome of *Brassica napus* phloem sap. *Proteomics* 6, 896–909. doi: 10.1002/pmic.200500155

Gogorcena, Y., Larbi, A., Andaluz, S., Carpena, R. O., Abadía, A., and Abadía, J. (2011). Effects of cadmium on cork oak (*Quercus suber* L.) plants grown in hydroponics. *Tree Physiol.* 31, 1401–1412. doi: 10.1093/treephys/tpr114

Gong, J. M., Lee, D. A., and Schroeder, J. I. (2003). Long distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10118–10123. doi: 10.1073/pnas.1734072100

Grillet, L., Ouerdane, L., Flis, P., Hoang, M. T. T., Isaure, M. P., Lobiński, R., et al. (2014). Ascorbate efflux as a new strategy for iron reduction and transport in plants. *J. Biol. Chem.* 289, 2515–2525. doi: 10.1074/jbc.M113.514828

Grusak, M. A. (1994). Iron transport to developing ovules of *Pisum sativum*. I. Seed import characteristics and phloem iron-loading capacity of source regions. *Plant Physiol.* 104, 649–655.

Guelke-Stelling, M., and von Blanckenburg, F. (2007). Fractionation of stable iron isotopes in higher plants. *Environ. Sci. Technol.* 41, 1896–1901. doi: 10.1021/es062288j

Guelke-Stelling, M., and von Blanckenburg, F. (2012). Fe isotope fractionation caused by translocation of iron during growth of bean and oat as models of strategy I and II plants. *Plant Soil* 352, 217–231. doi: 10.1007/s11104-011-0990-9

Han, D. G., Wang, L., Wang, Y., Yang, G. H., Gao, C., Yu, Z. Y., et al. (2013). Overexpression of Malus xiaojinensis CS1 gene in tobacco affects plant development and increases iron stress tolerance. *Sci. Hortic.* 150, 65–72. doi: 10.1016/j.scienta.2012.10.004

Han, D. G., Wang, Y., Zhang, L., Ma, L., Zhang, X. Z., Xu, X. F., et al. (2012). Isolation and functional characterization of *MxCS1*: a gene encoding a citrate synthase in *Malus xiaojinensis*. *Biol. Plantarum* 56, 50–56. doi: 10.1007/s10535-012-0015-4

Harris, N. S., and Taylor, G. J. (2001). Remobilization of cadmium in maturing shoots of near isogenic lines of durum wheat that differ in grain cadmium accumulation. *J. Exp. Bot.* 52, 1473–1481. doi: 10.1093/jexbot/52.360.1473

Harris, W. R., Sammons, D. R., and Grabiak, R. C. (2012). A speciation model of essential trace elements in phloem. *J. Inorg. Biochem.* 116, 140–150. doi: 10.1016/j.jinorgbio.2012.07.011

Haydon, M. J., and Cobbett, C. S. (2007). Transporters of ligands for essential metal ions in plants. *New Phytol.* 174, 499–506. doi: 10.1111/j.1469-8137.2007.02051.x

Haydon, M. J., Kawachi, M., Wirtz, M., Hillmer, S., Hell, R., and Krämer, U. (2012). Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in *Arabidopsis*. *Plant Cell* 24, 724–737. doi: 10.1105/tpc.111.095042

Heise, J., Krejci, S., Miersch, J., Krauss, G. J., and Humbeck, K. (2007). Gene expression of metallothioneins in barley during senescence and heavy metal treatment. *Crop Sci.* 47, 1111–1118. doi: 10.2135/cropsci2006.03.0183

Hocking, P. J. (1980). The composition of phloem exudate and xylem sap from tree tobacco (*Nicotiana glauca* Grah.). *Ann. Bot.* 45, 633–643.

Hocking, P. J. (1983). The dynamics of growth and nutrient accumulation by fruits of *Grevillea leucopteris* Meissn., a proteaceous shrub, with special reference to the composition of xylem and phloem sap. *New Phytol.* 93, 511–529. doi: 10.1111/j.1469-8137.1983.tb02702.x

Huguet, S., Bert, V., Laboudigue, A., Barthes, V., Isaure, M. P., Llorens, I., et al. (2012). Cd speciation and localization in the hyperaccumulator *Arabidopsis halleri*. *Environ. Exp. Bot.* 82, 54–65. doi: 10.1016/j.envexpbot.2012.03.011

Husted, S., Persson, D. P., Laursen, K. H., Hansen, T. H., Pedas, P., Schiller, M., et al. (2011). Review: the role of atomic spectrometry in plant science. *J. Anal. At. Spectrom.* 26, 52–79. doi: 10.1039/c0ja00058b

Ingle, R. A., Mugford, S. T., Rees, J. D., Campbell, M. M., and Smith, J. C. (2005). Constitutively high expression of the histidine biosynthetic pathway contributes to nickel tolerance in hyperaccumulator plants. *Plant Cell* 17, 2089–2106. doi: 10.1105/tpc.104.030577

Irtelli, B., Petrucci, W. A., and Navari-Izzo, F. (2009). Nicotianamine and histidine/proline are, respectively, the most important copper chelators in xylem sap of *Brassica carinata* under conditions of copper deficiency and excess. *J. Exp. Bot.* 60, 269–277. doi: 10.1093/jxb/ern286

Ishikawa, S., Suzui, N., Ito-Tanabata, S., Ishii, S., Igura, M., Abe, T., et al. (2011). Real-time imaging and analysis of differences in cadmium dynamics in rice cultivars (*Oryza sativa*) using positron-emitting ^{107}Cd tracer. *BMC Plant Biol.* 11:172. doi: 10.1186/1471-2229-11-172

Ishimaru, Y., Kakei, Y., Shimo, H., Bashir, K., Sato, Y., Sato, Y., et al. (2011). A rice phenolic efflux transporter is essential for solubilizing precipitated apoplasmic iron in the plant stele. *J. Biol. Chem.* 286, 24649–24655. doi: 10.1074/jbc.M111.221168

Jelali, N., Wissal, M. S., Dell'orto, M., Abdelly, C., Gharsalli, M., and Zocchi, G. (2010). Changes of metabolic responses to direct and induced Fe deficiency of two *Pisum sativum* cultivars. *Environ. Exp. Bot.* 68, 238–246. doi: 10.1016/j.envexpbot.2009.12.003

Kakei, Y., Yamaguchi, I., Kobayashi, T., Takahashi, T., Nakanishi, H., Yamakawa, T., et al. (2009). A highly sensitive, quick and simple quantification method for nicotianamine and 2′-deoxymugineic acid from minimum samples using LC/ESI-TOF-MS achieves functional analysis of these components in plants. *Plant. Cell Physiol.* 50, 1988–1993. doi: 10.1093/pcp/pcp141

Kato, M., Ishikawa, S. Inagaki, K., Chiba, K., Hayashi, H., Yanagisa, S., and Yoneyama, T. (2010). Possible chemical forms of cadmium and varietal differences in cadmium concentrations in the phloem sap of rice plants (*Oryza sativa* L.). *Soil Sci. Plant Nutr.* 56, 839–847. doi: 10.1111/j.1747-0765.2010.00514.x

Kawai, S., Kamei, S., Matsuda, Y., Ando, R., Kondo, S., Ishizawa, A., et al. (2001). Concentrations of iron and phytosiderophores in xylem sap of iron-deficient barley plants. *Soil Sci. Plant Nutr.* 47, 265–272. doi: 10.1080/00380768.2001.10408390

Kerkeb, L., and Krämer, U. (2003). The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiol.* 131, 716–724. doi: 10.1104/pp102.010686

Khouzam, R. B., Szpunar, J., Holeman, M., and Lobiński, R. (2012). Trace element speciation in food: state of the art of analytical techniques and methods. *Pure Appl. Chem.* 84, 169–179. doi: 10.1351/PAC-CON-11-08-14

Kiczka, M., Wiederhold, J. G., Kraemer, S. M., Bourdon, B., and Kretzschmar, R. (2010). Iron isotope fractionation during Fe uptake and translocation in alpine plants. *Environ. Sci. Technol.* 44, 6144–6150. doi: 10.1021/es100863b

Kim, S. A., and Guerinot M. L. (2007). Mining iron: iron uptake and transport in plants. *FEBS Lett.* 581, 2273–2280. doi: 10.1016/j.febslet.2007.04.043

Klatte, M., Schuler, M., Wirtz, M., Fink-Straube, C., Hell, R., and Bauer, P. (2009). The analysis of Arabidopsis nicotianamine synthase mutants reveals functions for nicotianamine in seed iron loading and iron deficiency responses. *Plant Physiol.* 150, 251–271. doi: 10.1104/pp.109.136374

Kobayashi, N. I., Tanoi, K., Hirose, A., and Nakanishi, T. M. (2013). Characterization of rapid intervascular transport of cadmium in rice stem by radioisotope imaging. *J. Exp. Bot.* 64, 507–517. doi: 10.1093/jxb/ers344

Köster, J., Hayen, H., von Wirén, N., and Weber, G. (2011b). Isoelectric focusing of small non-covalent metal species from plants. *Electrophoresis* 32, 772–781. doi: 10.1002/elps.201000529

Köster, J., Shi, R., von Wirén, N., and Weber, G. (2011a). Evaluation of different column types for the hydrophilic interaction chromatographic separation of iron-citrate and copper-histidine species from plants. *J. Chromatogr. A* 1218, 4934–4943. doi: 10.1016/j.chroma.2011.03.036

Krämer, U. (2010). Metal hyperaccumulation in plants. *Annu. Rev. Plant. Biol.* 61, 517–534. doi: 10.1146/annurev-arplant-042809-112156

Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. M., and Smith, J. A. C. (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379, 635–638. doi: 10.1038/379635a0

Krämer, U., Talke, I. N., and Hanikenne, M. (2007). Transition metal transport. *FEBS Lett.* 581, 2263–2272. doi: 10.1016/j.febslet.2007.04.010

Krüger, C., Berkowitz, O., Stephan, U., and Hell, R. (2002). A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of *Ricinus communis* L. *J. Biol. Chem.* 277, 25062–25069. doi: 10.1074/jbc.M201896200

Küpper, H., Goetz, B., Mijovilovich, A., Kupper, F. C., and Meyer-Klaucke, W. (2009). Complexation and toxicity of copper in higher plants. I.

Characterization of copper accumulation, speciation, and toxicity in *Crassula helmsii* as a new copper accumulator. *Plant Physiol.* 151, 702–714. doi: 10.1104/pp.109.139717

Küpper, H., Mijovilovich, A., Meyer-Klaucke, W., and Kroneck, P. M. (2004). Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by x-ray absorption spectroscopy. *Plant Physiol.* 134, 748–757. doi: 10.1104/pp.103.032953

Larbi, A., Morales, F., Abadía, A., and Abadía, J. (2010). Changes in iron and organic acid concentrations in xylem sap and apoplastic fluid of iron-deficient *Beta vulgaris* plants in response to iron resupply. *J. Plant Physiol.* 167, 255–260. doi: 10.1016/j.jplph.2009.09.007

Larbi, A., Morales, F., Abadía, J., and Abadía, A. (2003). Effects of branch solid Fe sulphate implants on xylem sap composition in field-grown peach and pear: changes in Fe, organic anions and pH. *J. Plant Physiol.* 160, 1473–1481. doi: 10.1078/0176-1617-01010

Lasat, M. M., Baker, A. L. J. M., and Kochian, L. V. (1998). Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiol.* 118, 875–883. doi: 10.1104/pp.118.3.875

Lattanzio, G., Andaluz, S., Andrea Matros, A., Calvete, J. J., Kehr, J., Abadía, A., et al. (2013). Protein profile of *Lupinus texensis* phloem sap exudates: searching for Fe and Zn containing proteins. *Proteomics* 13, 2283–2296. doi: 10.1002/pmic.201200515

Leitenmaier, B., and Küpper H. (2011). Cadmium uptake and sequestration kinetics in individual leaf cell protoplasts of the Cd/Zn hyperaccumulator *Thlaspi caerulescens*. *Plant Cell Environ.* 34, 208–219. doi: 10.1111/j.1365-3040.2010.02236.x

Leitenmaier, B., and Küpper, H. (2013). Compartmentation and complexation of metals in hyperaccumulator plants. *Front. Plant Sci.* 4:374. doi: 10.3389/fpls.2013.00374

Li, Y., Dankher, O. P., Carreira, L., Smith, A. P., and Meagher, R. B. (2006). The shoot-specific expression of γ-glutamylcysteine synthetase directs the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. *Plant Physiol.*141, 288–298. doi: 10.1104/pp.105.074815

Liao, M. T., Hedley, M. J., Woolley, D. J., Brooks, R. R., and Nichols, M. A. (2000). Copper uptake and translocation in chicory (*Cichorium intybus* L. cv. Grasslands Puna) and tomato (*Lycopersicon esculentum* Mill. cv. Rondy) plants grown in NFT system. I. Copper uptake and distribution in plants. *Plant Soil* 221, 135–142. doi: 10.1023/A:1004731415931

Lohaus, G., Pennewis, K., Sattelmacher, B., Hussman, M., and Muehling, K. H. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? *A critical evaluation with different plant species. Physiol. Plant.* 111, 457–465. doi: 10.1034/j.1399–3054.2001.1110405.x

Lombi, E., Scheckel, K. G., and Kempson, I. M. (2011). *In situ* analysis of metal(loid)s in plants: state of the art and artefacts. *Environ. Exp. Botany*. 72, 3–17. doi: 10.1016/j.envexpbot.2010.04.005

López-Millán, A. F., Grusak, M. A., and Abadía, J. (2012). Carboxylate metabolism changes induced by Fe deficiency in barley, a Strategy II plant species. *J. Plant Physiol.* 169, 1121–1124. doi: 10.1016/j.jplph.2012.04.010

López-Millán, A. F., Morales, F., Abadía, A., and Abadía, J. (2000). Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. *Plant Physiol.* 124, 873–884. doi: 10.1104/pp.124.2.873

López-Millán, A. F., Morales, F., Abadía, A., and Abadía, J. (2001). Iron deficiency-associated changes in the composition of the leaf apoplast fluid from field-grown pear (*Pyrus communis* L.) trees. *J. Exp. Bot.* 52, 1489–1498. doi: 10.1093/jexbot/52.360.1489

López-Millán, A. F., Morales, F., Gogorcena, Y., Abadía, A., and Abadía, J. (2009). Organic acid metabolism in iron deficient tomato plants. *J. Plant Physiol.* 166, 375–384. doi: 10.1016/j.jplph.2008.06.011

Lu, L., Tian, S., Zhang, J., Yang, X., Labavitch, J. M., Webb, S. M., et al. (2013). Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant species *Sedum alfredii*. *New Phytol.* 198, 721–731. doi: 10.1111/nph.12168

Ma, J. F., and Hiradate, S. (2000). Form of aluminium for uptake and translocation in buckwheat (*Fagopyrum esculentum* Moench). *Planta* 211, 355–360. doi: 10.1007/s004250000292

Marentes, E., and Grusak, M. A. (1998). Iron transport and storage within the seed coat of developing embryos of pea (*Pisum sativum* L). *Seed Sci. Res.* 8, 367–375. doi: 10.1017/S0960258500004293

Mari, S., Gendre, D., Pianelli, K., Ouerdane, L., Lobinski, R., Briat, J. F., et al. (2006). Root-to-shoot long-distance circulation of nicotianamine and nicotianamine-nickel chelates in the metal hyperaccumulator *Thlaspi caerulescens*. *J. Exp. Bot.* 57, 4111–4122. doi: 10.1093/jxb/erl184

Martínez-Cuenca, M. R., Iglesias, D. J., Talón, M., Abadía, J., López-Millán, A. F., Primo-Millo, E., et al. (2013). Metabolic responses to iron deficiency in roots of Carrizo citrange (*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L) Raf.). *Tree Physiol.* 33, 320–329. doi: 10.1093/treephys/tpt011

McNear, D. H., Afton, S. E., and Caruso, J. A. (2012). Exploring the structural basis for selenium/mercury antagonism in *Allium fistulosum*. *Metallomics* 4, 267–276. doi: 10.1039/c2mt00158f

McNear, D. H., Chaney, R. L., and Sparks, D. L. (2010). The metal hyperaccumulator *Alyssum murale* uses nitrogen and oxygen donor ligands for Ni transport and storage. *Phytochemistry* 71, 188–200. doi: 10.1016/j.phytochem.2009.10.023

Meija, J., Montes-Bayón, M., Caruso, J. A., and Sanz-Medel, A. (2006). Integrated mass spectrometry in (semi) metal speciation and its potential in phytochemistry. *Trends Anal. Chem.* 25, 44–51. doi: 10.1016/j.trac.2005.04.003

Mendoza-Cózatl, D. G., Butko, E., Springer, F., Torpey, J. W., Komives, E. A., Kehr, J., et al. (2008). Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. *Plant J.* 54, 249–259. doi: 10.1111/j.1365-313X.2008.03410.x

Mendoza-Cózatl, D. G., Jobe, T. O., Hauser, F., and Schroeder, J. I. (2011). Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Curr. Opin. Plant Biol.* 14, 554–562. doi: 10.1016/j.pbi.2011.07.004

Mesko, M. F., Hartwig, C. A., Bizzi, C. A., Pereira, J. S. F., Mello, P. A., and Flores, E. M. M. (2011). Sample preparation strategies for bioinorganic analysis by inductively coupled plasma mass spectrometry. *Int. J. Mass Spectrom.* 307, 123–136. doi: 10.1016/j.ijms.2011.03.002

Meyer, S., Angeli, A. D., Fernie, A. R., and Martinoia, E. (2010). Intra- and extra-cellular excretion of carboxylates. *Trends Plant Sci.* 15, 40–47. doi: 10.1016/j.tplants.2009.10.002

Mijovilovich, A., Leitenmaier, B., Mayer-Klaucke, W., Kroneck, P. M. H., Götz, B., and Küpper, H. (2009). Complexation and toxicity of copper in higher plants II. Different mechanisms for copper vs. cadmium detoxification in the copper-sensitive cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype). *Plant Physiol.* 151, 715–731. doi: 10.1104/pp.109.144675

Milner, M. J., and Kochian, L. V. (2008). Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Ann. Bot.* 102, 3–13. doi: 10.1093/aob/mcn063

Monicou, S., Szpunar, J., and Lobinski, R. (2009). Metallomics: the concept and methodology. *Chem. Soc. Rev.* 38, 1119–1138. doi: 10.1039/b713633c

Monsant, A. C., Kappen, P., Wang, Y., Pigram, P. J., Baker, A. J. M., and Tang, C. (2011). *In vivo* speciation of zinc in *Noccaea caerulescens* in response to nitrogen form and zinc exposure. *Plant Soil*. 348, 167–183. doi: 10.1007/s11104-011-0887-7

Mori, S., Uraguchi, S., Ishikawa, S., and Arao, T. (2009). Xylem loading process is a critical factor in determining Cd accumulation in the shoots of *Solanum melongena* and *Solanum torvum*. *Environ. Exp. Bot.* 67, 127–132. doi: 10.1016/j.envexpbot.2009.05.006

Morita, A., Horie, H., Fujii, Y., Takatsu, S., Watanabe, N., Yagi, A., et al. (2004). Chemical forms of aluminum in xylem sap of tea plants (*Camellia sinensis* L.). *Phytochemistry* 65, 2775–2780. doi: 10.1016/j.phytochem.2004.08.043

Morrissey, J., and Guerinot, M. L. (2009). Iron uptake and transport in plants: the good, the bad and the ionome. *Chem. Rev.* 109, 4553–4567. doi: 10.1021/cr900112r

Moynier, F., Fujii, T.,Wang, K., and Foriel, J. (2013). Ab initio calculations of the Fe(II) and Fe(III) isotopic effects in citrates, nicotianamine, and phytosiderophore, and new Fe isotopic measurements in higher plants. *Comp. Rend. Geosci.* 345, 230–240. doi: 10.1016/j.crte.2013.05.003

Moynier, F., Pichat, S., Pons, M. L., Fike, D., Balter, V., and Albarède, F. (2009). Isotopic fractionation and transport mechanisms of Zn in plants. *Chem. Geol.* 267, 125–130. doi: 10.1016/j.chemgeo.2008.09.017

Mullins, G. L., Sommers, L. E., and Housley, T. L. (1986). Metal speciation in xylem and phloem exudates. *Plant Soil* 96, 377–391. doi: 10.1007/BF02375142

Murakami, T., Ise, K., Hayakawa, M., Kamei, S., and Takagi, S. (1989). Stabilities of metal complexes of mugineic acid and their specific affinities for iron(III). *Chem Lett. (Jpn.)* 18, 2137–2140. doi: 10.1246/cl.1989.2137

Nakamura, S., Akiyama, C., Sasaki, T., Hattori, H., and Chino, M. (2008). Effect of cadmium on the chemical composition of xylem exudates from oilseed rape plants (*Brassica napus* L.). *Soil Sci. Plant Nutr.* 54, 118–127. doi: 10.1111/j.1747-0765.2007.00214.x

Nishiyama, R., Kato, M., Nagata, S., Yanagisawa, S., and Yoneyama, T. (2012). Identification of Zn–nicotianamine and Fe–2′-deoxymugineic acid in the phloem sap from rice plants (*Oryza sativa* L.). *Plant Cell Physiol.* 53, 381–390. doi: 10.1093/pcp/pcr188

Orera, I., Rodríguez-Castrillón, J. A., Moldovan, M., García-Alonso, J. I., Abadía, A., Abadía, J., et al. (2010). Using a dual-stable isotope tracer method to study the uptake, xylem transport and distribution of Fe and its chelating agent from stereoisomers of an Fe(III)-chelate used as fertilizer in Fe-deficient Strategy I plants. *Metallomics* 2, 646–657. doi: 10.1039/c0mt00018c

Ouerdane, L., Mari, S., Czernic, P., Lebrun, M., and Lobiński, R. (2006). Speciation of non-covalent nickel species in plant tissue extracts by electrospray Q-TOFMS/MS after their isolation by 2D size exclusion-hydrophilic interaction LC (SEC-HILIC) monitored by ICP-MS. *J. Anal. At. Spectrom*. 21, 676–683. doi: 10.1039/b602689c

Pal, R., and Rai, J. P. N. (2010). Phytochelatins: peptides involved in heavy metal detoxification. *Appl. Biochem. Biotechnol.* 160, 945–963. doi: 10.1007/s12010-009-8565-4

Palmer, C. M., and Guerinot, M. L. (2009). Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nat. Chem. Biol*. 5, 333–340. doi: 10.1038/nchembio.166

Palmer, L. J., Palmer, L. T., Pritchard, J., Graham, R. D., and Stangoulis, J. C. R. (2013). Improved techniques for measurement of nanolitre volumes of phloem exudate from aphid stylectomy. *Plant Methods* 9, 18. doi: 10.1186/1746-4811-9-18

Persans, M. W., Yan, X., Patnoe, J. M. L., Krämer, U., and Salt, D. E. (1999). Molecular dissection of the role of histidine in nickel hyperaccumulation in *Thlaspi goesingense* (Haìlaìcsy). *Plant Physiol.* 121, 1117–1126. doi: 10.1104/pp.121.4.1117

Pich, A., and Scholz, G. (1996). Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum* Mill.): nicotianamine-stimulated copper transport in the xylem. *J. Exp. Bot.* 47, 41–47. doi: 10.1093/jxb/47.1.41

Pich, A., Scholz, G., and Stephan, U. (1994). Iron-dependent changes of heavy-metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes- nicotianamine as a possible copper translocator. *Plant Soil* 165, 189–196. doi: 10.1007/BF00008061

Pittman, J. K. (2005). Managing the manganese: molecular mechanisms of manganese transport and homeostasis. *New Phytol.* 167, 733–742. doi: 10.1111/j.1469-8137.2005.01453.x

Puig, S., and Peñarrubia, L. (2009). Placing metal micronutrients in context: transport and distribution in plants. *Curr. Opin. Plant Biol.* 12, 299–306. doi: 10.1016/j.pbi.2009.04.008

Rellán-Álvarez, R., Abadía, J., and Álvarez-Fernández, A. (2008). Formation of metal-nicotianamine complexes as affected by pH, ligand exchange with citrate and metal exchange. A study by electrospray ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Sp.*. 22, 1553–1562. doi: 10.1002/rcm.3523

Rellán-Álvarez, R., El-Jendoubi, H., Wohlgemuth, G., Abadía, A., Fiehn, O., Abadía, A., et al. (2011a). Metabolite profile changes in xylem sap and leaf extracts of strategy I plants in response to iron deficiency and resupply. *Front. Plant Sci.* 2:66. doi: 10.3389/fpls.2011.00066

Rellán-Álvarez, R., Giner-Martínez-Sierra, J., Orduna, J., Orera, I., Rodríguez-Castrillón, J. A., García-Alonso, J. I., et al. (2010). Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol*. 51, 91–102. doi: 10.1093/pcp/pcp170

Rellán-Álvarez, R., López-Gomollón, S., Abadía, J., and Álvarez-Fernández, A. (2011b). Development of a new high-performance liquid chromatography-electrospray ionization Time-of-Flight mass spectrometry method for the determination of low molecular mass organic acids in plant tissue extracts. *J. Agric. Food Chem.* 59, 6864–6870. doi: 10.1021/jf200482a

Rodríguez-Castrillón, J. A., Moldovan, M., García-Alonso, J. I., Lucena, J. J., García-Tome, M. L., and Hernández-Apaolaza, L. (2008). Isotope pattern deconvolution as a tool to study iron metabolism in plants. *Anal. Bioanal. Chem.* 390, 579–590. doi: 10.1007/s00216-007-1716-y

Rodríguez-Medina, C., Atkins, C. A., Mann, A. J., Jordan, M. E., and Smith, P. M. C. (2011). Macromolecular composition of phloem exudate from white lupin (*Lupinus albus* L.). *BMC Plant Biol*. 11:36. doi: 10.1186/1471-2229-11-36

Rojas, C. L., Romera, F. J., Alcántara, E., Pérez-Vicente, R., Sariego, C., García-Alonso, J. I., et al. (2008). Efficacy of Fe(*o*,*o*-EDDHA) and Fe(*o*,*p*-EDDHA) isomers in supplying Fe to Strategy I plants differs in nutrient solution and calcareous soil. *J. Agric. Food Chem.* 56, 10774–10778. doi: 10.1021/jf80 22589

Rombolà, A. D., Brüggemann, W., López-Millán, A. F., Tagliavini, M., Abadía, J., Marangoni, B., et al. (2002). Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). *Tree Physiol.* 22, 869–875. doi: 10.1093/treephys/22.12.869

Roschzttardtz, H., Séguéla-Arnaud, M., Briat, J. F., Vert, G., and Curie, C. (2011). The FRD3 citrate effluxer promotes iron nutrition between symplastically disconnected tissues throughout Arabidopsis development. *Plant Cell* 23, 2725–2737. doi: 10.1105/tpc.111.088088

Ryan, B. M., Kirby, J. K., Degryse, F., Harris, H., McLaughlin, M. J., and Scheiderich, K. (2013). Copper speciation and isotopic fractionation in plants: uptake and translocation mechanisms. *New Phytol.* 199, 367–378. doi: 10.1111/nph.12276

Sagardoy, R. (2012). "Effects of Zn and Cd toxicity on metal concentrations in the xylem sap of *B. vulgaris* and *S. esculentum*," in *Study of Zn and Cd Homeostasis in Higher Plants*. Ph.D. Thesis, Zaragoza: University of Zaragoza.

Sagardoy, R., Morales, F., Rellán-Álvarez, R., Abadía, J., Abadía, A., and López-Millán, A. F. (2011). Carboxylate metabolism in sugar beet plants grown with excess Zn. *J. Plant Physiol*. 168, 730–733. doi: 10.1016/j.jplph.2010.10.012

Salt, D. E., Prince, R. C., Baker, A. J. M., Raskin, I., and Pickering, I. J. (1999). Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environ. Sci. Technol*. 33, 713–717. doi: 10.1021/es980825x

Salt, D. E., Prince, R. C., Pickering, I. J., and Raskin, I. (1995). Mechanisms of cadmium mobility and accumulation in indian mustard. *Plant Physiol*. 109, 1427–1433.

Sarret, G., Pilon Smits, E. A. H., Castillo Michel, H., Isaure, M. P., Zhao, F. J., and Tappero, R. (2013). Use of synchrotron-based techniques to elucidate metal uptake and metabolism in plants. *Adv. Agron.* 119, 1–82. doi: 10.1016/B978-0-12-407247-3.00001-9

Sasaki, A., Yamaji, N., Xia, J., and Ma, J. F. (2011). OsYSL6 Is involved in the detoxification of excess manganese in rice. *Plant Physiol.* 157, 1832–1840. doi: 10.1104/pp.111.186031

Sattelmacher, B. (2001). The apoplast and its significance for plant mineral nutrition. *New Phytol*. 149, 167–192. doi: 10.1046/j.1469-8137.2001.00034.x

Schaumlöffel, D., Ouerdane, L., Bouyssiere, B., and £obin, R. (2003). Speciation analysis of nickel in the latex of a hyperaccumulating tree *Sebertia acuminate* by HPLC and CZE with ICP MS and electrospray MS-MS detection. *J. Anal. At. Spectrom*. 18, 120–127. doi: 10.1039/b209819a

Schmidke, I., Krüger, C., Frömmichen, R., Scholz, G., and Stephan, U. W. (1999). Phloem loading and transport characteristics of iron in interaction with plant-endogenous ligands in castor bean seedlings. *Physiol. Plant.* 106, 82–89. doi: 10.1034/j.1399-3054.1999.106112.x

Schmidke, I., and Stephan, U. W. (1995). Transport of metal micronutrients in the phloem of castor bean (*Ricinus communis*) seedlings. *Physiol. Plant.* 95, 147–153. doi: 10.1111/j.1399-3054.1995.tb00821.x

Schmidt, H., Böttcher, C., Trampczynska, A., and Schuler, S. C. (2011). Use of recombinantly produced $^{15}N_3$-labelled nicotianamine for fast and sensitive stable isotope dilution ultra-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry. *Anal. Bioanal. Chem*. 399, 1355–1361. doi: 10.1007/s00216-010-4436-7

Schuler, M., Rellán-Álvarez, R., Fink-Straube, C., Abadía, J., and Bauer, P. (2012). Nicotianamine functions in the phloem-based transport of iron to sink organs, in pollen development and in pollen tube growth in *Arabidopsis*. *Plant Cell* 24, 2380–2400. doi: 10.1105/tpc.112.099077

Shelp, B. J. (1987). The composition of phloem exudate and xylem sap from Broccoli (*Brassica oleracea* var. italica) supplied with NH^{4+}, NO_3^- or NH_4NO_3. *J. Exp. Bot.* 38, 1619–1636. doi: 10.1093/jxb/38.10.1619

Silva, A. M., Kong, X., Parkin, M. C., Cammack, R., and Hider, R. C. (2009). Iron(III) citrate speciation in aqueous solution. *Dalton Trans*. 40, 8616–8625. doi: 10.1039/b910970f

Sinclair, S. A., and Krämer, U. (2012). The zinc homeostasis network of land plants. *Biochim. Biophys. Acta* 1823, 1553–1567. doi: 10.1016/j.bbamcr.2012.05.016

Singh, U. M., Sareen, P., Sengar, R. S., and Kumar, A. (2013). Plant ionomics: a newer approach to study mineral transport and its regulation. *Acta Physiol. Plant.* 35, 2641–2653. doi: 10.1007/s11738-013-1316-8

Song, J., Yang, Y. Q., Zhu, S. H., Chen, G. C., Yuan, X. F., Liu, T. T., et al. (2013). Spatial distribution and speciation of copper in root tips of cucumber revealed by μ-XRF and μ-XANES. *Biol. Plant.* 57, 581–586. doi: 10.1007/s10535-013-0317-1

Sperotto, R. A., Ricachenevsky, F. K., Waldow Vde A., and Fett, J. P. (2012). Iron biofortification in rice: it's a long way to the top. *Plant. Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Suzuki, M., Takahashi, M., Tsukamoto, T., Watanabe, S., Matsuhashi, S., Yazaki, J., et al. (2006). Biosynthesis and secretion of mugineic acid family phytosiderophores in zinc-deficient barley. *Plant J.* 48, 85–97. doi: 10.1111/j.1365-313X.2006.02853.x

Suzuki, M., Tsukamoto, T., Inoue, H., Watanabe, S., Matsuhashi, S., Takahashi, M., et al. (2008). Deoxymugineic acid increases Zn translocation in Zn-deficient rice plants. *Plant Mol. Biol.* 66, 609–617. doi: 10.1007/s11103-008-9292-x

Takahashi, M., Terada, Y., Nakai, I., Nakanishi, H., Yoshimura, E., Mori, S., et al. (2003). Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. *Plant Cell* 15, 1263–1280. doi: 10.1105/tpc.010256

Tanaka, K., Fujimaki, S., Fujiwara, T., Yoneyama, T., and Hayashi, H. (2007). Quantitative estimation of the contribution of the phloem in cadmium transport to grains in rice plants (*Oryza sativa* L.). *Soil Sci. Plant Nutr.* 53, 72–77. doi: 10.1111/j.1747-0765.2007.00116.x

Tehseen, M., Cairns, N., Sherson, S., and Cobbett, C. S. (2010). Metallochaperone-like genes in *Arabidopsis thaliana*. *Metallomics* 2, 556–564. doi: 10.1039/c003484c

Terzano, R., Mimmo, T., Vekemans, B., Vincze, L., Falkenberg, G., Tomasi, N., et al. (2013). Iron (Fe) speciation in xylem sap by XANES at a high brilliant synchrotron X-ray source: opportunities and limitations. *Anal. Bioanal. Chem.* 405, 5411–5419. doi: 10.1007/s00216-013-6959-1

Thimm, O., Essigmann, B., Kloska, S., Altmann, T., and Buckhout, T. J. (2001). Response of *Arabidopsis* to iron deficiency stress as revealed by microarray analysis. *Plant Physiol.* 127, 1030–1043. doi: 10.1104/pp.010191

Thomine, S., and Vert, G. (2013). Iron transport in plants: better be safe than sorry. *Curr. Opin. Plant Biol.* 16, 322–327. doi: 10.1016/j.pbi.2013.01.003

Tian, S., Lu, L., Yang, X., Webb, S. M., Du, Y., and Brown, P. A. (2010). Spatial imaging and speciation of lead in the accumulator plant *Sedum alfredii* by microscopically focused synchrotron x-ray investigation. *Environ. Sci. Technol.* 44, 5920–5926. doi: 10.1021/es903921t

Tiffin, L. O. (1966). Iron translocation. I. Plant culture exudate sampling iron-citrate analysis. *Plant Physiol.* 41, 510–514. doi: 10.1104/pp.41.3.510

Trampczynska, A., Küpper, H., Meyer-Klaucke, W., Schmidt, H., and Clemens, S. (2010). Nicotianamine forms complexes with Zn(II) *in vivo*. *Metallomics* 2, 57–66. doi: 10.1039/b913299f

Tsednee, M., Mak, Y. W., Chen, Y. R., and Yeh, K. C. (2012). Sensitive LC-ESI-Q-TOF-MS method reveals novel phytosiderophores and phytosiderophore–iron complexes in barley. *New Phytol.* 195, 951–961. doi: 10.1111/j.1469-8137.2012.04206.x

Tsukamoto, T., Nakanishi, H., Kiyomiya, S., Watanabe, S., Matsuhashi, S., Nishizawa, N. K., et al. (2006). ^{52}Mn translocation in barley monitored using a positron-emitting tracer imaging system. *Soil Sci. Plant Nutr.* 52, 717–725. doi: 10.1111/j.1747-0765.2006.00096.x

Tsukamoto, T., Nakanishi, H., Uchida, H., Watanabe, S., Matsuhashi, S., Mori, S., et al. (2009). ^{52}Fe translocation in barley as monitored by a positron- emitting tracer imaging system (PETIS): evidence for the direct translocation of Fe from roots to young leaves via phloem. *Plant Cell Physiol.* 50, 48–57. doi: 10.1093/pcp/pcn192

Ueno, D., Iwashita, T., Zhao, F. J., and Ma, J. F. (2008). Characterization of Cd translocation and identification of the Cd form in xylem sap of the Cd-hyperaccumulator *Arabidopsis halleri*. *Plant Cell Physiol.* 49, 540–548. doi: 10.1093/pcp/pcn026

Uraguchi, S., Mori, S., Kuramata, M., Kawasaki, A., Arao, T., and Ishikawa, S. (2009). Root-to-shoot Cd translocation via the xylem is the major process determining shoot and grain cadmium accumulation in rice. *J. Exp. Bot.* 60, 2677–2688. doi: 10.1093/jxb/erp119

Vacchina, V., Mari, S., Czernic, P., Marques, L., Pianelli, K., Schaumloffel, D., et al. (2003). Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray MS/MS assisted by cloning using yeast complementation. *Anal. Chem.* 75, 2740–2745. doi: 10.1021/ac020704m

van Belleghem, F., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, J., d'Haen, J., et al. (2007). Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytol.* 173, 495–508. doi: 10.1111/j.1469-8137.2006.01940.x

van Goor, B. J., and Wiersma, D. (1976). Chemical forms of manganese and zinc in phloem exudate. *Physiol. Plant.* 36, 213–216. doi: 10.1111/j.1399-3054.1976.tb03938.x

Vilaine, F., Palauqui, J. C., Amselem, J., Kusiak, C., Lemoine, R., and Dinant, S. (2003). Towards deciphering phloem: a transcriptome analysis of the phloem of *Apium graveolens*. *Plant J.* 36, 67–81. doi: 10.1046/j.1365-313X.2003.01855.x

Vogel-Mikus, K., Arcon, I., and Kodre, A. (2010). Complexation of cadmium in seeds and vegetative tissues of the cadmium hyperaccumulator *Thlaspi praecox* as studied by X-ray absorption spectroscopy. *Plant Soil* 331, 439–451. doi: 10.1007/s11104-009-0264-y

von Blanckenburg, F., von Wirén, N., Guelke, M., Weiss, D. J., and Bullen, T. D. (2009). Fractionation of metal stable isotopes by higher plants. *Elements* 5, 375–380. doi: 10.2113/gselements.5.6.375

von Wirén, N., Khodr, H., and Hider, R. C. (2000). Hydroxylated phytosiderophore species possess an enhanced chelate stability and affinity for iron(III). *Plant Physiol.* 124, 1149–1157. doi: 10.1104/pp.124.3.1149

von Wirén, N., Klair, S., Bansal, S., Briat, J. F., Khodr, H., Shioiri, T., et al. (1999). Nicotianamine chelates both Fe-III and Fe-II. Implications for metal transport in plants. *Plant Physiol.* 119, 1107–1114. doi: 10.1104/pp.119.3.1107

Wada, Y., Yamaguchi, I., Takahashi, M., Nakanishi, H., Mori, S., and Nishizawa, N. K. (2007). Highly sensitive quantitative analysis of nicotianamine using LC/ESI-TOF-MS with an internal standard. *Biosci. Biotechnol. Biochem.* 71, 435–441. doi: 10.1271/bbb.60496

Watanabe, T., and Osaki, M. (2001). Influence of aluminum and phosphorus on growth and xylem sap composition in *Melastoma malabathricum* L. *Plant Soil* 237, 63–70. doi: 10.1023/A:1013395814958

Waters, B. M., and Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

Watmough, S. A., Hutchinson, T. C., and Evans, R. D. (1999). The distribution of ^{67}Zn and ^{207}Pb applied to white spruce foliage at ambient concentrations under different pH regimes. *Environ. Exp. Bot.* 41, 83–92. doi: 10.1016/S0098-8472(98)00056-2

Weber, G., von Wirén, N., and Hayen, H. (2006). Analysis of iron(II)/iron(III) phytosiderophore complexes by nano-electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Commun. Mass* Spectrom. 20, 973–980. doi: 10.1002/rcm.2402

Wei, Z. G., Wong, J. W., Hong, F., Zhao, H. Y., Li, H. X., and Hu, F. (2007a). Determination of inorganic and organic anions in xylem saps of two contrasting oilseed rape (*Brassica juncea L.*) varieties: roles of anions in long-distance transport of cadmium. *Microchem. J.* 86, 53–59. doi: 10.1016/j.microc.2006.10.003

Wei, Z. G., Wong, J. W., Zhao, H. Y., Zhang, H. J., Li, H. X., and Hu, F. (2007b). Separation and determination of heavy metals associated with low molecular weight chelators in xylem xaps of Indian Mustard (*Brassica juncea*) by size exclusion chromatography and atomic absorption spectrometry. *Biol. Trace Elem. Res.* 118, 146–158. doi: 10.1007/s12011-007-0022-z

White, M. C., Decker, A. M., and Chaney, R. L. (1981). Metal complexation in xylem fluid. *Plant Physiol.* 67, 292–300. doi: 10.1104/pp.67.2.292

Wiersma, D., and van Goor, B. J. (1979). Chemical forms of nickel and cobalt in phloem of *Ricinus communis*. *Physiol. Plant.* 45, 440–442. doi: 10.1111/j.1399-3054.1979.tb02610.x

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., et al. (2009). Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnol. J.* 7, 631–644. doi: 10.1111/j.1467-7652.2009.00430.x

Wood, B. A., and Feldmann, J. (2012). Quantification of phytochelatins and their metal(loid) complexes: critical assessment of current. *Anal. Bioanal. Chem.* 402, 3299–3309. doi: 10.1007/s00216-011-5649-0

Wu, B., Chen, Y. X., and Becker, J. S. (2009). Study of essential element accumulation in the leaves of a Cu-tolerant plant *Elsholtzia splendens* after Cu treatment by imaging laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). *Anal. Chimica Acta* 633, 165–172. doi: 10.1016/j.aca.2008.11.052

Wycisk, K., Kim, E. J., Schroeder, J. I., and Krämer, U. (2004). Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in *Arabidopsis thaliana*. *FEBS Lett.* 578, 128–134. doi: 10.1016/j.febslet.2004.10.086

Xuan, Y., Scheuermann, E. B., Meda, A. R., Hayen, H., von Wirén, N., and Weber, G. (2006). Separation and identification of phytosiderophores and their metal complexes in plants by zwitterionic hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry. *J. Chromatogr. A* 1136, 73–81 doi: 10.1016/j.chroma.2006.09.060

Xuan, Y., Scheuermann, E. B., Meda, A. R., Jacob, P., von Wirén, N., and Weber, G. (2007). CE of phytosiderophores and related metal species in plants. *Electrophoresis* 28, 3507–3519. doi: 10.1002/elps.200700117

Yamaguchi, N., Ishikawa, S., Abe, T., Baba, K., Arao, T., and Terada, Y. (2012). Role of the node in controlling traffic of cadmium, zinc, and manganese in rice. *J. Exp. Bot*. 63, 2729–2737. doi: 10.1093/jxb/err455

Ye, W. L., Wood, B. A., Stroud, J. L., Andralojc, P. J., Raab, A., McGrath, S. P., et al. (2010). Arsenic speciation in phloem and xylem exudates of castor bean. *Plant Physiol.* 54, 1505–1513. doi: 10.1104/pp.110.163261

Yokosho, K., Yamaji, N., Ueno, D., Mitani, N., and Ma, J. F. (2009). OsFRDL1 is a citrate transporter required for efficient translocation of iron in rice. *Plant Physiol*. 149, 297–305. doi: 10.1104/pp.108.128132

Yoneyama, T. Gosho, T. Kato, M., Goto, S., and Hayashi, H. (2010). Xylem and phloem transport of Cd, Zn and Fe into the grains of rice plants (*Oryza sativa* L.) grown in continuously flooded Cd-contaminated soil. *Soil Sci. Plant Nutr.* 56, 445–453. doi: 10.1111/j.1747-0765.2010.00481.x

Yoshimura, E., Sakaguchi, T., Nakanishi, H., Nishizawa, N. K., Nakai, I., and Mori, S. (2000). Characterization of the chemical state of iron in the leaves of wild-type tomato and of a nicotianamine-free mutant chloronerva by X-ray Absorption Near-edge Structure (XANES). *Phytochem. Anal.* 11, 160–162. doi: 10.1002/(SICI)1099-1565(200005/06)11:3<160::AID-PCA500>3.0.CO;2-C

Yruela, I. (2009). Copper in plants: acquisition, transport and interactions. *Funct. Plant Biol.* 36, 409–430. doi: 10.1071/FP08288

Yun, W., Pratt, S. T., Miller, R. M., Cai, Z., Hunter, D. B., Jarstfer, A. G., et al. (1998). X-ray imaging and microspectroscopy of plants and fungi. *J. Synchrotron Radiat.* 5, 1390–1395. doi: 10.1107/S0909049598007225

Zhang, C., Yu, X., Ayre, B. G., and Turgeon, R. (2012). The origin and composition of curcubit "phloem" exudate. *Plant Physiol.* 158, 1873–1882. doi: 10.1104/pp.112.194431

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Generation of boron-deficiency-tolerant tomato by overexpressing an *Arabidopsis thaliana* borate transporter *AtBOR1*

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Nutrient deficiency in soil poses a widespread agricultural problem. Boron (B) is an essential micronutrient in plants, and its deficiency causes defects in both vegetative and reproductive growth in various crops in the field. In *Arabidopsis thaliana*, increased expression of a major borate transporter gene *AtBOR1* or boric acid channel gene *AtNIP5;1* improves plant growth under B-deficient conditions. In this study, we examined whether high expression of a borate transporter gene increases B accumulation in shoots and improves the growth of tomato plant, a model of fruit-bearing crops, under B-deficient conditions. We established three independent transgenic tomato plants lines expressing *AtBOR1* using *Agrobacterium*-mediated transformation of tomato (*Solanum lycopersicum* L. cv. Micro-Tom). Reverse transcription-polymerase chain reaction (RT-PCR) analysis confirmed that two lines (Line 1 and Line 2) more strongly expressed *AtBOR1* than Line 3. Wild-type plants and the transgenic plants were grown hydroponically under B-sufficient and B-deficient conditions. Wild-type and Line 3 (weakly expressing transgenic line) showed a defect in shoot growth under B-deficient conditions, especially in the development of new leaves. However, seedlings of Line 1 and Line 2, the transgenic lines showing strong *AtBOR1* expression, did not show the B-deficiency phenotype in newly developing leaves. In agreement with this phenotype, shoot biomass under low-B conditions was higher in the strongly expressing *AtBOR1* line. B concentrations in leaves or fruits were also higher in Line 2 and Line 1. The present study demonstrates that strong expression of *AtBOR1* improved growth in tomato under B-deficient conditions.

Keywords: *AtBOR1*, boron-deficiency, nutrient, tomato, transgenic, transporter

INTRODUCTION

Boron (B) is an essential micronutrient for plants (Warington, 1923). B mediates cross-linking of rhamnogalacturonan-II (RG-II), a component of the pectic polysaccharide, and proper formation of the RG-II dimer is essential for maintenance of the cell wall structure and plasticity (Hu and Brown, 1994; Matoh et al., 1996; O'Neill et al., 1996, 2001). Hence, B is important for root elongation (Kouchi and Kumazawa, 1975), leaf expansion (Kirk and Loneragan, 1988; Huang et al., 1996; Dell and Huang, 1997), viable pollen grain production, and pollen tube elongation (Garg et al., 1979; Cheng and Rerkasem, 1993). B-deficient growth conditions impair vegetative and/or reproductive growth (Dell and Huang, 1997; Shorrocks, 1997), and B-deficiency has been observed in various agricultural soils, which limits crop production globally (Shorrocks, 1997). Application of B fertilizer is one approach to grow crops under B-deficient conditions in the field (Schon and Blevins, 1990), but excess B is also toxic to plants (Nable et al., 1997). A narrow B concentration range exists between deficient and toxic levels for plants, which complicates B fertilizer application (Francois, 1984; Gupta et al., 1985; Schon and Blevins, 1990). Thus, understanding the plant B transport mechanisms is important to improve B nutrition of crops.

Passive diffusion was believed to be the major process of transmembrane B transport prior to the identification of B-transporting molecules (Takano et al., 2008). Physiological evidence of preferential B transport is suggestive of the contribution of transporter molecules to B transport in plants (Dannel et al., 2000; Matoh and Ochiai, 2005; Uraguchi and Fujiwara, 2011). In *Arabidopsis thaliana* roots, AtNIP5;1, a boric acid channel, plays a role in B uptake (Takano et al., 2006). AtBOR1, an efflux B transporter, mediates xylem loading of B (Takano et al., 2002). Another *A. thaliana* B transporter, AtBOR2, contributes to RG-II dimer formation in roots subjected to limited B environments (Miwa et al., 2013). AtNIP6;1, another boric acid channel in *A. thaliana*, mediates preferential B distribution to developing leaves under B-deficiency (Tanaka et al., 2008). These findings suggest that long-distance B delivery and local B distribution/supply are important for plant growth under B-deficient conditions.

Using the identified transporters, transgenic plants tolerant to low-B conditions have been generated by artificially upregulating expression of B transporters in *A. thaliana* plants. Overexpression

of AtNIP5;1, a boric acid channel for root B uptake, and/or AtBOR1, an efflux B transporter for xylem loading, improves the vegetative and reproductive growth of *A. thaliana* under B-deficient conditions (Miwa et al., 2006; Kato et al., 2009). The generation of B-deficiency-tolerant *A. thaliana* plants suggests that upregulating B-transporter expression can improve the growth of crops under B-deficiency.

AtBOR1 homologous genes have been isolated from crops such as rice (Nakagawa et al., 2007; Tanaka et al., 2013), grapevine (Pérez-Castro et al., 2012), wheat (Leaungthitikanchana et al., 2013), and *Brassica napus* (Sun et al., 2012). In addition, B-deficiency-tolerant cultivars of rice, maize, and wheat show increased *BOR1* transcript levels (Leaungthitikanchana et al., 2014). This indicates that *BOR1* homologs are highly conserved in crops and can be used to improve B-deficiency-tolerance. However, the effects of overexpressing *BOR1* on B-deficiency-tolerance have not been examined in crops.

In this study, we introduced the *AtBOR1* gene into a tomato model cultivar (*Solanum lycopersicum* L. cv. Micro-tom) and established *AtBOR1*-overexpressing tomato plants. Tomato is a model plant of fruit-bearing crops. The occurrence of B-deficiency in tomato cultivation fields has been reported in several countries from Europe, Asia, South America, and Africa (Shorrocks, 1997). We examined the growth and B accumulation in these plants subjected to different B conditions. Two independent lines strongly expressing *AtBOR1* showed normal leaf development, even under B-deficient conditions, and higher B accumulatioin in shoots/fruits compared with non-transgenic tomato plants. These results suggest that upregulating B-transporter expression may improve the growth of fruit-bearing crops under B-deficient conditions.

MATERIALS AND METHODS

PLANT MATERIAL AND CONSTRUCTION

Seeds of tomato cv. Micro-Tom, a dwarf tomato cultivar (Scott and Harbaugh, 1989) were obtained from Kazusa DNA Research Institute (Chiba, Japan) and University of Tsukuba (Ibaraki, Japan). A plasmid carrying the CaMV 35S RNA promoter (P35S):*AtBOR1* was constructed for the transformation of tomato plants based on hygromycin selection. The P35S:*AtBOR1* fragment of pTF469 (Miwa et al., 2006) was amplified by polymerase chain reaction (PCR) using the primers 5′-CACCAGATTAGCCTTTTCAATTTCAG-3′ and 5′-GATCTAGTAACATAGATGACACCGC-3′. The amplified fragment was subcloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). P35S:*AtBOR1* was then subcloned into pMDC99 (Curtis and Grossniklaus, 2003) using the LR recombination reaction. The resulting plasmid was named pHH104.

PLANT TRANSFORMATION

pTF469 and pHH104 were used for transformation of tomato plants to obtain plants expressing *AtBOR1*. Kanamycin (Wako Pure Chemicals, Osaka, Japan) and hygromycin (Roche Diagnostics, Basel, Switzerland) were used for selecting the transformants carrying pTF467 and pHH104, respectively. *Agrobacterium*-mediated transformation of Micro-Tom was performed as described previously (Sun et al., 2006) with minor modifications. Briefly, for hygromycin selection, 5 mg/L of hygromycin was added to the callus induction and shoot induction media. Antibiotic selection was not applied during the rooting step.

PCR-BASED CONFIRMATION OF THE T-DNA INSERTION

Genomic DNA was extracted from the leaf (about 5 mg) as described previously (Kasajima et al., 2004). Obtained DNA was used as template to confirm T-DNA integration into the genome by PCR. Primers specific to *AtBOR1* and NOS-terminator in the T-DNA were designed as 5′-CGTGGAAACCGTTCCATTC-3′ and 5′-GCCAAATGTTTGAACGATCGG-3′, and were used to amplify the T-DNA fragment. Tomato EST SGN-E341940 was selected as a gene with homology to *AtACTIN2* (At3G18780) and was referred to as the *Actin-like* gene. The primers specific to *Actin-like* were 5′-TGTTGCTATTCAGGCTGTGC-3′ and 5′-AATCACGACCAGCAAGATCC-3′.

REVERSE TRANSCRIPTION (RT)-PCR ANALYSIS

Seeds of non-transgenic and transgenic plants (T_1) were germinated on vermiculite and grown for 14 days (22°C, 16 h light/8 h dark). T-DNA insertion was then examined by PCR as described above. PCR-positive transgenic plants and non-transgenic plants were transferred to MGRL hydroponic solution (Fujiwara et al., 1992) containing 100 μM boric acid. The solution was replaced weekly. Fourteen days after transfer, roots of the plants were harvested for RNA extraction. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNase-Free DNase Kit (Qiagen) was used to eliminate DNA contamination. Reverse transcription was conducted using PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). PCR was performed using obtained cDNA as template. Primers 5′-AATCTCGCAGCGGAAACG-3′ and 5′-TGGAGTCGAACTTGAACTTGTC-3′ were used for *AtBOR1* expression analysis. The expression of the *Actin-like* gene was also examined as a control using the primers described above.

PLANTS CULTURED UNDER DIFFERENT B SUPPLIES

Plants were incubated at 25°C under a 16 h light/8 h dark cycle. Seeds of non-transgenic and transgenic plants (T_1) were sown on vermiculite and grown for 19 days, after which the T-DNA insertion was examined by PCR as described above. PCR-positive transgenic plants and non-transgenic plants were transferred to MGRL hydroponic solution supplemented with 0.1 or 100 μM boric acid. The solution was renewed twice a week for the 0.1 μM boric acid treatment and once a week for the 100 μM boric acid treatment.

DETERMINATION OF BORON CONCENTRATIONS IN PLANT TISSUES

To determine the shoot B concentration, whole shoots were harvested after 20 days of hydroponic culture and then dried at 60°C for at least 3 days. After determination of total shoot dry weight, samples were acid-digested and subjected to inductively coupled plasma mass spectrometry (ICP-MS; SPQ-9000, Seiko Instruments Inc., Chiba, Japan) for quantifying the B concentration (Takano et al., 2002). Plants were also grown hydroponically

until fruit ripening and harvest. After drying and acid digestion, B concentrations in the fruits were determined by ICP-MS.

STATISTICAL ANALYSES

To verify the statistical significance of differences among the lines, the data were analyzed using the Student's *t*-test ($p < 0.05$).

RESULTS

GENERATION OF TRANSGENIC TOMATO EXPRESSING *AtBOR1*

After several batches of transformation, 11 independent candidate lines were obtained. Among them, three lines showed good fertility and more than 100 seeds were obtained from each line. These three lines were used for the following experiments. Genomic DNA was extracted from a leaf of regenerated plants (T_0). The T-DNA integration into the genome was examined on these plants by PCR analysis using the specific primers for T-DNA (**Figure 1A**). All tested regenerated T_0 plants showed a band at the expected size. No band corresponding to the T-DNA fragment was obtained from non-transgenic plants using PCR. The *Actin-like* gene was used as a positive control and all plants (including non-transgenic plants) showed a band at the expected size. These results indicated that the regenerated plants contained the T-DNA insertion in their genome. These three lines were named L1, L2, and L3.

Reverse transcription-polymerase chain reaction analysis was conducted to investigate whether the introduced *AtBOR1* gene was expressed in these transgenic lines (**Figure 1B**). *AtBOR1* expression was clearly observed after 20 cycles of PCR in L1 and L2, whereas no signal was detected in non-transgenic plants and L3. A relatively weak signal of *AtBOR1* was obtained from L3 after 30 cycles of PCR compared to L1 and L2, but not from non-transgenic plants. These results suggest that introduced *AtBOR1* is expressed relatively strongly in L1 and L2 compared to L3.

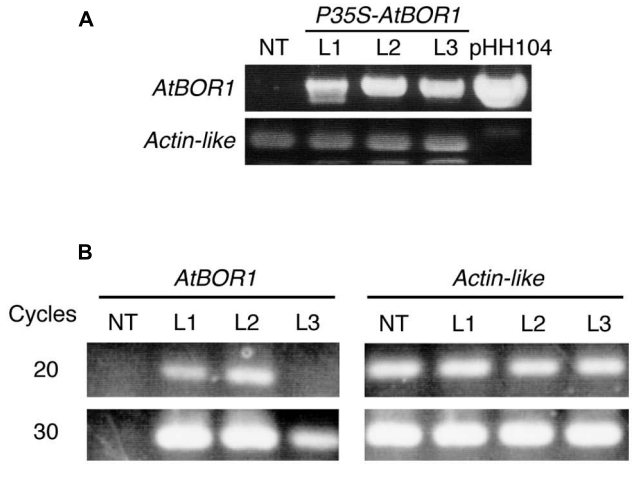

FIGURE 1 | Establishment of transgenic tomato expressing *AtBOR1*. **(A)** Genomic DNA was extracted from non-transgenic (NT) and regenerated T_0 plants (L1, L2, and L3). PCR was performed using genomic DNA as a template with primers specific to the T-DNA or tomato *Actin*-like gene. pHH104 was also used as a control for T-DNA. **(B)** Total RNA was extracted from the roots of non-transgenic plants (NT) and transgenic T1 plants (L1, L2, and L3). Semiquantitative RT-PCR was performed using primers specific to *AtBOR1* or the *Actin*-like gene.

TRANSGENIC TOMATO PLANTS WITH STRONG EXPRESSION OF *AtBOR1* DID NOT SHOW B-DEFICIENCY SYMPTOMS UNDER LOW-B GROWTH CONDITIONS

To investigate the effect of heterologous *AtBOR1* expression on growth of tomato plants under B-deficiency, non-transgenic plants and three transgenic T_1 lines were grown hydroponically in the presence of 0.1 or 100 μM boric acid. Experiments were performed with at least four replications and representative individuals are shown in **Figure 2**. Under B-sufficient conditions (100 μM B), shoot growth and development were normal in all tested lines (**Figures 2A,D,G,J**), although L3 showed relatively small shoots (**Figure 2J**) compared to other samples (**Figures 2A,D,G**). Phenotypic differences among lines were clear under the low-B conditions (0.1 μM B). In non-transgenic plants under the low-B conditions, shoot growth was retarded compared to the control (**Figures 2A,B**). Curly leaves were observed and development of newly growing leaves was often inhibited by the B-deficient treatment (**Figure 2C**). These phenotypes were also observed in L3 subjected to the low-B treatment (**Figures 2K,L**), but not observed in L1 and L2 plants (**Figures 2E,F,H,I**). In L1 and L2, development of new leaves was normal, even under the low-B condition (**Figures 2F,I**). These results indicated that heterologous expression of *AtBOR1* increases tolerance of tomato plants to B-deficiency stress.

ENHANCED BORON ACCUMULATION IN TOMATO PLANTS EXPRESSING *AtBOR1* UNDER THE LOW-B GROWTH CONDITION

Shoot dry weight and B concentration were measured after 20 days of hydroponic culture under the B-sufficient and B-deficient conditions. L1 was not tested in this experiment due to the limited seed numbers. Shoot dry weight of L2, which showed improved B-deficiency-tolerance (**Figures 2H,I**), was slightly higher than those of the non-transgenic plants and L3, although not significantly (**Figure 3A**). This tendency was not observed in the 100 μM boric acid treatment.

The B concentration in shoots was compared among the lines (**Figure 3B**). Under the low-B treatment, shoot B concentration was 1.4-fold higher in L2 than in non-transgenic plants and L3. When plants were supplied with 100 μM boric acid, B accumulation in shoots did not differ significantly between the non-transgenic plants and L2. L3 tended to accumulate less B than non-transgenic plants.

We also examined fruit yields of non-transgenic and transgenic plants. In all lines, low-B treatment impaired fruit yield; however, no consistent result was obtained due to the large variation among plants (data not shown). We next measured B concentration in fruits harvested from the plants grown under B-sufficient and B-deficient conditions (**Figure 4**). For the low-B treatment, the fruits of L1 accumulated significantly higher B than non-transgenic plants (**Figure 4A**). Fruit B concentration under the B-deficient condition appeared higher in L2 than in non-transgenic plants, although the difference was not significant. This tendency of an increased B concentration in L1 and L2 was not observed under B-sufficient conditions (**Figure 4B**). The B concentrations in fruits of L3 was relatively lower than in non-transgenic plants under both B treatments. These results suggest that B accumulation in shoots and fruits is enhanced in the stronger *AtBOR1* expression lines.

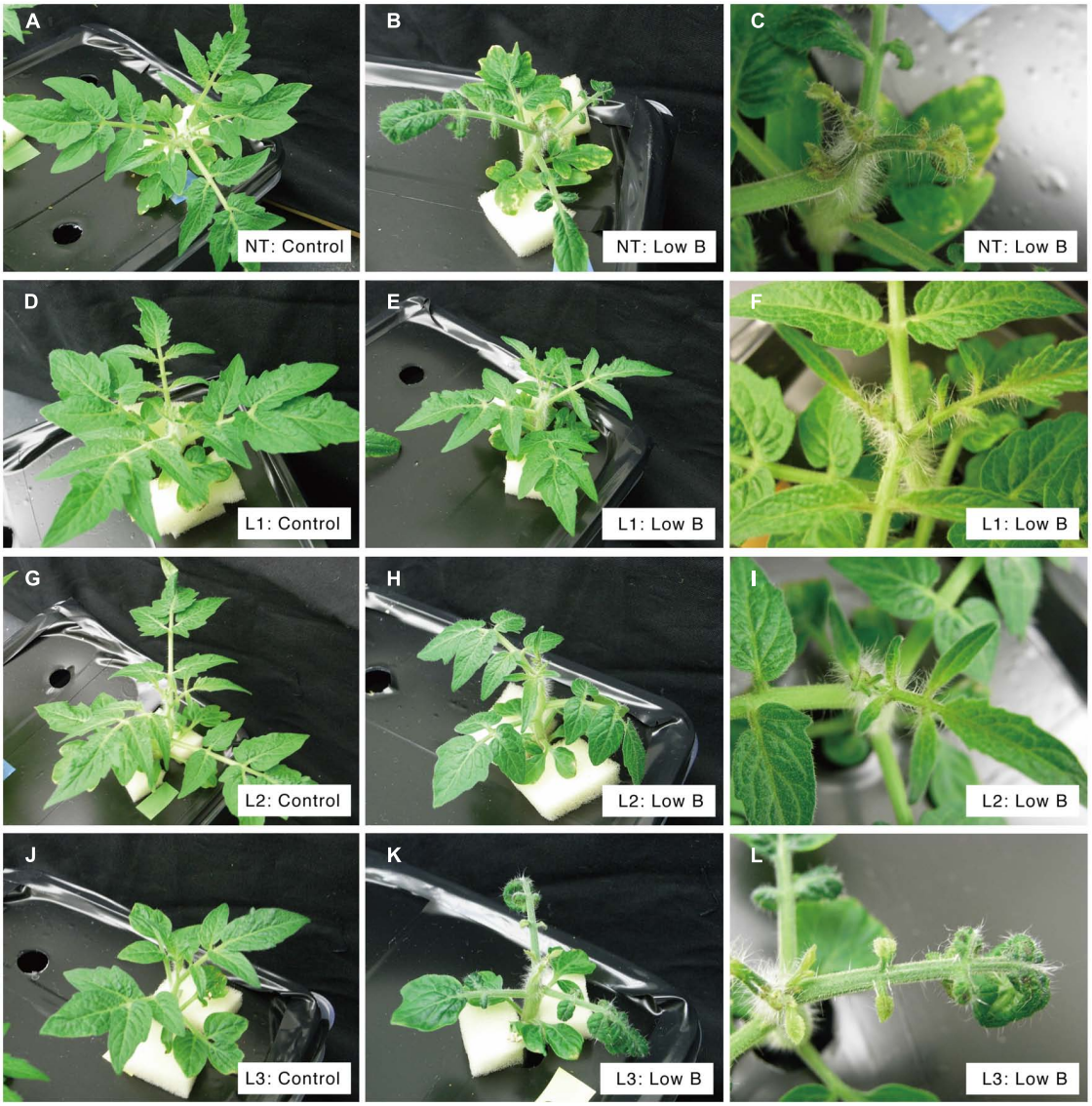

FIGURE 2 | Shoot development of transgenic tomato plants expressing *AtBOR1* under B-deficiency and sufficiency. Non-transgenic (NT) plants **(A–C)**, transgenic plants L1 **(D–F)**, L2 **(G–I)**, and L3 **(J–L)** were grown for 15 days with a hydroponic solution supplied containing 100 μM (control) or 0.1 μM (low-B) boric acid. Representative plants under control **(A,D,G,J)** and low-B **(B,E,H,K)** conditions and their developing leaves under low-B conditions **(C,F,I)** are shown.

DISCUSSION

As discussed in a recent review (Schroeder et al., 2013), regulation and manipulation of plant membrane transporters can be used to improve crop production under various soil-derived stresses such as aluminum toxicity and nutrient deficiency. B-deficiency occurs in various fields globally (Shorrocks, 1997). Based on the molecular mechanisms of B transport, we generated *A. thaliana* plants tolerant to low-B conditions by upregulating B-transporter genes (Miwa et al., 2006; Kato et al., 2009). Increased expression of *AtNIP5;1* and/or *AtBOR1* significantly improved vegetative and reproductive growth of *A. thaliana* with limited B supply. Since the concentration range between B-deficiency and toxicity is rather narrow in many plants, improper B fertilization can lead to an excess dosage and negatively affect crop growth (Francois, 1984; Gupta et al., 1985; Schon and Blevins, 1990). Therefore, as established in *A. thaliana*, molecular breeding of crops with enhanced B-transport activity is a promising approach to address B-deficiency (Miwa et al., 2006; Kato et al., 2009). However, no attempt has been made to establish B-deficiency-tolerant crops by enhancing *BOR1* expression.

In this study, tomato was selected as a test plant because it is a common model of fruit-bearing crops. The occurrence of B-deficiency has been reported for tomato cultivation in many countries (Shorrocks, 1997). Genotypic variation of B-uptake activity and B-deficiency-tolerance among tomato cultivars has been observed (Brown and Jones, 1971; Bellaloui and Brown,

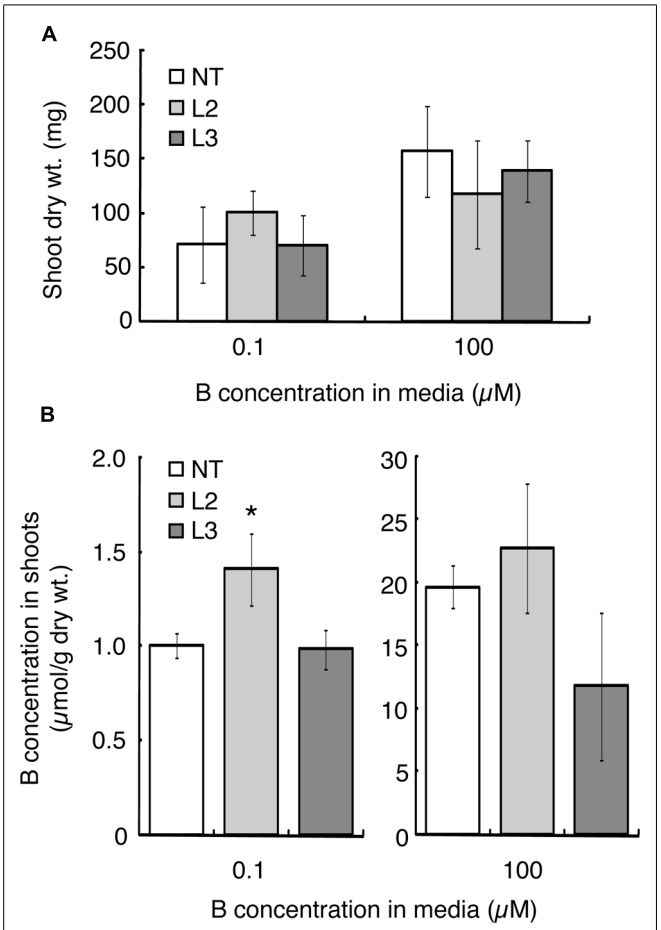

FIGURE 3 | Shoot dry weights and B concentrations in transgenic tomato plants expressing *AtBOR1* under B-deficiency and sufficiency. Non-transgenic (NT) and transgenic (L2 and L3) plants were grown for 20 days in a hydroponic solution containing 0.1 or 100 μM boric acid. Shoot dry weight **(A)** and B concentration in shoots **(B)** were measured. Values represent the means ± standard deviation ($n = 3$–4). An asterisk indicates significant differences from non-transgenic plants (Student's *t*-test, $p < 0.05$).

1998), which indicates that enhancement of B-transport efficiency confers B-deficiency-tolerance in tomato. B-deficiency symptoms of tomato plants are represented by shoot growth inhibition, curly and yellowish leaves of young seedlings, and defects in quality fruit setting during the reproductive growth stages (Johnston and Fisher, 1930; Brown and Jones, 1971; Yamauchi et al., 1986).

In the present study, wild-type tomato plants showed retarded whole shoot growth, curled leaves, and abnormal and poor development of new leaves in the presence of 0.1 μM boric acid (**Figures 2A–C** and **3A**). These phenotypes are typical B-deficiency symptoms reported in early tomato seedlings, suggesting that our experimental condition was suitable for evaluating the effects of *AtBOR1* overexpression in young tomato plants grown under limited B availability. We performed several batches of transformation experiments and obtained three independent transgenic tomato lines carrying P35S-*AtBOR1* (**Figure 1A**) with substantial seed yields. Expression analysis of introduced *AtBOR1* in these lines demonstrated that L1 and L2 were strongly expressing lines and L3 was a rather weakly expressing line (**Figure 1B**). These strongly expressing lines did not show severe B-deficiency symptoms, which were observed in non-transgenic plants as well as in weakly expressing L3 (**Figure 2**). Furthermore, shoot and fruit B accumulation under the low-B conditions significantly increased in L1 and L2, respectively, compared to non-transgenic plants, but not in L3 (**Figures 3B** and **4A**). These results suggest that

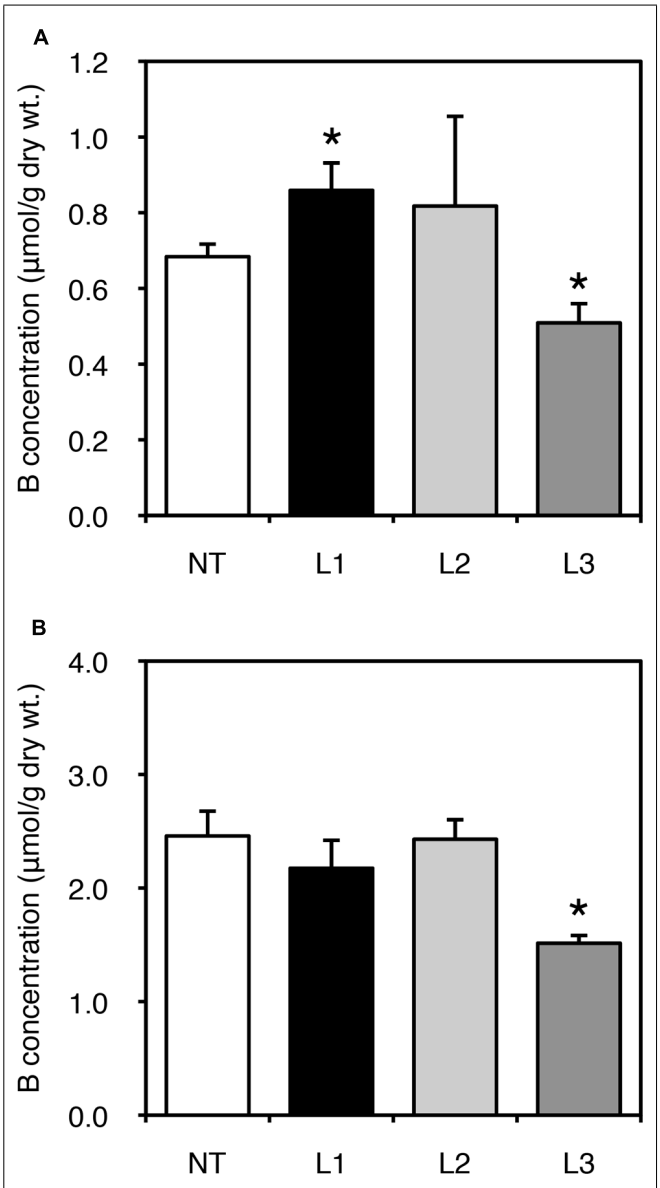

FIGURE 4 | Fruit B concentrations in transgenic tomato plants expressing *AtBOR1* under B-deficiency and sufficiency. Non-transgenic (NT) and transgenic (L1, L2, and L3) plants were grown in a hydroponic solution supplied with 0.1 or 100 μM boric acid until fruit setting. Fruits were harvested from plants grown under 0.1 μM **(A)** or 100 μM **(B)** B treatment. B concentrations in the fruits were measured using ICP-MS. Values represent the means ± standard deviation ($n = 3$–4). An asterisk indicates significant differences from the non-transgenic plants (Student's *t*-test, $p < 0.05$).

enhanced expression of a B transporter improves the root-to-shoot translocation of B in tomato under low-B availability, which contributes to maintaining proper shoot development. Since the effect of *AtBOR1* expression on yield remains unclear due to the considerable variation observed under our hydroponic conditions, this should be further examined with an improved cultivation system and moderate B-deficiency conditions.

Optimal B concentrations in culture media are narrow, and overdose of B leads to increased B accumulation in plant tissues and plant growth inhibition (Francois, 1984; Gupta et al., 1985; Schon and Blevins, 1990). Excess B increases DNA damage, which may cause B toxicity in plants (Sakamoto et al., 2011). Concentrations of B in shoot and fruits under B-sufficient conditions were similar between the non-transgenic and *AtBOR1*-expressing plants (**Figures 3B** and **4B**). Growth under the 100 μM boric acid condition was comparable among the non-transgenic and transgenic plants (**Figures 2** and **3A**), suggesting that overexpression of *AtBOR1* has a negligible adverse effect on tomato young seedlings under B-sufficient conditions. In *A. thaliana* roots, AtBOR1 protein levels are regulated by post-translational mechanisms, and protein degradation is promoted under B-sufficient conditions through ubiquitination of AtBOR1 (Takano et al., 2005; Kasai et al., 2011). This regulation of AtBOR1 likely prevents B overaccumulation in plants. In fact, AtBOR1-overexpressing *A. thaliana* accumulates wild-type levels of B under excess B treatments (Miwa et al., 2006). In this study, we examined the effects of heterologous *AtBOR1* expression in tomato, but similar mechanisms may regulate AtBOR1 levels in tomato under B-sufficient conditions.

CONCLUSION

In the present study, we established transgenic tomato plants tolerant to B-deficient environments by upregulating a B-transporter gene. Since B-deficiency is a serious issue in various crop productions, our findings suggest that application of AtBOR1 or its ortholog to crop breeding may improve growth under B-deficient cultivation conditions.

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REFERENCES

Bellaloui, N., and Brown, P. (1998). Cultivar differences in boron uptake and distribution in celery (*Apium graveolens*), tomato (*Lycopersicon esculentum*) and wheat (*Triticum aestivum*). *Plant Soil* 198, 153–158. doi: 10.1023/A:1004343031242

Brown, J. C., and Jones, W. E. (1971). Differential transport of boron in tomato (*Lycopersicon esculenlum* Mill.). *Physiol. Plant.* 25, 279–282. doi: 10.1111/j.1399-3054.1971.tb01443.x

Cheng, C., and Rerkasem, B. (1993). Effects of boron on pollen viability in wheat. *Plant Soil* 155, 313–315. doi: 10.1007/BF00025045

Curtis, M. D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 133, 462–469. doi: 10.1104/pp.103.027979

Dannel, F., Pfeffer, H., and Romheld, V. (2000). Characterization of root boron pools, boron uptake and boron translocation in sunflower using the stable isotopes B-10 and B-11. *Aust. J. Plant Physiol.* 27, 397–405. doi: 10.1071/PP99086

Dell, B., and Huang, L. B. (1997). Physiological response of plants to low boron. *Plant Soil* 193, 103–120. doi: 10.1023/A:1004264009230

Francois, L. E. (1984). Effect of excess boron on tomato yield, fruit size, and vegetative growth. *J. Am. Soc. Hortic. Sci.* 109, 322–324.

Fujiwara, T., Hirai, M. Y., Chino, M., Komeda, Y., and Naito, S. (1992). Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia. *Plant Physiol.* 99, 263–268. doi: 10.1104/pp.99.1.263

Garg, O., Sharma, A., and Kona, G. (1979). Effect of boron on the pollen vitality and yield of rice plants (*Oryza sativa* L. var. Jaya). *Plant Soil* 52, 591–594. doi: 10.1007/BF02277956

Gupta, U. C., Jame, Y. W., Campbell, C. A., Leyshon, A. J., and Nicholaichuk, W. (1985). Boron toxicity and deficiency: a review. *Can. J. Soil Sci.* 65, 381–409. doi: 10.4141/cjss85-044

Huang, L., Ye, Z., and Bell, R. (1996). The importance of sampling immature leaves for the diagnosis of boron deficiency in oilseed rape (*Brassica napus* cv. Eureka). *Plant Soil* 183, 187–198. doi: 10.1007/BF00011434

Hu, H. I., and Brown, P. H. (1994). Localization of boron in cell-walls of squash and tobacco and Its association with pectin – evidence for a structural role of boron in the cell-wall. *Plant Physiol.* 105, 681–689. doi: 10.1104/pp.105.2.681

Johnston, E. S., and Fisher, P. L. (1930). The essential nature of boron to the growth and fruiting of the tomato. *Plant Physiol.* 5, 387–392. doi: 10.1104/pp.5.3.387

Kasai, K., Takano, J., Miwa, K., Toyoda, A., and Fujiwara, T. (2011). High boron-induced ubiquitination regulates vacuolar sorting of the *BOR1* borate transporter in *Arabidopsis thaliana*. *J. Biol. Chem.* 286, 6175–6183. doi: 10.1074/jbc.M110.184929

Kasajima, I., Ide, Y., Ohkama-Ohtsu, N., Hayashi, H., Yoneyama, T., and Fujiwara, T. (2004). A protocol for rapid DNA extraction from *Arabidopsis thaliana* for PCR analysis. *Plant Mol. Biol. Rep.* 22, 49–52. doi: 10.1007/BF02773348

Kato, Y., Miwa, K., Takano, J., Wada, M., and Fujiwara, T. (2009). Highly boron deficiency-tolerant plants generated by enhanced expression of NIP5;1, a boric acid channel. *Plant Cell Physiol.* 50, 58–66. doi: 10.1093/Pcp/Pcn168

Kirk, G. J., and Loneragan, J. F. (1988). Functional boron requirement for leaf expansion and Its use as a critical value for diagnosis of boron deficiency in soybean. *Agron. J.* 80, 758–762. doi: 10.2134/agronj1988.00021962008000050013x

Kouchi, H., and Kumazawa, K. (1975). Anatomical responses of root tips to boron deficiency II. Effect of boron deficiency on the cellular growth and development in root tips. *Soil Sci. Plant Nutr.* 21, 137–150. doi: 10.1080/00380768.1975.10432630

Leaungthitikanchana, S., Fujibe, T., Tanaka, M., Wang, S., Sotta, N., Takano, J., et al. (2013). Differential expression of three *BOR1* genes corresponding to different genomes in response to boron conditions in hexaploid wheat (*Triticum aestivum* L.). *Plant Cell Physiol.* 54, 1056–1063. doi: 10.1093/pcp/pct059

Leaungthitikanchana, S., Tanaka, M., Lordkaew, S., Jamjod, S., Rerkasem, B., and Fujiwara, T. (2014). Comparison of *BOR1*-like gene expression in two genotypes with different boron efficiencies in commercial crop plants in Thailand. *Soil Sci. Plant Nutr.* doi: 10.1080/00380768.2014.881707

Matoh, T., Kawaguchi, S., and Kobayashi, M. (1996). Ubiquity of a borate rhamnogalacturonan II complex in the cell walls of higher plants. *Plant Cell Physiol.* 37, 636–640. doi: 10.1093/oxfordjournals.pcp.a028992

Matoh, T., and Ochiai, K. (2005). Distribution and partitioning of newly taken-up boron in sunflower. *Plant Soil* 278, 351–360. doi: 10.1007/s11104-005-0372-2

Miwa, K., Takano, J., and Fujiwara, T. (2006). Improvement of seed yields under boron-limiting conditions through overexpression of *BOR1*, a boron transporter for xylem loading, in *Arabidopsis thaliana*. *Plant J.* 46, 1084–1091. doi: 10.1111/J.1365-313x.2006.02763.X

Miwa, K., Wakuta, S., Takada, S., Ide, K., Takano, J., Naito, S., et al. (2013). Roles of BOR2, a boron exporter, in cross linking of rhamnogalacturonan II and root elongation under boron limitation in *Arabidopsis*. *Plant Physiol.* 163, 1699–1709. doi: 10.1104/pp.113.225995

Nable, R. O., Bañuelos, G. S., and Paull, J. G. (1997). Boron toxicity. *Plant Soil* 193, 181–198. doi: 10.1023/A:1004272227886

Nakagawa, Y., Hanaoka, H., Kobayashi, M., Miyoshi, K., Miwa, K., and Fujiwara, T. (2007). Cell-type specificity of the expression of Os *BOR1*, a rice efflux boron transporter gene, is regulated in response to boron availability for efficient boron uptake and xylem loading. *Plant Cell* 19, 2624–2635. doi: 10.1105/tpc.106.049015

O'Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001). Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* 294, 846–849. doi: 10.1126/science.1062319

O'Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A.G., et al. (1996). Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. In vitro conditions for the formation and hydrolysis of the dimer. *J. Biol. Chem.* 271, 22923–22930. doi: 10.1074/jbc.271.37.22923

Pérez-Castro, R., Kasai, K., Gainza-Cortés, F., Ruiz-Lara, S., Casaretto, J. A., Peña-Cortés, H., et al. (2012). *VvBOR1*, the grapevine ortholog of *AtBOR1*, encodes an efflux boron transporter that is differentially expressed throughout reproductive development of *Vitis vinifera* L. *Plant Cell Physiol.* 53, 485–494. doi: 10.1093/pcp/pcs001

Sakamoto, T., Inui, Y. T., Uraguchi, S., Yoshizumi, T., Matsunaga, S., Mastui, M., et al. (2011). Condensin II alleviates DNA damage and is essential for tolerance of boron overload stress in *Arabidopsis*. *Plant Cell* 23, 3533–3546. doi: 10.1105/tpc.111.086314

Schon, M. K., and Blevins, D. G. (1990). Foliar boron applications increase the final number of branches and pods on branches of field-grown soybeans. *Plant Physiol.* 92, 602–607. doi: 10.1104/pp.92.3.602

Schroeder, J. I., Delhaize, E., Frommer, W. B., Guerinot, M. L., Harrison, M. J., Herrera-Estrella, L., et al. (2013). Using membrane transporters to improve crops for sustainable food production. *Nature* 497, 60–66. doi: 10.1038/nature11909

Scott, J. W., and Harbaugh, B. K. (1989). Micro-Tom. A miniature dwarf tomato. *Florida Agric. Exp. Station Circ.* 370, 1–6.

Shorrocks, V. M. (1997). The occurrence and correction of boron deficiency. *Plant Soil* 193, 121–148. doi: 10.1023/A:1004216126069

Sun, H.-J., Uchii, S., Watanabe, S., and Ezura, H. (2006). A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiol.* 47, 426–431. doi: 10.1093/pcp/pci251

Sun, J., Shi, L., Zhang, C., and Xu, F. (2012). Cloning and characterization of boron transporters in *Brassica napus*. *Mol. Biol. Rep.* 39, 1963–1973. doi: 10.1007/s11033-011-0930-z

Takano, J., Miwa, K., and Fujiwara, T. (2008). Boron transport mechanisms: collaboration of channels and transporters. *Trends Plant Sci.* 13, 451–457. doi: 10.1016/J.Tplants.2008.05.007

Takano, J., Miwa, K., Yuan, L. X., Von Wiren, N., and Fujiwara, T. (2005). Endocytosis and degradation of *BOR1*, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12276–12281. doi: 10.1073/Pnas.0502060102

Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., et al. (2002). *Arabidopsis* boron transporter for xylem loading. *Nature* 420, 337–340. doi: 10.1038/Nature01139

Takano, J., Wada, M., Ludewig, U., Schaaf, G., Von Wiren, N., and Fujiwara, T. (2006). The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell* 18, 1498–1509. doi: 10.1105/tpc.106.041640

Tanaka, M., Wallace, I. S., Takano, J., Roberts, D. M., and Fujiwara, T. (2008). NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in *Arabidopsis*. *Plant Cell* 20, 2860–2875. doi: 10.1105/tpc.108.058628

Tanaka, N., Uraguchi, S., Saito, A., Kajikawa, M., Kasai, K., Sato, Y., et al. (2013). Roles of pollen-specific boron efflux transporter, OsBOR4, in the rice fertilization process. *Plant Cell Physiol.* 54, 2011–2019. doi: 10.1093/pcp/pct136

Uraguchi, S., and Fujiwara, T. (2011). Significant contribution of boron stored in seeds to initial growth of rice seedlings. *Plant Soil* 340, 435–442. doi: 10.1007/s11104-010-0614-9

Warington, K. (1923). The effect of boric acid and borax on the broad bean and certain other plants. *Ann. Bot.* os-37, 629–672.

Yamauchi, T., Hara, T., and Sonoda, Y. (1986). Effects of boron deficiency and calcium supply on the calcium metabolism in tomato plant. *Plant Soil* 93, 223–230. doi: 10.1007/BF02374224

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Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants

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Manganese (Mn), an essential trace element, is important for plant health. In plants, Mn serves as a cofactor in essential processes such as photosynthesis, lipid biosynthesis and oxidative stress. Mn deficient plants exhibit decreased growth and yield and are more susceptible to pathogens and damage at freezing temperatures. Mn deficiency is most prominent on alkaline soils with approximately one third of the world's soils being too alkaline for optimal crop production. Despite the importance of Mn in plant development, relatively little is known about how it traffics between plant tissues and into and out of organelles. Several gene transporter families have been implicated in Mn transport in plants. These transporter families include NRAMP (natural resistance associated macrophage protein), YSL (yellow stripe-like), ZIP (zinc regulated transporter/iron-regulated transporter [ZRT/IRT1]-related protein), CAX (cation exchanger), CCX (calcium cation exchangers), CDF/MTP (cation diffusion facilitator/metal tolerance protein), P-type ATPases and VIT (vacuolar iron transporter). A combination of techniques including mutant analysis and Synchrotron X-ray Fluorescence Spectroscopy can assist in identifying essential transporters of Mn. Such knowledge would vastly improve our understanding of plant Mn homeostasis.

Keywords: manganese, metal transport, Arabidopsis, rice, synchrotron x-ray fluorescence

INTRODUCTION

With an increasing human population, there is a growing demand for improvements in crop production. Mineral nutrients such as iron (Fe), copper (Cu), and zinc (Zn) are essential for both plants and animals, but maintaining optimal micronutrient levels presents challenges in both the plant and animal kingdoms. To combat human nutrient deficiency, efforts are being made to increase the bioavailability of nutrients in the edible portions of plants (Schroeder et al., 2013). Another concern is contamination of the soil with non-essential metal(oid)s such as arsenic (As), lead (Pb), cadmium (Cd), and mercury (Hg) (Salt et al., 1998). These elements are detrimental to both plants (through direct uptake from the soil) and humans (through consumption of contaminated plant products). One strategy for remediating soil contaminated with metals is using hyperaccumulator plants, which can tolerate and store remarkably high levels of metals in the aerial portion of the plant (Kramer, 2010). Mn is an example of an element that is both required in humans but can potentially be toxic. In excess, Mn can induce neurological symptoms that resemble Parkinson's disease (Martinez-Finley et al., 2013). Therefore, reducing human exposure to high levels of Mn is a major worldwide health concern. By better understanding the molecular mechanisms, specifically which transport proteins allow plants to take up and store metals, steps can be taken toward improved crop growth and the engineering of biofortified plants for improved human nutrition.

This review will focus on the micronutrient Mn. Despite its importance in cellular processes, such as photosynthesis and protecting cells against reactive oxygen species (ROS), little is known about transporters essential for Mn uptake and storage in the cell. We describe common and emerging techniques for assaying Mn localization and accumulation within the plant. In addition, we detail the protein families implicated in Mn transport. Particular emphasis will be placed on the model plant species *Arabidopsis thaliana* and *Oryza sativa* (rice), on which the majority of characterization work has been carried out. The transporter families will be organized based on their putative function of transporting Mn into or out of the cytoplasm. **Table 1** summarizes the transporters discussed in this review. The subcellular localization (in *A. thaliana* and rice) and tissue localization (in *A. thaliana*) of the transporters is shown in **Figures 1** and **2**, respectively.

SYMPTOMS OF Mn DEFICIENCY AND Mn TOXICITY

Mn is a micronutrient element required for normal plant growth and development. It is essential for most photosynthetic organisms as a component of the oxygen-evolving complex in photosystem II (PSII), which catalyzes the water-splitting reaction to produce oxygen and provides electrons for the photosynthetic electron transport chain (Goussias et al., 2002; Nickelsen and Rengstl, 2013). Mn is also required for multiple steps in carbohydrate, lipid and lignin biosynthesis in plants (Marschner, 2012). Mn acts as a direct cofactor of a variety of enzymes

Table 1 | Putative Mn transporters referenced in the text.

Transporter	Tissue expression	Subcellular localization	Transcript response to Mn deficiency	Transcript response to Mn toxicity	Other proposed substrates	References
NRAMP FAMILY						
AtNRAMP1	Root (all tissues) > shoot	PM	Up in root	- -	Fe, Cd	Curie et al., 2000; Thomine et al., 2000; Cailliatte et al., 2010
AtNRAMP3	Shoot and root vasculature, developing seed	VM	None	- -	Fe, Cd	Thomine et al., 2000
AtNRAMP4	Shoot vasculature > root vasculature, developing seed	VM	None	- -	Fe, Zn, Cd	Thomine et al., 2000
OsNRAMP3	Nodes > root and leaf vasculature, panicle, husk, flower	PM	None	None (PTM)		Yamaji et al., 2013
OsNRAMP5	Root exodermis and endodermis	PM	None	–	Cd	Ishimaru et al., 2012; Sasaki et al., 2012
DMTI	Root, leaf, stems	PM/peri-bacteroid membrane	- -	- -	Fe, Zn, Cu	Kaiser et al., 2003
LeNRAMP1	Root	VM	- -	- -		Bereczky et al., 2003
LeNRAMP3	Root > shoot	VM	- -	- -		Bereczky et al., 2003
YSL FAMILY						
AtYSL4	Shoot, silique, root, flower, developing seed	VM/EM/CM	None	- -	Ni, Fe**	Conte et al., 2013; Divol et al., 2013
AtYSL6	Shoot, flower, silique, developing seed	VM/EM/CM	None	- -	Ni, Fe**	Conte et al., 2013; Divol et al., 2013
OsYSL2	Leaf and leaf sheath phloem, root phloem, developing seed	PM	None	- -	Fe	Koike et al., 2004
OsYSL6	Root, shoot	Un-determined	None	- -	- -	Sasaki et al., 2011
ZmYS1	Root epidermis, leaf mesophyll	PM	- -	- -	Fe, Zn, Cu, Ni, Cd	Roberts et al., 2004; Schaaf et al., 2004; Ueno et al., 2009
ZIP FAMILY						
AtIRT1	Root epidermis, flower	PM	None	- -	Fe, Zn, Cu, Cd, Co	Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002
HvIRT1	Root	PM, ER	None	- -	Fe, Zn	Pedas et al., 2008
PsIRT1	Root	PM	- -	- -	Fe, Zn, Cd	Cohen et al., 2004
LeIRT1	Root, flowers	- -	- -	- -	Fe, Zn, Cd	Eckhardt et al., 2001
LeIRT2	Root	- -	- -	- -	Fe, Zn, Cd	Eckhardt et al., 2001
AtZIP1	Root vasculature > shoot vasculature	VM	Up in shoot	- -	Zn	Milner et al., 2013
AtZIP2	Root vasculature > shoot vasculature	PM	Down in shoot	- -	Zn	Milner et al., 2013
AtZIP5	Root > shoot	- -	- -	- -		Milner et al., 2013
AtZIP6	Root > shoot	- -	- -	- -		Milner et al., 2013
AtZIP7	Shoot > root	- -	- -	- -	Zn, Fe	Milner et al., 2013
AtZIP9	Root, shoot	- -	- -	- -		Milner et al., 2013
MtZIP4	Leaf, root*	MM (predicted)	Down in leaf	- -		Lopez-Millan et al., 2004

(Continued)

Table 1 | Continued

Transporter	Tissue expression	Subcellular localization	Transcript response to Mn deficiency	Transcript response to Mn toxicity	Other proposed substrates	References
MtZIP7	Leaf	PM (predicted)	None	- -		Lopez-Millan et al., 2004
CAX FAMILY						
AtCAX2	Root, shoot and flower vasculature, fruit, stem	VM	- -	None	Ca, Cd, Zn	Hirschi et al., 2000; Schaaf et al., 2002; Shigaki et al., 2003; Pittman et al., 2004; Edmond et al., 2009
AtCAX4	Root, leaf, stem, flower, silique	VM	- -	Up	Cd	Cheng et al., 2002
AtCAX5	Root, stem, fruit, flower, leaf	VM	- -	Up		Edmond et al., 2009
OsCAX1a	Root, shoot, flower	- -	- -	- -	Ca	Kamiya and Maeshima, 2004; Kamiya et al., 2005
OsCAX3	Root, shoot, flower	- -	- -	- -	Ca	Kamiya and Maeshima, 2004; Kamiya et al., 2005
LeCAX2	Leaf, fruit	- -	- -	- -	Ca	Edmond et al., 2009
HvCAX2	Root, shoot, seed	- -	- -	None	Ca	Edmond et al., 2009
CCX FAMILY						
AtCCX3	Flowers, stem, leaf, root	VM, EM	- -	Up in root and flowers	Na, K	Morris et al., 2008
CDF/MTP FAMILY						
AtMTP11	Leaf hydathodes, guard cells, root tip	Golgi/PVC	- -	None		Delhaize et al., 2007; Peiter et al., 2007
OsMTP8.1	Shoot	VM	None	Up in shoot		Chen et al., 2013
ShMTP8	- -	Internal organelle	- -	- -	- -	Delhaize et al., 2003
PtMTP11.1	- -	GLC	- -	- -		Peiter et al., 2007
PtMTP11.2	- -	GLC	- -	- -		Peiter et al., 2007
BmMTP10	Root, shoot	Golgi	- -	Up in root and shoot		Erbasol et al., 2013
BmMTP11	Root, shoot	Golgi	- -	None		Erbasol et al., 2013
P-type ATPase FAMILY						
AtECA1	Root vasculature, flower, leaf vasculature, stem, silique	ER	- -	- -	Ca, Zn, Ni	Wu et al., 2002
AtECA3	Root vasculature and tip, Leaf vasculature, hydathodes, guard cells, flower, stem, silique	Golgi/EM	None	- -	Ca	Mills et al., 2008
LeLCA1	- -	- -	- -	- -	Ca	Johnson et al., 2009
VIT/CCC1-LIKE FAMILY						
AtVIT1	Developing seed, vasculature	VM	- -	- -	Fe	Kim et al., 2006
OsVIT1	Leaf > root, stem, panicle, embryo	VM	- -	- -	Fe, Zn	Zhang et al., 2012
OsVIT2	Leaf > root, stem, panicle, embryo	VM	- -	- -	Fe, Zn	Zhang et al., 2012

**Only during Zn deficiency. **Not observed by Conte et al. (2013). At, Arabidopsis thaliana; Hv, Hordeum vulgare; Ps, Pisum sativum; Le, Lycopersicon esculentum (Solanum lycopersicum); Mt, truncatula; Os, Oryza sativa; Zm, Zea mays; Bm, Beta vulgaris; PM, Plasma Membrane; ER, Endoplasmic Reticulum; VM, Vacuolar Membrane; MM, Mitochondrial Membrane; EM, Endomembrane Compartment; PVC, Pre-Vacuolar Compartment; GLC, Golgi-Like Compartment; PTM, Post-Translational Modification; - -, not tested.*

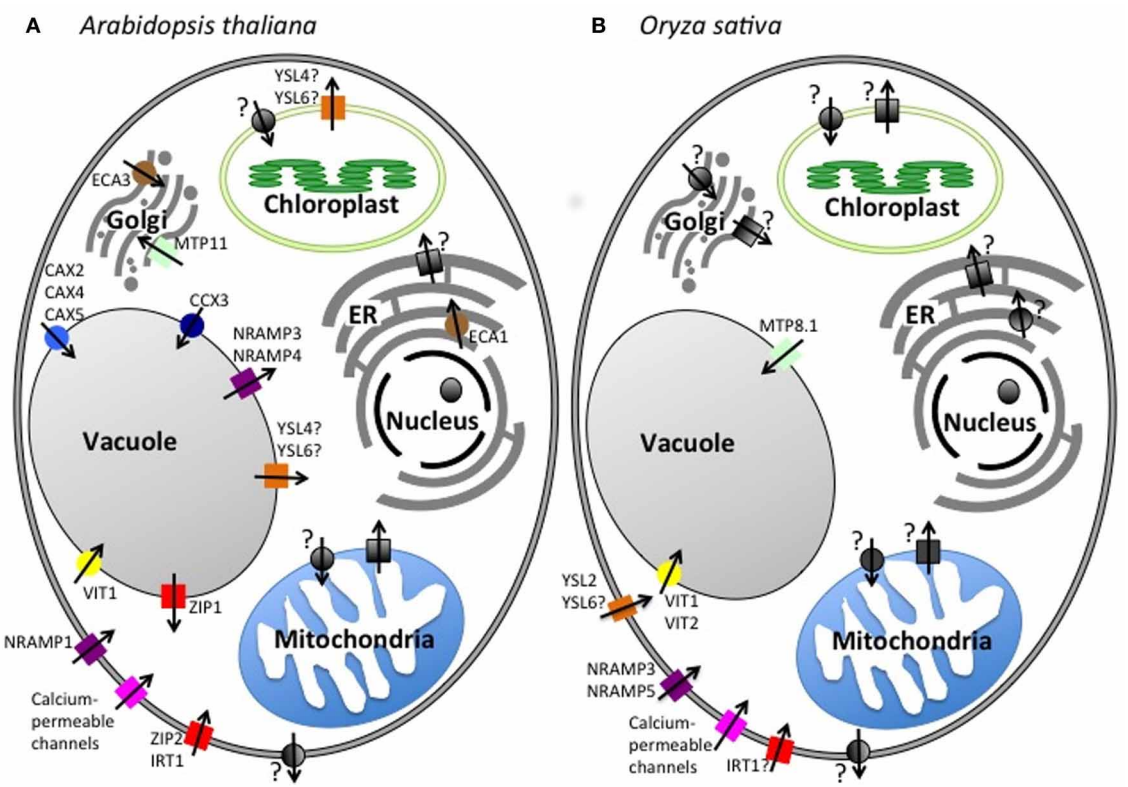

FIGURE 1 | Subcellular localization of putative Mn transporters. A diagram of a plant cell showing the Mn transport pathways in **(A)** *A. thaliana* and **(B)** *O. Sativa*. Squares, import into the cytosol; Circles, export out of the cytosol; Gray, unknown; Red, ZIP family; Magenta, Calcium-permeable channels; Orange, YSL family; Light blue, CAX family; Dark Blue, CCX family; Yellow, VIT family; Purple, NRAMP family; Brown, P2A-Type ATPase family; Green, CDF/MTP family.

(∼35 in plants), which include decarboxylases of the TCA cycle, RNA polymerases and numerous glycosyl transferases. In some enzymes, other metals can replace Mn as an enzymatic cofactor (Hebbern et al., 2009). Typically magnesium (Mg) replaces Mn because it is 50–100 times more abundant in the cell. Conversely, when Mn is in excess, it can replace Mg, which can have detrimental effects on the cellular processes in which Mg is involved. There are some enzymes that specifically require Mn such as those involved in cellular redox reactions. Mn is an indispensable component of Mn superoxide dismutase (MnSOD), a principal antioxidant enzyme of the mitochondria. A recent study analyzing the effect of Mn deficiency on Chlamydomonas showed that MnSOD activity decreases before that of PSII (Allen et al., 2007). This finding suggests intracellular regulation of Mn to support PSII function in the chloroplast in preference to MnSOD function in the mitochondria.

Plant uptake of Mn is a function of the Mn oxidation state in the soil. While Mn can exist in a range of oxidation states (Mn^{1+}, Mn^{2+}, Mn^{3+}, Mn^{4+}, Mn^{6+}, and Mn^{7+}), the most commonly found forms in biological systems are Mn^{2+}, Mn^{3+} and $Mn^{4+}$with Mn^{4+} being the least stable (Marschner, 2012). The most soluble species in soil is the divalent cation, Mn^{2+}, which is also the form of Mn that is most efficiently accumulated in plants (Marschner, 2012). Soil pH is a major determinant of Mn oxidation state in soil. At neutral or higher pH, Mn^{3+} and Mn^{4+} predominate and insoluble Mn oxides will form (Rengel, 2000; Marschner, 2012). Mn solubility is also influenced by microorganisms, which can either reduce or oxidize Mn, thereby affecting its availability to the plant (Lovley et al., 2011; Geszvain et al., 2012).

Despite its necessity, Mn is required in relatively small amounts (∼20–40 milligrams per kilogram of dry weight in most crop species) (He et al., 2005; Jiang, 2006; Marschner, 2012). However, it is one of the most prevalent trace element deficiencies seen in cereals including wheat and barley (Jiang, 2006). Fertilizers containing Mn sulphate can be added to the soil, however this is costly and the added Mn can be oxidized making it unavailable for plant acquisition. A more cost-effective and eco-friendly alternative is to preferentially breed plants that are able to thrive in low Mn soil. There has been a significant effort to identify crop species that are considered Mn efficient and can tolerate growth on low Mn, either by storing more Mn or by having an increased ability to absorb Mn from the soil (Jiang, 2006; Pedas et al., 2008). The genetic basis of Mn efficiency is not well understood.

Mn-deficient plants exhibit inhibited growth and decreased biomass (Marschner, 2012). Interveinal chlorosis due to decreases in net photosynthesis and chlorophyll content are common. This is most likely because the Mn-complex is needed to stabilize the PSII reaction center protein, D1 (Krieger et al., 1998; Allen et al., 2007; Yanykin et al., 2010). Mn-deficient plants are also

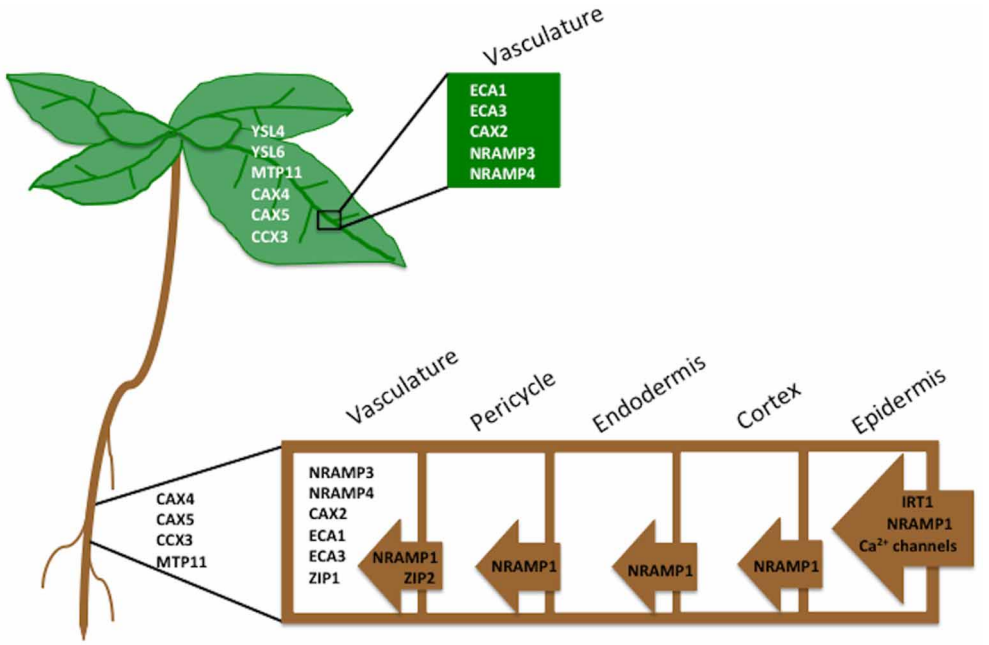

FIGURE 2 | Tissue localization of Mn transporters. The probable role of transporter family members in translocating Mn from the soil into the aerial portion of the plant in *A. thaliana*. The transporters listed to the left of the cells are not yet localized to a specific tissue.

characterized by tissue necrosis due to a decrease in MnSOD levels and an increase in oxygen free radicals (Allen et al., 2007; Marschner, 2012). Because Mn is a cofactor in the biosynthesis of cinnamic acid and its polymerization into lignin, decreased lignin concentration is especially prominent in the roots of Mn deficient plants (Marschner, 2012; Salvador et al., 2013). This is believed to be a contributing factor to an increased susceptibility to damage by freezing temperatures and root-infecting pathogens (Marschner, 2012). Mn deficiency can result from multiple factors including high concentrations of other minerals in the soil [i.e., Fe, Mg, calcium (Ca), phosphorus (P)] that can interfere with Mn absorption as well as soil alkalinity (pH >7.5) (Lynch and St. Clair, 2004; Marschner, 2012). Highly calcareous soils and soils found in arid and semi-arid regions are described as too alkaline for vegetative growth (Lynch and St. Clair, 2004).

Mn toxicity can also be detrimental to plants. Toxicity can occur in poorly drained acidic soils (pH < 5.5), where there is a predominance of Mn^{2+} (Lynch and St. Clair, 2004). Excess Mn can also prevent the uptake and translocation of other essential elements such as Ca, Mg, Fe, and P, presumably due to the similarity in ionic radius or binding strength for ligands (Marschner, 2012; Millaleo et al., 2013). In addition, toxicity can inhibit PSII and increase the accumulation of oxidized Mn and oxidized phenolic compounds in the leaf apoplast (Fecht-Christoffers et al., 2003; Marschner, 2012). As a result, common symptoms of Mn toxicity include interveinal chlorosis and tissue necrosis, which manifests as brown spots on mature leaves of plants, ultimately resulting in reduced plant biomass (Marschner, 2012). There are multiple mechanisms proposed to combat the deleterious effects of excess Mn. For example, plants can sequester Mn in the apoplast or vacuole (Horst and Maier, 1999; Hirschi et al., 2000; Schaaf et al., 2002). In fact, ectopic expression of vacuolar Mn transporters can increase the plant's tolerance to excess Mn (Delhaize et al., 2003). Free Mn ions can be chelated in metabolically inactive Mn^{2+}-organic acid complexes (Horst and Maier, 1999; Pittman, 2005; Fernando et al., 2010). In addition, Mn^{2+} can be mobilized into the endoplasmic reticulum (ER), thereby reducing cytoplasmic Mn (Wu et al., 2002).

TECHNIQUES FOR STUDYING METAL TRANSPORT AND ACCUMULATION IN PLANTS

The range of metals transported by a particular transport protein can be determined by expressing the gene in a simpler model, usually yeast. Some of the yeast strains commonly used to test metal transport capabilities are *smf1*, *fet3fet4*, *ctr1*, and *zrt1zrt2*, which are unable to transport Mn, Fe, Cu, and Zn, respectively, across the plasma membrane (Dancis et al., 1994; Dix et al., 1994; Supek et al., 1996; Zhao and Eide, 1996a,b). Specific metal transport by a protein of interest is indicated by rescued growth of these yeast strains in a low Mn, Fe, Cu or Zn media or in the presence of a divalent metal chelator. For example, the *smf1* mutant strain is unable to grow on media containing the divalent cation chelator EGTA. Another commonly used yeast strain to assay metal transport is *pmr1*. The P_2-type Ca-ATPase, PMR1 (plasma membrane ATPase related 1) pumps both Ca^{2+} and Mn^{2+} in the Golgi for detoxification purposes or for use as a cofactor for Golgi-localized proteins (Rudolph et al., 1989; Durr et al., 1998). When PMR1 is defective, yeast are more sensitive to high concentrations of Mn^{2+} (Durr et al., 1998). Therefore, complementation of *pmr1*

yeast with a Mn efflux transporter should restore growth when Mn in the media is high. Indirect studies using yeast, such as competition assays, are sometimes used to determine if a transporter has broad specificity (examples can be found in Grotz et al., 1998; Kaiser et al., 2003). However, further studies are necessary to confirm biological function.

Xenopus oocytes, immature eggs of an aquatic frog, are used to study the physiological function of a transporter. Electrophysiological measurements can be recorded in this system as well as uptake of radioactively labeled metals like ^{54}Mn. This system also allows for the addition of any potential metal chelators necessary for Mn translocation across the membrane. While it is not clear whether Mn-specific metallochaperones exist in plants, Mn can complex with nicotianamine (NA), phytosiderophores (PS), phytate and organic acids (Koike et al., 2004; Haydon and Cobbett, 2007; Fernando et al., 2010).

Mn content in plant tissues or in yeast expressing a plant transporter is measured to demonstrate difference in Mn transport efficiency. To date, the most accurate and sensitive method to measure metal content in a sample is Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Baxter et al., 2008; Donner et al., 2012). However, because ICP-MS requires the total digestion of a sample, it does not collect spatial information about an element *in vivo*. For some metals, ion specific fluorophores exist, which, in conjunction with confocal microscopy, can image the subcellular localization of a metal (Burdette et al., 2001; Miller et al., 2006, 2008; Yoon et al., 2007), but there is currently no Mn-specific fluorescent probe. Therefore, other techniques are needed to spatially resolve Mn localization in a cell.

Quantitative *in vivo* cryo-scanning electron microscopy (SEM)/energy dispersive X-ray analysis (EDAX) has been used to provide detailed electron micrographs of tissue from hyperaccumulator plants along with energy dispersive X-ray spectra from regions of interest (Fernando et al., 2006b).The samples are prepared by rapidly freezing them in liquid nitrogen, which preserves the metal location during processing and microbeam exposure (Fernando et al., 2013). Further analysis using Particle-Induced X-ray Emission induced by a focused ion beam (μPIXE) was used to confirm the cryo-SEM/EDAX results (Fernando et al., 2006a). Synchrotron X-Ray Fluorescence (SXRF) technology is a method used to localize metals *in vivo* at resolutions down to 250 nm. For a review on how SXRF can be used to study gene function see Punshon et al. (2013). It is important to recognize that no one method stands alone in determining the role of a protein in Mn translocation and multiple methods must be used in parallel.

MANGANESE LOCALIZATION *IN PLANTA*

Intracellular Mn is found in multiple locations in the cell including the chloroplast, cell wall, mitochondria and Golgi apparatus (Pittman, 2005). Mn is also located in the vacuole, an organelle that is critical for cellular metal homeostasis, where it serves as an intracellular sink when metals are in excess and as a source when metals are limited (Pittman, 2005; Fernando et al., 2006a; Lanquar et al., 2010). Much of the Mn imaging in plant tissues has been performed on Mn hyperaccumulators, due to their unique metabolism and ease of imaging Mn-enriched tissues (Leitenmaier and Kupper, 2013). To date, there are around 22 Mn hyperaccumulators identified around the world (Fernando et al., 2013). The majority of hyperaccumulator plants' primary sequestration sites are in non-photosynthetic tissues such as trichomes (leaf hairs) and epidermal tissue. However, Mn hyperaccumulators can sequester excess Mn in photosynthetic tissues as well as non-photosynthetic tissues. In previous studies of five different Mn hyperaccumulators, excess foliar Mn was concentrated in the double layer of chloroplast containing palisade mesophyll cells in the leaf (Fernando et al., 2006a,b). In contrast, the Mn hyperaccumulator, *Maytenus founieri*, exhibited increased Mn accumulation in the non-photosynthetic leaf epidermal tissues, which is reminiscent of other metal hyperaccumulators such as the Zn/Cd hyperaccumulator *A. halleri* (Fernando et al., 2008).

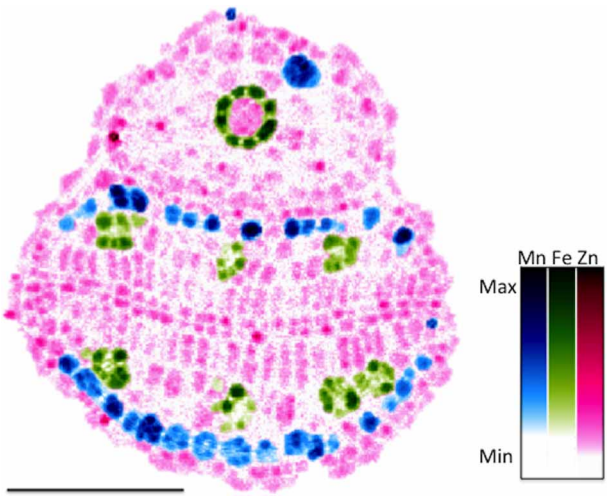

FIGURE 3 | Elemental distribution of Mn, Zn and Fe in *A. thaliana* seeds collected using Synchrotron X-Ray Florescence. Two dimensional map of a cross-section of an *A. thaliana* seed collected at 1 micron resolution at the Advanced Photon Source. Mn (blue), Zn (magenta), and Fe (green) are shown.

SXRF can be used to spatially localize and quantify Mn in *A. thaliana* seeds at a resolution high enough to resolve metals at a subcellular level (Donner et al., 2012). Recent studies using μPIXE confirmed SXRF data that Mn is localized to spongy mesophyll on the abaxial side of the embryonic leaf (Kim et al., 2006; Schnell Ramos et al., 2013) (**Figure 3**). Donner et al. (2012) pointed out that, due to its requirement for photosynthesis, Mn needs to relocate to the palisade mesophyll cells during germination.

MANGANESE TRANSPORTERS

Studying the metal transport mechanisms of other species can greatly assist our understanding of how plants take up and distribute Mn. While Mn transport is relatively understudied in plants, it is well understood in bacterial and yeast systems (Jakubovics and Jenkinson, 2001; Culotta et al., 2005; Papp-Wallace and Maguire, 2006; Reddi et al., 2009). In the yeast, *Saccharaomyces cerevisiae*, Mn is transported into the cell through the high affinity Mn^{2+} transporter SMF1 of the NRAMP family (Supek et al., 1996) and the high affinity phosphate transporter, PHO84 (Jensen et al., 2003). PHO84-type phosphate transporters prefer neutral metal-phosphate complexes; therefore the substrate

is presumed to be $MnHPO_4$ (Jensen et al., 2003). The photosynthetic alga *Chlamydomonas* also encodes an NRAMP protein that is predicted to be involved in high affinity Mn^{2+} influx (Allen et al., 2007). Photosynthetic autotrophs such as cyanobacteria require Mn for PSII assembly and a gene product with sequence similarity to an ABC (ATP binding cassette)-type transporter is implicated in Mn uptake in this organism (Bartsevich and Pakrasi, 1995).

There are few Mn-only transporters identified in plants. One possible explanation is that Mn shares many of the same transporters as other divalent cations such as Fe. One study found that it might not be possible to uncouple Mn and Fe transport in one high affinity Fe transporter after systematically introducing point mutations into the metal binding domain of IRT1 (Rogers et al., 2000). In fact, the majority of transporters implicated in Mn translocation have broad specificity for several divalent cations including Fe, Zn, Cu, Cd, Ca, Co (cobalt), Ni (nickel). The transporter families discussed below are organized based on transport of Mn into or out of the cytosol.

TRANSPORT INTO THE CYTOSOL

NRAMP family

NRAMP1 was first characterized in mice as a gene involved in resistance to intracellular pathogens (Nevo and Nelson, 2006). Like many of the transporter families implicated in Mn transport, the NRAMPs transport a broad range of metals and can transport Mn^{2+} as well as Fe^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Al^{3+} (Nevo and Nelson, 2006; Xia et al., 2010). The plant NRAMP proteins are predicted to be ion transporters based on sequence and predicted structural homology to the NRAMPs characterized in organisms such as humans and yeast. NRAMPs have between 10 and 12 transmembrane domains and a consensus transport sequence between transmembrane domains 8 and 9 that is shared with other ion channels and transporters (Cellier et al., 1995).

NRAMP proteins have been characterized in a number of plant species including *Solanum lycopersicum*, *Glycine max*, *Malas baccata*, *Thlaspi japonicum* and hyperaccumulators *A. halleri* and *Thlaspi caerulescens* (Bereczky et al., 2003; Kaiser et al., 2003; Mizuno et al., 2005; Xiao et al., 2008; Oomen et al., 2009). There are 6 and 7 NRAMP transporter proteins in *A. thaliana* and *Oryza sativa*, respectively. The first NRAMP genes cloned from plants were from rice in 1997 (Belouchi et al., 1997). However, not all are functionally characterized (Bennetzen, 2002; Nevo and Nelson, 2006).

Functional characterization of AtNRAMP1, AtNRAMP3, and AtNRAMP4 was first reported in 2000 (Thomine et al., 2000). AtNRAMP1 is believed to be a high affinity Mn transporter in the root due to the transcriptional upregulation of *AtNRAMP1* in the root under Mn deficiency and its PM localization (Cailliatte et al., 2010). In addition, *AtNRAMP1* expression increases in Fe deficiency and the protein complements yeast defective in Fe or Mn transport (Curie et al., 2000). Studies show that Arabidopsis *Atnramp1* knockout lines are more susceptible to Mn deficiency, accumulate less Mn in the shoot when grown without Mn and have decreased Mn in the root when grown under Mn replete conditions (Cailliatte et al., 2010). Plants overexpressing *AtNRAMP1* are more resistant to Mn deficiency. Elemental images of Mn in *Atnramp1–1* mutant seeds shows wild type distribution of Mn, therefore it is unlikely that these genes are necessary for Mn loading into the embryonic cotyledons (Donner et al., 2012).

AtNRAMP3 and AtNRAMP4 are also able to complement yeast deficient in either Mn or Fe uptake. *Atnramp3–1* mutants show increased Mn in the roots of plants grown in Fe limited conditions while Mn accumulation decreased when *AtNRAMP3* is expressed in the cell under control of the strong 35S promoter (Thomine et al., 2003). This phenotype is possibly due to the vacuolar localization of this transporter. Expression data and transcriptional fusions to the promoters of *AtNRAMP3* and *AtNRAMP4* show that the transporters are expressed in the root stele and the vasculature of leaves and cotyledons and are highly upregulated during Fe deficiency (Thomine et al., 2000, 2003; Lanquar et al., 2005). AtNRAMP4 was identified as part of the vacuolar proteome in *A. thaliana* mesophyll cells (Carter et al., 2004). Translational fusions of both AtNRAMP3 and AtNRAMP4 proteins show that they are localized to the vacuolar membrane; therefore they are thought to be important for metal remobilization from the vacuole, *in planta* (Thomine et al., 2003).

AtNRAMP3 and AtNRAMP4 transporters are functionally redundant; only *Atnramp3nramp4* double mutants exhibit a strong phenotype in response to metal deficiency. Both AtNRAMP3 and AtNRAMP4 are considered necessary for Fe remobilization during early germination due to their high expression levels at this growth stage (Lanquar et al., 2005). Lanquar et al. (2010) demonstrated that AtNRAMP3 and AtNRAMP4 are necessary for Mn export from the vacuole in the mesophyll cells of adult plant leaves. Double mutants have increased Mn accumulation in vacuoles. Under Mn deficiency, double mutants exhibit stunted growth and chlorosis when compared to wild type. Expression of either AtNRAMP3 or AtNRAMP4 in the double mutant can restore growth in Mn deficiency. Interestingly, it has been shown in other photosynthetic organisms that MnSOD activity decreases before there is a loss of function PSII in response to Mn deficiency (Allen et al., 2007). However, in Arabidopsis *Atnramp3nramp4* double mutants, the level of active PSII decreases while there is no loss of MnSOD activity (Lanquar et al., 2010). These results suggest intra-organellar crosstalk between the chloroplast and vacuole that mediate proper Mn distribution in the cell.

To date, it is not known whether AtNRAMP2, AtNRAMP5 or AtNRAMP6 are important for Mn homeostasis.

Of the 7 NRAMPs in rice, only 4 have been functionally characterized. *O. sativa* plants utilize OsNRAMP3 to respond to environmental changes in Mn availability. *OsNRAMP3* is constitutively expressed in the node of rice, which connects the vasculature of the root to aerial tissues of the plant (including the leaves, panicles and stems) (Yamaji et al., 2013). Under Mn deficiency, the PM localized OsNRAMP3 transports Mn from the transpiration stream in the xylem of enlarged vascular bundles to the younger tissues and panicles to meet minimal growth requirements. In contrast, when Mn is in excess, OsNRAMP3 is internalized in vesicles and rapidly degraded. Then, Mn is preferentially loaded into the older leaves, which are directly connected to the xylem-enlarged vascular bundles, thereby protecting developing

tissue from Mn toxicity. This study demonstrates the importance of post-translational regulation in response to environmental nutrient availability.

OsNRAMP5, on the other hand, does not respond to varying levels of environmental Mn, although gene expression increases slightly in the roots when plants are under Fe or Zn deficiency (Sasaki et al., 2012). Expression is seen in the mature zones of the root, more specifically at the PM of the exodermal and endodermal layers. Knockdown and RNAi lines accumulate less Mn in the roots and shoots and growth is stunted in response to Mn deficiency (Ishimaru et al., 2012; Sasaki et al., 2012). This phenotype is specific to Mn and not to other metal deficiencies tested, such as Fe-deficiency (Sasaki et al., 2012). Supplementing the plant with excess Mn does not rescue the growth defect. Therefore, it is likely that OsNRAMP5 is essential for Mn uptake from the soil in rice.

YSL family

The YSL transporters belong to a poorly characterized family of oligopeptide transporters (OPTs), which can transport amino acid-containing compounds and their derivatives (Yen et al., 2001). They are not found in all kingdoms, rather they are only found in plants, bacteria, fungi and archaea (Yen et al., 2001). The family is named after maize YS1 (ZmYS1), which accumulates in the root PM when Fe is scarce (Von Wiren et al., 1994; Roberts et al., 2004; Schaaf et al., 2004; Ueno et al., 2009). The characteristic interveinal chlorosis in the leaf of *ys1* knockdown mutants prompted the name "yellow stripe." Transport studies in both yeast and oocytes suggest that it can transport metals such as Mn, Zn, Cu, Ni, Cd as well as Fe (Schaaf et al., 2004). Therefore, it is reasonable to hypothesize that YSLs can transport Mn in other plant species.

YSL proteins mediate cellular uptake of metals complexed to non-proteinogenic amino acids: PS or its biosynthetic precursor NA (Yen et al., 2001). There is evidence that PS and NA are ligands for Mn (Schaaf et al., 2004; Haydon and Cobbett, 2007). Graminaceous plants (grasses) that include many crop species, use PS and NA for metal uptake and translocation, respectively (Palmer and Guerinot, 2009). PS is not produced by non-graminaceous plants (such as *A. thaliana*). Therefore, YSLs in non-grasses most likely use NA for intercellular and intracellular metal transport (Bashir et al., 2011).

There are eight predicted *YSL* genes in *A. thaliana* based on sequence similarity to YS1 (Curie et al., 2001). Knocking out a single *AtYSL* gene does not produce a strong visible phenotype (Didonato et al., 2004; Waters et al., 2006; Curie et al., 2009; Conte et al., 2013; Divol et al., 2013). However, it was discovered that if crosses are made between *Atysl* knockouts whose genes are in the same AtYSL subclass, a visible phenotype is observed. Examples include *Atysl1ysl3, Atysl4ysl6*, and *Atysl5ysl7* (Curie et al., 2009; Conte et al., 2013; Divol et al., 2013). Proteomics studies of the Arabidopsis vacuole predicted AtYSL4 and AtYSL6 to be vacuolar-localized (Jaquinod et al., 2007). Evidence shows that they are either located at the vacuole/endocompartments (shown via translational fusions) or the chloroplast (shown via immunofluorescence) (Conte et al., 2013; Divol et al., 2013). Data suggests that these proteins may be effluxers of heavy metals from an internal cellular compartment. Conte et al. (2013) observed that in comparison to wild type and single mutants, the double mutant is more resistant to prolonged growth on excess Mn. This phenotype was not reported by Divol et al. (2013). Thus far, the physiological role of AtYSL4 and AtYSL6 in Mn homeostasis remains to be clarified.

In rice, there are 18 putative *YSL* genes (Koike et al., 2004; Conte et al., 2013). OsYSL2 and OsYSL6 are implicated in Mn homeostasis. Electrophysiological and localization studies suggest the OsYSL2 is involved in lateral movement of Mn^{2+}-NA complexes via the phloem and loading into developing seeds (Koike et al., 2004). OsYSL6 is required for Mn detoxification when soil Mn is in excess (Sasaki et al., 2011). OsYSL6 is expressed constitutively in all cells of the roots and the shoots, particularly in senescing leaves. GFP-fusions of the protein disrupt localization, but due to successful complementation of the *smf1* yeast mutant, it is hypothesized to localize to the PM. When expressed in yeast, OsYSL6 translocates Mn^{2+}-NA. In *Osysl6* knockouts, plants exhibit symptoms of toxicity and excess Mn accumulates in the apoplast of the roots and shoots. When Mn^{2+} accumulates in the apoplast, it is oxidized into Mn^{3+}, which in turn will oxidize proteins and lipids (Fecht-Christoffers et al., 2003). Therefore, it is probable that when plants are exposed to high levels of Mn, OsYSL6 translocates Mn from the apoplast to the symplast where it is properly sequestered.

ZIP family

The *ZIP* gene family members are known to transport a broad range of metals including Fe^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Mn^{2+} in both eukaryotes and prokaryotes (Korshunova et al., 1999; Guerinot, 2000). Structurally, they are composed of eight transmembrane domains with the N- and C- termini facing outside the PM. Their length and amino acid composition varies within the cytoplasmic variable region between transmembrane domains 3 and 4. The variable region contains a conserved histidine motif that is believed to be important for metal binding and point mutations of inter-membrane domains can alter metal selectivity (Guerinot, 2000; Rogers et al., 2000).

The ZIP family of metal transporters is named for its founding members in both *S. cerevisae* and *A. thaliana*, respectively (Grotz et al., 1998). Of the 16 ZIP transporters in Arabidopsis, few have been functionally characterized (Maser et al., 2001; Rampey et al., 2013). IRT1 (Iron Regulated Transporter 1) is a high affinity Fe transporter that also has low affinity for other metals including Mn (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002; Yang et al., 2008). It is upregulated in Fe deficient roots. In addition, exogenous Mn cannot rescue the chlorotic phenotype of *irt1* mutants, which only grow in the presence of excess Fe (Vert et al., 2002). However, *irt1* mutant plants accumulate less Mn in their tissues during Fe deficiency, suggesting that IRT1 is a major pathway for Mn^{2+} uptake when Fe is scarce (Connolly et al., 2002). Because Mn uptake is still required for the plant during Fe replete conditions, there is likely another PM membrane localized transporter on the root epidermis that can transport Mn. Interestingly, under Mn^{2+} deficiency, IRT1 transcript levels decrease and Fe transport increases as evidenced by high Fe concentrations in the plant (Yang et al., 2008).

A more recent study found that in addition to AtIRT1, six *A. thaliana* ZIPs could restore growth to the Mn uptake defective *smf1* mutant: AtZIP1, AtZIP2, AtZIP5, AtZIP6, AtZIP7, and AtZIP9 (Milner et al., 2013). AtZIP3, AtZIP4, AtZIP10, AtZIP11, AtZIP12 did not. *AtZIP8* is a pseudogene in Arabidopsis and this did not complement any of the yeast strains used in the study. Under normal conditions, AtZIP1, AtZIP2, and AtZIP6 are more highly expressed in the root while AtZIP7 transcript is more highly concentrated in the shoot. *AtZIP5* and *AtZIP9* transcripts are not very abundant and can be found in the root and shoot. The physiological roles of AtZIP6, AtZIP7, and AtZIP9 in Mn homeostasis *in planta* remain to be explored.

AtZIP1 is most highly expressed in the root stele and is localized to the vacuole (Milner et al., 2013). AtZIP1 is believed to remobilize Mn from vacuoles into the cytoplasm to allow for their translocation to the shoot. This function is supported by the phenotype of *Atzip1* T-DNA insertion lines, which are more sensitive to low Mn and accumulate more Mn in the root. AtZIP2 is proposed to supply Mn to the aerial tissues in the plant via the root vasculature and not through direct uptake from the soil based on it localization to the PM in the root stele. In addition, mutant plants lacking functional AtZIP2 are more tolerant to Mn toxicity and less tolerant to Mn-deficiency, suggesting their importance in Mn translocation to the shoot for sequestration of excess Mn or normal plant health. However, it is unlikely that AtZIP2 is the primary transporter of Mn in the root because gene expression decreases during Mn deficiency.

To date, the literature does not detail any OsZIPs that are able to transport Mn. However, *OsZIP5* transcript was slightly induced in the root upon Mn deficiency (Lee et al., 2010). Yet, it was not able to rescue *smf1* mutants and is therefore unlikely to be a direct transporter of Mn. ZIPs are described in other plant species, aside from Arabidopsis, that transport Mn. These species include *Solanum lycopersicum*, *Pisum sativa*, *Hordeum vulgare*, and *Medicago truncatula* (Eckhardt et al., 2001; Bereczky et al., 2003; Cohen et al., 2004; Lopez-Millan et al., 2004; Pedas et al., 2008). Tomato LeIRT1 and LeIRT2 along with PsIRT1, HvIRT1, MtZIP4, and MtZIP7 can restore growth to *smf1* mutants in Mn limited media. In barley, Mn efficiency correlates with increased expression of HvIRT1, an OsIRT1 ortholog, in roots (Pedas et al., 2008).

Calcium-permeable channels

Calcium-permeable channels located in the PM of the root cells are a Ca^{2+} influx pathway that may be permeable to Mn^{2+} (White et al., 2002). Initial studies of these channels used biochemical and electrophysiological experiments to characterize their role in catalyzing cation influx because the genes encoding these channels had not been identified. A subset of cation channels that may be permeable to Mn^{2+} (as well as a broad range of other cations) are found in the apical PM of Arabidopsis root hairs (Very and Davies, 2000), protoplasts from the endodermis, cortex and root elongation zone and the epidermis of a developing root tip (Kiegle et al., 2000). They are also identified in the stele of maize roots (White et al., 2002). In addition, *LCT1*, a gene in wheat roots that is potentially a Ca^{2+}-permeable channel, was cloned and heterologously expressed in a yeast mutant where the transport of Ca^{2+} is blocked by the addition of Mn^{2+} to the media (Clemens et al., 1998). There is further evidence based on competition assays that Ca^{2+}-permeable channels transport Mn^{2+} in *A. thaliana* root hair tips (Wymer et al., 1997) and maize roots (Marshall et al., 1994).

EXPORT FROM THE CYTOSOL

CAX family

The CAX proteins are one of five transporter families that constitute the Ca^{2+}/cation antiporters (CaCA) superfamily (Shigaki and Hirschi, 2006; Emery et al., 2012). Although the CAX family members were originally identified as Ca^{2+} transporters, further study revealed their ability to transport a wide array of ions, hence their name modification from "calcium exchanger" to "cation exchanger." Typically, the CAXs contain 11 transmembrane domains and are found in plants, fungi and bacteria and in lower vertebrates. They facilitate the redistribution of cations across a membrane using electrochemical energy generated by a proton pump in order to maintain optimal ionic concentrations in the cell.

There are six CAX proteins in *A. thaliana*, which are split into two distinct phylogenetic groups: AtCAX1, AtCAX3, and AtCAX4 are part of the Type IA subgroup and AtCAX2, AtCAX5, and AtCAX6 are part of the Type 1B subgroup (Shigaki et al., 2006). The biological significance of the subgroups is unclear. In plants, the CAXs mediate efflux of ions into the vacuole. AtCAX2 contains a three-amino acid Mn^{2+} binding domain (Cys-Ala-Phe) between transmembrane domain 4 and transmembrane domain 5 (Shigaki et al., 2003). Expression of AtCAX2 in yeast can confer tolerance to Mn^{2+} toxicity (Schaaf et al., 2002; Shigaki et al., 2003). Furthermore, ectopic expression of AtCAX2 in tobacco (*Nicotiana tabacum*) can mediate vacuolar sequestration of Mn and confer resistance to high Mn stress (Hirschi et al., 2000), indicating a Mn transport function. In *A. thaliana*, *AtCAX2* is expressed at low levels in all tissues and does not respond to changes in Mn availability (Hirschi et al., 2000). However, Arabidopsis *Atcax2* mutants accumulate significantly less Mn in the vacuole compared to wild type (Pittman et al., 2004). The *Atcax2* knockout mutants display no obvious phenotype, even under Mn stress, and some Mn does continue to accumulate in the vacuole, suggesting that there are other vacuolar transporters that can compensate for the absence of AtCAX2 (Pittman et al., 2004). Candidates include AtCAX4 and AtCAX5 along with the AtCCX3 and AtVIT1 transporters, which will be discussed below.

AtCAX4 and AtCAX5 are likely involved in Mn^{2+}/H^{+} antiport activity. Like AtCAX2, AtCAX4, and AtCAX5 are located at the vacuolar membrane and are constitutively expressed at low levels in all tissues (Cheng et al., 2002; Edmond et al., 2009). AtCAX5 is most highly expressed in the stem and root and AtCAX4 is more highly expressed in the roots (determined via qRT-PCR) and plays a role in root growth under ion stress, such as Mn^{2+} toxicity (Edmond et al., 2009; Mei et al., 2009). Interestingly, AtCAX4 and AtCAX5 RNA levels increase when plants are exposed to high Mn^{2+} (Cheng et al., 2002; Edmond et al., 2009; Mei et al., 2009). AtCAX4 increases Mn stress tolerance when expressed in tobacco and AtCAX5 can rescue Mn-sensitive yeast, indicating

their ability to transport Mn (Korenkov et al., 2007; Edmond et al., 2009).

Thus far, single *Atcax* mutants do not exhibit strong phenotypes, even when exposed to Mn stress. This is possibly due to functional redundancy of the AtCAX proteins. A subset of double mutants in Arabidopsis are reported in the literature: *Atcax1cax3*, *Atcax2cax3*, and *Atcax1cax2* (Connorton et al., 2012; Punshon et al., 2012). AtCAX1 appears to be important for determining the phenotype of a double mutant plant. A study by Connorton et al. (2012), found that *Atcax2* and *Atcax2cax3* mutants are both more sensitive to high Mn than wild type. However, the *Atcax1cax2* double mutant does not display any signs of Mn toxicity, suggesting that by deleting *Atcax1* from the *Atcax2* mutants, Mn is once again able to accumulate in the vacuole. The phenotype of the *Atcax1cax2* mutant is consistent with the phenotype of the plants lacking a functional *AtCAX1* gene, which are also more tolerant to Mn toxicity. Therefore, it will be important to understand the apparent cross-talk that occurs between the AtCAX proteins, which confers their Mn transport capabilities.

There are five CAX proteins in the rice genome, all of which have been cloned and their transport specificities assessed in yeast (Kamiya et al., 2005). OsCAX1a and OsCAX3 confer Mn tolerance in yeast, therefore they are potentially Mn^{2+}/H^{+} exchangers *in planta* (Kamiya and Maeshima, 2004; Kamiya et al., 2005). It is important to note that CAX proteins can also transport Mn in other plant species. Examples include HvCAX2 in barley and LeCAX2 in tomato (Edmond et al., 2009). Future work is needed to confirm that they are also localized to the vacuole *in planta,* where they mediate Mn tolerance.

CCX family

CCXs are one of five families of transporters, (along with the CAXs) which make up the CaCA superfamily (Shigaki et al., 2006; Emery et al., 2012). There are five CCX proteins (CCX1-5), which were previously identified as CAX7-11. They were reclassified due to their higher sequence homology to the mammalian PM K^{+}-dependent Na^{+}/Ca^{2+} exchangers (NCXs). Expression of AtCCX3 in yeast rescues mutants defective in either PM or vacuolar import of Mn^{2+} (Morris et al., 2008). However, GFP and HA tagged versions of the protein support localization to intracellular compartments. Wild type yeast expressing AtCCX3 had nearly double the Mn concentration when compared to yeast expressing the control vector. Also, when ectopically expressed in tobacco, plant Mn^{2+} concentration significantly increased as leaves matured, which led to tissue necrosis. This phenotype is likely due to the defects in ion homeostasis in the plant resulting in increased ROS as evidenced by higher oxidation of proteins compared to controls. In plants, treatment with Mn induces *AtCCX3* expression in roots and flowers (Morris et al., 2008). Like *Atcax* single mutants, there are no apparent growth defects in Arabidopsis *Atccx3* mutant plants, which could be due to functional redundancy. Interestingly, AtCCX3 can transport both monovalent and divalent cations. However, the only divalent cation that can be transported by AtCCX3 is Mn^{2+}. Whether other AtCCXs can translocate Mn^{2+} remains to be studied.

CDF/MTP family

The CDF family, also known as MTP, is ubiquitous among all kingdoms of life (Maser et al., 2001; Montanini et al., 2007). The CDFs act as proton antiporters, which efflux metals such as Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Mn^{2+}out of the cytoplasm or into subcellular compartments (Gustin et al., 2011). However, one CDF protein is reported to be responsible for Zn uptake into the cytoplasm (Cragg et al., 2002). The plant CDFs are clustered into three functional groups based on phylogenetic analysis: Zn-CDFs, Fe/Zn-CDFs, and Mn-CDFs (Montanini et al., 2007; Gustin et al., 2011). Metal selectivity of the proteins is inferred from metal transport activity (either confirmed or hypothesized) of the respective members of the three subgroups. Mn-CDFs also contain amino acid residues that may predict metal specificity. The CDFs typically have six transmembrane domains and Mn-CDFs contain the highly conserved consensus sequence DXXXD (where X = any amino acid) in transmembrane domains 2 and 4, which is not found in the Zn- or Fe/Zn-CDFs (Montanini et al., 2007). Studies show that even single point mutations within key structural sites can alter metal ion specificity of the MTPs. For example, single point mutations in AtMTP1 (Podar et al., 2012) and OsMTP1 (Menguer et al., 2013) allow Mn uptake, which is not observed with the wild type protein.

In plants, the CDFs are identified as MTPs due to their role in detoxification of heavy metals (Ricachenevsky et al., 2013). The first characterized Mn-CDF transporter was identified in the Mn hyperaccumulating tropical legume, *Stylosanthes hamata* (Delhaize et al., 2003). ShMTP8 (previously annotated ShMTP1) can confer Mn^{2+} tolerance when ectopically expressed in Arabidopsis or yeast by sequestering excess metal in the vacuole. Of the twelve identified CDFs in *A. thaliana*, there are four in the Mn-CDF subgroup, which are also highly similar to ShMTP8, suggesting similar function. These proteins are AtMTP8, AtMTP9, AtMTP10, AtMTP11. The Mn-CDFs are further divided into subgroup 8 (including AtMTP8 and ShMTP8) and subgroup 9 (including AtMTP9, AtMTP10, and AtMTP11). To date, AtMTP11 is the only functionally characterized Arabidopsis Mn-CDF. Yeast transformed with AtMTP11are more tolerant to Mn^{2+} (Delhaize et al., 2007; Peiter et al., 2007). In addition, increased Mn^{2+} dependent proton-transport activity was recorded in yeast microsomal vesicles prepared from yeast expressing AtMTP11 in comparison to vesicles prepared from the control yeast strain (Delhaize et al., 2007). These data further support the role of AtMTP11 as an Mn^{2+}/H^{+} antiporter. Two independent studies localized AtMTP11 to the pre-vacuolar and Golgi-like compartments. Surprisingly, *AtMTP11* is most highly expressed in the leaf hydathodes and the root tip rather than in tissues that typically accumulate excess Mn such as the trichomes (Peiter et al., 2007). Hydathodes are involved in secretion of water containing salts and metals from the leaf. Therefore, Peiter et al. (2007) hypothesized that AtMTP11 is involved in vesicular trafficking and exocytosis of excess Mn at secretory tissues where Mn will be excreted rather than stored. This hypothesis is supported by the phenotype of both knockout mutants and plants overexpressing AtMTP11. Arabidopsis *Atmtp11* mutants are hypersensitive to high Mn while overexpression lines are hypertolerant to high Mn (Delhaize et al., 2007; Peiter et al.,

2007). Also, the *Atmtp11* mutants accumulate more Mn in the shoot and root, presumably due to their inability to secrete Mn from tissues (Peiter et al., 2007).

In rice there are five Mn-CDFs: OsMTP8 and OsMTP8.1 from Group 8 and OsMTP9, OsMTP11, and OsMTP11.1 from group 9. Recently, OsMTP8.1 was identified in a screen of rice shoot cDNAs that conferenced Mn tolerance in yeast (Chen et al., 2013). *pmr1* yeast mutants expressing OsMTP8.1 also accumulated more Mn. In rice, OsMTP8.1 is mainly expressed in shoots under all conditions tested, and expression increases in response to excess Mn. *Osmtp8.1* knockdown and knockout mutants exhibited toxicity symptoms in the presence of elevated Mn and Mn accumulation in the roots and shoot compared to wild type was reduced. The protein is localized to the vacuole, resulting in the hypothesis that OsMTP8.1 is important for Mn detoxification by sequestering Mn into the vacuole in rice plants.

Two MTP11 orthologs (PtMTP11.1 and PtMTP11.2) in poplar are believed to have a similar function as AtMTP11 due to their localization to Golgi-like compartments and ability to complement Arabidopsis *Atmtp11* plants (Peiter et al., 2007). Another study in beets (*Beta vulgaris*) identified two genes, *BmMTP10* and *BmMTP11*, which were named after their orthologs in *A. thaliana*. Much like AtMTP11, these proteins are associated with the Golgi and are hypothesized to efflux excess Mn via the secretory pathway. The conservation of Mn-CDF function among multiple plant species supports the importance of these transporters in Mn detoxification and cellular homeostasis.

P-type ATPase family

The endomembrane system is essential for coordinating ion homeostasis in the cell. Several transporters that localize to the vacuolar membrane are described to facilitate metal uptake and release. However, there are also transporters in other endomembrane compartments that are equally important. Currently, there are two characterized proteins in Arabidopsis, AtECA1 (ER-type calcium ATPases) and AtECA3, which are localized to the ER and Golgi Apparatus, respectively (Liang et al., 1997; Wu et al., 2002; Li et al., 2008; Mills et al., 2008). AtECA1 and AtECA3 function as Mn^{2+} pumps that remove Mn^{2+} from the cytosol and deliver it into their respective endomembrane compartment.

The ECAs belong to the Ca^{2+}-ATPase subfamily within the P-type ATPase superfamily of transporters, which use energy from ATP hydrolysis to catalyze the translocation of cations across membranes (Baxter et al., 2003; Huda et al., 2013). Plant Ca^{2+}-ATPases are categorized into P_{2A} and P_{2B}-types, both of which are generally described as Ca^{2+} pumps (Evans and Williams, 1998; Mills et al., 2008). There are four predicted P_{2A}-type ECA proteins in *A. thaliana* (AtECA1-4) and three in rice (OsECA1-3) (Baxter et al., 2003). P_{2A}-type ATPases show sequence homology to the sarcoplasmic/ER Ca^{2+}-ATPases (SERCA type) found in mammals. SERCAs, PMCAs (plasma membrane Ca^{2+}-ATPases) and SPCAs (secretory pathway Ca^{2+}-ATPases) comprise three distinct subfamilies of P_2-type Ca-ATPases in animal cells (Pittman et al., 1999; Mills et al., 2008). PMCAs are homologous to P_{2B}-type PMCAs, while there are no known SPCAs in plants.

Both AtECA1 and AtECA3 are able to restore the growth of *pmr1* yeast when Mn is high (Wu et al., 2002; Li et al., 2008; Mills et al., 2008). Proteomics analysis of Arabidopsis organelles, translational fusions and co-localization with marker proteins supports the ER localization of AtECA1 as well as the Golgi localization of AtECA3 (Wu et al., 2002; Dunkley et al., 2006; Mills et al., 2008; Nikolovski et al., 2012). AtECA1 is expressed in all major organs (especially in the root and flower) where it is believed to play a major role in managing Mn toxicity in the cell (Wu et al., 2002). *Ateca1* mutants appear wild type when grown under standard conditions, yet are smaller and chlorotic in the presence of high Mn. Two different *Ateca3* mutants exhibit opposite phenotypes to Mn stress. *Ateca3-2* mutants are more susceptible to Mn deficiency (Mills et al., 2008). When grown without Mn, they display stunted growth and leaf chlorosis, which can be rescued by the addition of even trace amounts of Mn. The *Ateca3-4* mutant allele is more sensitive to Mn toxicity (Li et al., 2008). The phenotypes of plants carrying these alleles suggest that AtECA3 is important for pumping Mn into the Golgi for proper plant nutrition as well as detoxification via the Golgi compartments. Further investigation is needed to understand the variation between the alleles that uncouples the proposed dual function of the AtECA3 transporter.

There is no evidence that the Arabidopsis AtECA2 or AtECA4 transporters play a role in Mn homeostasis. In fact, *Ateca2* mutants appear similar to wild type when grown under high or low Mn (Mills et al., 2008). A tomato ECA, LCA1 (Lycopersicon esculentum Ca^{2+}-ATPases), shows high amino acid sequence similarity to ECA2 (77%) in comparison to ECA1 and ECA3 (Pittman et al., 1999). LCA1 can also complement the *pmr1* yeast mutant, suggesting that it is also an endocompartment-localized Mn^{2+} pump (Johnson et al., 2009). Additional study of ECAs *in A. thaliana*, rice and other crop species is required to clarify their role in Mn homeostasis *in planta*.

VIT/CCC1-like family

CCC1 (Ca^{2+}-sensitive cross complementer 1), which transports Fe and Mn into the vacuole in yeast (Li et al., 2001), has six orthologs in Arabidopsis referred to as CCC1-like (Rampey et al., 2006; Gollhofer et al., 2011). To date, AtVIT1 is the best characterized CCC1-like transporter and is likely involved in Mn uptake into the vacuole. As hypothesized, *ccc1* mutant yeast, when expressing AtVIT1, could accumulate Mn in the vacuoles (Kim et al., 2006). High-resolution SXRF images of *Atvit1* mutant seeds display highly localized Mn in the Arabidopsis embryo in a pattern identical to that seen in wild type. This is not wholly surprising considering the multitude of transporters that are implicated in Mn transport in the absence of AtVIT1. Therefore, although AtVIT1 is a potential route for Mn transport in planta, it does not appear to be essential for localization of Mn in the seed. The functional orthologs of AtVIT1 in rice (OsVIT1 and OsVIT2) are localized to the vacuole. They transport Mn, Fe and Zn in yeast, however physiological studies of mutants suggest that they are only Fe and Zn transporters *in planta* (Zhang et al., 2012).

CONCLUSIONS AND FUTURE PERSPECTIVES

Mn, although essential for plant survival, can be toxic to plants. Over the past few decades, several transporter families have been identified that play a role in Mn homeostasis. Interestingly, many

of the transporters that translocate Mn have broad specificity for other ions, particularly divalent cations. For example, transporters such as IRT1, originally described as an Fe transporter in the root, can also transport Mn into the plant. In addition, CAX family members along with ECA proteins were originally thought to transport Ca; it was only subsequent research that discovered their roles in Mn transport as well. Despite the advancements in identifying Mn transporters, there is still a lot unknown regarding the molecular mechanisms controlling Mn homeostasis in plants. Many Mn transporters are not differentially regulated at the transcriptional level in response to Mn stress. Further research can explore the possibility that many of the transporters are under post-transcriptional or post-translational control. The degradation of OsNRAMP3 in response to Mn toxicity in rice may be a common mechanism used by plants to tolerate stress conditions (Yamaji et al., 2013).

Further characterization of orthologs of Mn transporters found in *A. thaliana* in crop species is a necessary step toward agronomic advancement. Research involving identification of genes involved in Mn homeostasis can be exploited to generate plants that are able to thrive in suboptimal soil conditions which would increase crop production and guarantee food security. For example, if we breed plants with high Mn uptake efficiency in the root, we can grow plants in highly alkaline soils where plants usually succumb to Mn deficiency. In conjunction, developing plants with increased Mn storage capability can assist in either soil Mn detoxification or improved growth of plants in acidic soils.

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REFERENCES

Allen, M. D., Kropat, J., Tottey, S., Del Campo, J. A., and Merchant, S. S. (2007). Manganese deficiency in Chlamydomonas results in loss of photosystem II and MnSOD function, sensitivity to peroxides, and secondary phosphorus and iron deficiency. *Plant Physiol.* 143, 263–277. doi: 10.1104/pp.106.088609

Bartsevich, V. V., and Pakrasi, H. B. (1995). Molecular identification of an ABC transporter complex for manganese: analysis of a cyanobacterial mutant strain impaired in the photosynthetic oxygen evolution process. *EMBO J.* 14, 1845–1853.

Bashir, K., Ishimaru, Y., Shimo, H., Nagasaka, S., Fujimoto, M., Takanashi, H., et al. (2011). The rice mitochondrial iron transporter is essential for plant growth. *Nat. Commun.* 2, 322. doi: 10.1038/ncomms1326

Baxter, I., Muthukumar, B., Park, H. C., Buchner, P., Lahner, B., Danku, J., et al. (2008). Variation in molybdenum content across broadly distributed populations of *Arabidopsis thaliana* is controlled by a mitochondrial molybdenum transporter (MOT1). *PLoS Genet.* 4:e1000004. doi: 10.1371/journal.pgen.1000004

Baxter, I., Tchieu, J., Sussman, M. R., Boutry, M., Palmgren, M. G., Gribskov, M., et al. (2003). Genomic comparison of P-type ATPase ion pumps in Arabidopsis and rice. *Plant Physiol.* 132, 618–628. doi: 10.1104/pp.103.021923

Belouchi, A., Kwan, T., and Gros, P. (1997). Cloning and characterization of the OsNramp family from *Oryza sativa*, a new family of membrane proteins possibly implicated in the transport of metal ions. *Plant Mol. Biol.* 33, 1085–1092. doi: 10.1023/A:1005723304911

Bennetzen, J. (2002). The rice genome. Opening the door to comparative plant biology. *Science* 296, 60–63. doi: 10.1126/science.1071402

Bereczky, Z., Wang, H. Y., Schubert, V., Ganal, M., and Bauer, P. (2003). Differential regulation of *NRAMP* and *IRT* metal transporter genes in wild type and iron uptake mutants of tomato. *J. Biol. Chem.* 278, 24697–24704. doi: 10.1074/jbc.M301365200

Burdette, S. C., Walkup, G. K., Spingler, B., Tsien, R. Y., and Lippard, S. J. (2001). Fluorescent sensors for $Zn^{(2+)}$based on a fluorescein platform: synthesis, properties and intracellular distribution. *J. Am. Chem. Soc.* 123, 7831–7841. doi: 10.1021/ja010059l

Cailliatte, R., Schikora, A., Briat, J. F., Mari, S., and Curie, C. (2010). High-affinity manganese uptake by the metal transporter NRAMP1 is essential for Arabidopsis growth in low manganese conditions. *Plant Cell* 22, 904–917. doi: 10.1105/tpc.109.073023

Carter, C., Pan, S., Zouhar, J., Avila, E. L., Girke, T., and Raikhel, N. V. (2004). The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. *Plant Cell* 16, 3285–3303. doi: 10.1105/tpc.104.027078

Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W., et al. (1995). Nramp defines a family of membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10089–10093. doi: 10.1073/pnas.92.22.10089

Chen, Z., Fujii, Y., Yamaji, N., Masuda, S., Takemoto, Y., Kamiya, T., et al. (2013). Mn tolerance in rice is mediated by MTP8.1, a member of the cation diffusion facilitator family. *J. Exp. Bot.* 64, 4375–4387. doi: 10.1093/jxb/ert243

Cheng, N. H., Pittman, J. K., Shigaki, T., and Hirschi, K. D. (2002). Characterization of CAX4, an Arabidopsis $H^{(+)}$/cation antiporter. *Plant Physiol.* 128, 1245–1254. doi: 10.1104/pp.010857

Clemens, S., Antosiewicz, D. M., Ward, J. M., Schachtman, D. P., and Schroeder, J. I. (1998). The plant cDNA LCT1 mediates the uptake of calcium and cadmium in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12043–12048. doi: 10.1073/pnas.95.20.12043

Cohen, C. K., Garvin, D. F., and Kochian, L. V. (2004). Kinetic properties of a micronutrient transporter from *Pisum sativum* indicate a primary function in Fe uptake from the soil. *Planta* 218, 784–792. doi: 10.1007/s00425-003-1156-7

Connolly, E. L., Fett, J. P., and Guerinot, M. L. (2002). Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell* 14, 1347–1357. doi: 10.1105/tpc.001263

Connorton, J. M., Webster, R. E., Cheng, N., and Pittman, J. K. (2012). Knockout of multiple Arabidopsis cation/$H^{(+)}$ exchangers suggests isoform-specific roles in metal stress response, germination and seed mineral nutrition. *PLoS ONE* 7:e47455. doi: 10.1371/journal.pone.0047455

Conte, S. S., Chu, H. H., Rodriguez, D. C., Punshon, T., Vasques, K. A., Salt, D. E., et al. (2013). *Arabidopsis thaliana* Yellow Stripe1-Like4 and Yellow Stripe1-Like6 localize to internal cellular membranes and are involved in metal ion homeostasis. *Front. Plant Sci.* 4:283. doi: 10.3389/fpls.2013.00283

Cragg, R. A., Christie, G. R., Phillips, S. R., Russi, R. M., Kury, S., Mathers, J. C., et al. (2002). A novel zinc-regulated human zinc transporter, hZTL1, is localized to the enterocyte apical membrane. *J. Biol. Chem.* 277, 22789–22797. doi: 10.1074/jbc.M200577200

Culotta, V. C., Yang, M., and Hall, M. D. (2005). Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*. *Eukaryot. Cell* 4, 1159–1165. doi: 10.1128/EC.4.7.1159-1165.2005

Curie, C., Alonso, J. M., Le Jean, M., Ecker, J. R., and Briat, J. F. (2000). Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem. J.* 347, 749–755. doi: 10.1042/0264-6021:3470749

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S. L., Briat, J. F., and Walker, E. L. (2001). Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409, 346–349. doi: 10.1038/35053080

Dancis, A., Haile, D., Yuan, D. S., and Klausner, R. D. (1994). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J. Biol. Chem.* 269, 25660–25667.

Delhaize, E., Gruber, B. D., Pittman, J. K., White, R. G., Leung, H., Miao, Y., et al. (2007). A role for the *AtMTP11* gene of Arabidopsis in manganese transport and tolerance. *Plant J.* 51, 198–210. doi: 10.1111/j.1365-313X.2007.03138.x

Delhaize, E., Kataoka, T., Hebb, D. M., White, R. G., and Ryan, P. R. (2003). Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. *Plant Cell* 15, 1131–1142. doi: 10.1105/tpc.009134

Didonato, R. J. Jr., Roberts, L. A., Sanderson, T., Eisley, R. B., and Walker, E. L. (2004). Arabidopsis *Yellow Stripe-Like2 (YSL2)*: a metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metal complexes. *Plant J.* 39, 403–414. doi: 10.1111/j.1365-313X.2004.02128.x

Divol, F., Couch, D., Conejero, G., Roschzttardtz, H., Mari, S., and Curie, C. (2013). The Arabidopsis Yellow Stripe LIKE4 and 6 transporters control iron release from the chloroplast. *Plant Cell* 25, 1040–1055. doi: 10.1105/tpc.112.107672

Dix, D. R., Bridgham, J. T., Broderius, M. A., Byersdorfer, C. A., and Eide, D. J. (1994). The *FET4* gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 269, 26092–26099.

Donner, E., Punshon, T., Guerinot, M. L., and Lombi, E. (2012). Functional characterisation of metal(loid) processes in planta through the integration of synchrotron techniques and plant molecular biology. *Anal. Bioanal. Chem.* 402, 3287–3298. doi: 10.1007/s00216-011-5624-9

Dunkley, T. P., Hester, S., Shadforth, I. P., Runions, J., Weimar, T., Hanton, S. L., et al. (2006). Mapping the Arabidopsis organelle proteome. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6518–6523. doi: 10.1073/pnas.0506958103

Durr, G., Strayle, J., Plemper, R., Elbs, S., Klee, S. K., Catty, P., et al. (1998). The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with $Ca^{2+}$and Mn^{2+} required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *Mol. Biol. Cell* 9, 1149–1162. doi: 10.1091/mbc.9.5.1149

Eckhardt, U., Mas Marques, A., and Buckhout, T. J. (2001). Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. *Plant Mol. Biol.* 45, 437–448. doi: 10.1023/A:1010620012803

Edmond, C., Shigaki, T., Ewert, S., Nelson, M. D., Connorton, J. M., Chalova, V., et al. (2009). Comparative analysis of CAX2-like cation transporters indicates functional and regulatory diversity. *Biochem J.* 418, 145–154. doi: 10.1042/BJ20081814

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5624–5628. doi: 10.1073/pnas.93.11.5624

Emery, L., Whelan, S., Hirschi, K. D., and Pittman, J. K. (2012). Protein phylogenetic analysis of $Ca^{(2+)}$/cation Antiporters and insights into their evolution in plants. *Front. Plant Sci.* 3:1. doi: 10.3389/fpls.2012.00001

Erbasol, I., Bozdag, G. O., Koc, A., Pedas, P., and Karakaya, H. C. (2013). Characterization of two genes encoding metal tolerance proteins from Beta vulgaris subspecies maritima that confers manganese tolerance in yeast. *Biometals* 26, 795–804. doi: 10.1007/s10534-013-9658-7

Evans, D. E., and Williams, L. E. (1998). P-type calcium ATPases in higher plants - biochemical, molecular and functional properties. *Biochim. Biophys. Acta* 1376, 1–25. doi: 10.1016/S0304-4157(97)00009-9

Fecht-Christoffers, M. M., Braun, H. P., Lemaitre-Guillier, C., Vandorsselaer, A., and Horst, W. J. (2003). Effect of manganese toxicity on the proteome of the leaf apoplast in cowpea. *Plant Physiol.* 133, 1935–1946. doi: 10.1104/pp.103.029215

Fernando, D. R., Bakkaus, E. J., Perrier, N., Baker, A. J., Woodrow, I. E., Batianoff, G. N., et al. (2006a). Manganese accumulation in the leaf mesophyll of four tree species: a PIXE/EDAX localization study. *New Phytol.* 171, 751–757. doi: 10.1111/j.1469-8137.2006.01783.x

Fernando, D. R., Batianoff, G. N., Baker, A. J., and Woodrow, I. E. (2006b). *In vivo* localization of manganese in the hyperaccumulator *Gossia bidwillii* (Benth.) N. Snow & Guymer (Myrtaceae) by cryo-SEM/EDAX. *Plant Cell Environ.* 29, 1012–1020. doi: 10.1111/j.1365-3040.2006.01498.x

Fernando, D. R., Marshall, A., Baker, A. J., and Mizuno, T. (2013). Microbeam methodologies as powerful tools in manganese hyperaccumulation research: present status and future directions. *Front. Plant Sci.* 4:319. doi: 10.3389/fpls.2013.00319

Fernando, D. R., Mizuno, T., Woodrow, I. E., Baker, A. J., and Collins, R. N. (2010). Characterization of foliar manganese (Mn) in Mn (hyper)accumulators using X-ray absorption spectroscopy. *New Phytol.* 188, 1014–1027. doi: 10.1111/j.1469-8137.2010.03431.x

Fernando, D. R., Woodrow, I. E., Jaffre, T., Dumontet, V., Marshall, A. T., and Baker, A. J. (2008). Foliar manganese accumulation by *Maytenus founieri* (Celastraceae) in its native New Caledonian habitats: populational variation and localization by X-ray microanalysis. *New Phytol.* 177, 178–185. doi: 10.1111/j.1469-8137.2007.02253.x

Geszvain, K., Butterfield, C., Davis, R. E., Madison, A. S., Lee, S. W., Parker, D. L., et al. (2012). The molecular biogeochemistry of manganese(II) oxidation. *Biochem. Soc. Trans.* 40, 1244–1248. doi: 10.1042/BST20120229

Gollhofer, J., Shlawicke, C., Jungnick, N., Schmidt, W., and Buckhout, T. J. (2011). Members of a small family of nodulin-like genes are regulated under iron deficiency in roots of *Arabidopsis thaliana*. *Plant Physiol. Biochem.* 49, 557–564. doi: 10.1016/j.plaphy.2011.02.011

Goussias, C., Boussac, A., and Rutherford, A. W. (2002). Photosystem II and photosynthetic oxidation of water: an overview. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 357, 1369–1381. discussion: 1419–1320. doi: 10.1098/rstb.2002.1134

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., and Eide, D. (1998). Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7220–7224. doi: 10.1073/pnas.95.12.7220

Guerinot, M. L. (2000). The ZIP family of metal transporters. *Biochim. Biophys. Acta* 1465, 190–198. doi: 10.1016/S0005-2736(00)00138-3

Gustin, J. L., Zanis, M. J., and Salt, D. E. (2011). Structure and evolution of the plant cation diffusion facilitator family of ion transporters. *BMC Evol. Biol.* 11:76. doi: 10.1186/1471-2148-11-76

Haydon, M. J., and Cobbett, C. S. (2007). Transporters of ligands for essential metal ions in plants. *New Phytol.* 174, 499–506. doi: 10.1111/j.1469-8137.2007.02051.x

He, Z. L., Yang, X. E., and Stoffella, P. J. (2005). Trace elements in agroecosystems and impacts on the environment. *J. Trace Elem. Med. Biol.* 19, 125–140. doi: 10.1016/j.jtemb.2005.02.010

Hebbern, C. A., Laursen, K. H., Ladegaard, A. H., Schmidt, S. B., Pedas, P., Bruhn, D., et al. (2009). Latent manganese deficiency increases transpiration in barley (*Hordeum vulgare*). *Physiol. Plant* 135, 307–316. doi: 10.1111/j.1399-3054.2008.01188.x

Hirschi, K. D., Korenkov, V. D., Wilganowski, N. L., and Wagner, G. J. (2000). Expression of arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. *Plant Physiol.* 124, 125–133. doi: 10.1104/pp.124.1.125

Horst, W. J., and Maier P. (1999). "Compartmentalization of manganese in the vacuoles and in the apoplast of leaves in relation to genotypic manganese leaf-tissue tolerance in *Vigna unguiculata* (L.) Walp," in *Plant Nutrition- Molecular Biology and Genetics*, eds G. Gissel-Nielsen and A. Jensen (Dordrecht: Kluwer Academic Publishers), 222–234.

Huda, K. M., Banu, M. S., Tuteja, R., and Tuteja, N. (2013). Global calcium transducer P-type $Ca^{(2)(+)}$-ATPases open new avenues for agriculture by regulating stress signalling. *J. Exp. Bot.* 64, 3099–3109. doi: 10.1093/jxb/ert182

Ishimaru, Y., Takahashi, R., Bashir, K., Shimo, H., Senoura, T., Sugimoto, K., et al. (2012). Characterizing the role of rice NRAMP5 in manganese, iron and cadmium transport. *Sci. Rep.* 2:286. doi: 10.1038/srep00286

Jakubovics, N. S., and Jenkinson, H. F. (2001). Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. *Microbiology* 147, 1709–1718.

Jaquinod, M., Villiers, F., Kieffer-Jaquinod, S., Hugouvieux, V., Bruley, C., Garin, J., et al. (2007). A proteomics dissection of *Arabidopsis thaliana* vacuoles isolated from cell culture. *Mol. Cell Proteomics* 6, 394–412. doi: 10.1074/mcp.M600250-MCP200

Jensen, L. T., Ajua-Alemanji, M., and Culotta, V. C. (2003). The *Saccharomyces cerevisiae* high affinity phosphate transporter encoded by *PHO84* also functions in manganese homeostasis. *J. Biol. Chem.* 278, 42036–42040. doi: 10.1074/jbc.M307413200

Jiang, W. (2006). Mn use efficiency in different wheat cultivars. *Environ. Exp. Bot.* 57, 41–50. doi: 10.1016/j.envexpbot.2005.04.008

Johnson, N. A., Liu, F., Weeks, P. D., Hentzen, A. E., Kruse, H. P., Parker, J. J., et al. (2009). A tomato ER-type Ca^{2+}-ATPase, LCA1, has a low thapsigargin-sensitivity and can transport manganese. *Arch. Biochem. Biophys.* 481, 157–168. doi: 10.1016/j.abb.2008.11.010

Kaiser, B. N., Moreau, S., Castelli, J., Thomson, R., Lambert, A., Bogliolo, S., et al. (2003). The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. *Plant J.* 35, 295–304. doi: 10.1046/j.1365-313X.2003.01802.x

Kamiya, T., Akahori, T., and Maeshima, M. (2005). Expression profile of the genes for rice cation/H^+ exchanger family and functional analysis in yeast. *Plant Cell Physiol.* 46, 1735–1740. doi: 10.1093/pcp/pci173

Kamiya, T., and Maeshima, M. (2004). Residues in internal repeats of the rice cation/H^+ exchanger are involved in the transport and selection of cations. *J. Biol. Chem.* 279, 812–819. doi: 10.1074/jbc.M309726200

Kiegle, E., Gilliham, M., Haseloff, J., and Tester, M. (2000). Hyperpolarisation-activated calcium currents found only in cells from the elongation zone of *Arabidopsis thaliana* roots. *Plant J.* 21, 225–229. doi: 10.1046/j.1365-313x.2000.00659.x

Kim, S. A., Punshon, T., Lanzirotti, A., Li, L., Alonso, J. M., Ecker, J. R., et al. (2006). Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VIT1. *Science* 314, 1295–1298. doi: 10.1126/science.1132563

Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori, S., et al. (2004). OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J.* 39, 415–424. doi: 10.1111/j.1365-313X.2004.02146.x

Korenkov, V., Hirschi, K., Crutchfield, J. D., and Wagner, G. J. (2007). Enhancing tonoplast Cd/H antiport activity increases Cd, Zn, and Mn tolerance, and impacts root/shoot Cd partitioning in *Nicotiana tabacum* L. *Planta* 226, 1379–1387. doi: 10.1007/s00425-007-0577-0

Korshunova, Y. O., Eide, D., Clark, W. G., Guerinot, M. L., and Pakrasi, H. B. (1999). The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Mol. Biol.* 40, 37–44. doi: 10.1023/A:1026438615520

Kramer, U. (2010). Metal hyperaccumulation in plants. *Annu. Rev. Plant Biol.* 61, 517–534. doi: 10.1146/annurev-arplant-042809-112156

Krieger, A., Rutherford, A. W., Vass, I., and Hideg, E. (1998). Relationship between activity, D1 loss, and Mn binding in photoinhibition of photosystem II. *Biochemistry* 37, 16262–16269. doi: 10.1021/bi981243v

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., et al. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24, 4041–4051. doi: 10.1038/sj.emboj.7600864

Lanquar, V., Ramos, M. S., Lelievre, F., Barbier-Brygoo, H., Krieger-Liszkay, A., Kramer, U., et al. (2010). Export of vacuolar manganese by AtNRAMP3 and AtNRAMP4 is required for optimal photosynthesis and growth under manganese deficiency. *Plant Physiol.* 152, 1986–1999. doi: 10.1104/pp.109.150946

Lee, S., Jeong, H. J., Kim, S. A., Lee, J., Guerinot, M. L., and An, G. (2010). OsZIP5 is a plasma membrane zinc transporter in rice. *Plant Mol. Biol.* 73, 507–517. doi: 10.1007/s11103-010-9637-0

Leitenmaier, B., and Kupper, H. (2013). Compartmentation and complexation of metals in hyperaccumulator plants. *Front. Plant Sci.* 4:374. doi: 10.3389/fpls.2013.00374

Li, L., Chen, O. S., McVey Ward, D., and Kaplan, J. (2001). CCC1 is a transporter that mediates vacuolar iron storage in yeast. *J. Biol. Chem.* 276, 29515–29519. doi: 10.1074/jbc.M103944200

Li, X., Chanroj, S., Wu, Z., Romanowsky, S. M., Harper, J. F., and Sze, H. (2008). A distinct endosomal Ca^{2+}/Mn^{2+} pump affects root growth through the secretory process. *Plant Physiol.* 147, 1675–1689. doi: 10.1104/pp.108.119909

Liang, F., Cunningham, K. W., Harper, J. F., and Sze, H. (1997). ECA1 complements yeast mutants defective in Ca^{2+} pumps and encodes an endoplasmic reticulum-type Ca^{2+}-ATPase in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U.S.A.* 94, 8579–8584. doi: 10.1073/pnas.94.16.8579

Lopez-Millan, A. F., Ellis, D. R., and Grusak, M. A. (2004). Identification and characterization of several new members of the ZIP family of metal ion transporters in *Medicago truncatula*. *Plant Mol. Biol.* 54, 583–596. doi: 10.1023/B:PLAN.0000038271.96019.aa

Lovley, D. R., Ueki, T., Zhang, T., Malvankar, N. S., Shrestha, P. M., Flanagan, K. A., et al. (2011). Geobacter: the microbe electric's physiology, ecology, and practical applications. *Adv. Microb. Physiol.* 59, 1–100. doi: 10.1016/B978-0-12-387661-4.00004-5

Lynch, J. P., and St. Clair, S. B. (2004). Mineral stress: the missing link in understanding how global climate change will affect plants in real world soils. *Field Crop Res.* 90, 101–115. doi: 10.1016/j.fcr.2004.07.008

Marschner, P. (2012). *Marschner's Mineral Nutrition of Higher Plants.* Boston, MA: Academic Press.

Marshall, J., Corzo, A., Leigh, R. A., and Sanders, D. (1994). Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from *Zea mays* L. roots. *Plant J.* 5, 683–694. doi: 10.1111/j.1365-313X.1994.00683.x

Martinez-Finley, E. J., Gavin, C. E., Aschner, M., and Gunter, T. E. (2013). Manganese neurotoxicity and the role of reactive oxygen species. *Free Radic. Biol. Med.* 62, 65–75. doi: 10.1016/j.freeradbiomed.2013.01.032

Maser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., et al. (2001). Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiol.* 126, 1646–1667. doi: 10.1104/pp.126.4.1646

Mei, H., Cheng, N. H., Zhao, J., Park, S., Escareno, R. A., Pittman, J. K., et al. (2009). Root development under metal stress in *Arabidopsis thaliana* requires the H^+/cation antiporter CAX4. *New Phytol.* 183, 95–105. doi: 10.1111/j.1469-8137.2009.02831.x

Menguer, P. K., Farthing, E., Peaston, K. A., Ricachenevsky, F. K., Fett, J. P., and Williams, L. E. (2013). Functional analysis of the rice vacuolar zinc transporter OsMTP1. *J. Exp. Bot.* 64, 2871–2883. doi: 10.1093/jxb/ert136

Millaleo, R., Reyes-Diaz, M., Alberdi, M., Ivanov, A. G., Krol, M., and Huner, N. P. (2013). Excess manganese differentially inhibits photosystem I versus II in *Arabidopsis thaliana*. *J. Exp. Bot.* 64, 343–354. doi: 10.1093/jxb/ers339

Miller, E. W., He, Q., and Chang, C. J. (2008). Preparation and use of Leadfluor-1, a synthetic fluorophore for live-cell lead imaging. *Nat. Protoc.* 3, 777–783. doi: 10.1038/nprot.2008.43

Miller, E. W., Zeng, L., Domaille, D. W., and Chang, C. J. (2006). Preparation and use of Coppersensor-1, a synthetic fluorophore for live-cell copper imaging. *Nat. Protoc.* 1, 824–827. doi: 10.1038/nprot.2006.140

Mills, R. F., Doherty, M. L., Lopez-Marques, R. L., Weimar, T., Dupree, P., Palmgren, M. G., et al. (2008). ECA3, a Golgi-localized P2A-type ATPase, plays a crucial role in manganese nutrition in Arabidopsis. *Plant Physiol.* 146, 116–128. doi: 10.1104/pp.107.110817

Milner, M. J., Seamon, J., Craft, E., and Kochian, L. V. (2013). Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. *J. Exp. Bot.* 64, 369–381. doi: 10.1093/jxb/ers315

Mizuno, T., Usui, K., Horie, K., Nosaka, S., Mizuno, N., and Obata, H. (2005). Cloning of three ZIP/Nramp transporter genes from a Ni hyperaccumulator plant *Thlaspi japonicum* and their Ni^{2+}-transport abilities. *Plant Physiol. Biochem.* 43, 793–801. doi: 10.1016/j.plaphy.2005.07.006

Montanini, B., Blaudez, D., Jeandroz, S., Sanders, D., and Chalot, M. (2007). Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity. *BMC Genomics* 8:107. doi: 10.1186/1471-2164-8-107

Morris, J., Tian, H., Park, S., Sreevidya, C. S., Ward, J. M., and Hirschi, K. D. (2008). AtCCX3 is an Arabidopsis endomembrane H^+ -dependent K^+ transporter. *Plant Physiol.* 148, 1474–1486. doi: 10.1104/pp.108.118810

Nevo, Y., and Nelson, N. (2006). The NRAMP family of metal-ion transporters. *Biochim. Biophys. Acta* 1763, 609–620. doi: 10.1016/j.bbamcr.2006.05.007

Nickelsen, J., and Rengstl, B. (2013). Photosystem II assembly: from cyanobacteria to plants. *Annu. Rev. Plant Biol.* 64, 609–635. doi: 10.1146/annurev-arplant-050312-120124

Nikolovski, N., Rubtsov, D., Segura, M. P., Miles, G. P., Stevens, T. J., Dunkley, T. P., et al. (2012). Putative glycosyltransferases and other plant Golgi apparatus proteins are revealed by LOPIT proteomics. *Plant Physiol.* 160, 1037–1051. doi: 10.1104/pp.112.204263

Oomen, R. J., Wu, J., Lelievre, F., Blanchet, S., Richaud, P., Barbier-Brygoo, H., et al. (2009). Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. *New Phytol.* 181, 637–650. doi: 10.1111/j.1469-8137.2008.02694.x

Palmer, C. M., and Guerinot, M. L. (2009). Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nat. Chem. Biol.* 5, 333–340. doi: 10.1038/nchembio.166

Papp-Wallace, K. M., and Maguire, M. E. (2006). Manganese transport and the role of manganese in virulence. *Annu. Rev. Microbiol.* 60, 187–209. doi: 10.1146/annurev.micro.60.080805.142149

Pedas, P., Ytting, C. K., Fuglsang, A. T., Jahn, T. P., Schjoerring, J. K., and Husted, S. (2008). Manganese efficiency in barley: identification and characterization of the metal ion transporter HvIRT1. *Plant Physiol.* 148, 455–466. doi: 10.1104/pp.108.118851

Peiter, E., Montanini, B., Gobert, A., Pedas, P., Husted, S., Maathuis, F. J., et al. (2007). A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8532–8537. doi: 10.1073/pnas.0609507104

Pittman, J. K. (2005). Managing the manganese: molecular mechanisms of manganese transport and homeostasis. *New Phytol.* 167, 733–742. doi: 10.1111/j.1469-8137.2005.01453.x

Pittman, J. K., Mills, R. F., O'connor, C. D., and Williams, L. E. (1999). Two additional type IIA $Ca^{(2+)}$-ATPases are expressed in *Arabidopsis thaliana*: evidence that type IIA sub-groups exist. *Gene* 236, 137–147. doi: 10.1016/S0378-1119(99)00242-5

Pittman, J. K., Shigaki, T., Marshall, J. L., Morris, J. L., Cheng, N. H., and Hirschi, K. D. (2004). Functional and regulatory analysis of the *Arabidopsis thaliana* CAX2 cation transporter. *Plant Mol. Biol.* 56, 959–971. doi: 10.1007/s11103-004-6446-3

Podar, D., Scherer, J., Noordally, Z., Herzyk, P., Nies, D., and Sanders, D. (2012). Metal selectivity determinants in a family of transition metal transporters. *J. Biol. Chem.* 287, 3185–3196. doi: 10.1074/jbc.M111.305649

Punshon, T., Hirschi, K., Yang, J., Lanzirotti, A., Lai, B., and Guerinot, M. L. (2012). The role of CAX1 and CAX3 in elemental distribution and abundance in Arabidopsis seed. *Plant Physiol.* 158, 352–362. doi: 10.1104/pp.111.184812

Punshon, T., Ricachenevsky, F. K., Hindt, M. N., Socha, A. L., and Zuber, H. (2013). Methodological approaches for using synchrotron X-ray fluorescence (SXRF) imaging as a tool in ionomics: examples from *Arabidopsis thaliana*. *Metallomics* 5, 1133–1145. doi: 10.1039/c3mt00120b

Rampey, R. A., Baldridge, M. T., Farrow, D. C., Bay, S. N., and Bartel, B. (2013). Compensatory mutations in predicted metal transporters modulate auxin conjugate responsiveness in Arabidopsis. *G3* 3, 131–141. doi: 10.1534/g3.112.004655

Rampey, R. A., Woodward, A. W., Hobbs, B. N., Tierney, M. P., Lahner, B., Salt, D. E., et al. (2006). An Arabidopsis basic helix-loop-helix leucine zipper protein modulates metal homeostasis and auxin conjugate responsiveness. *Genetics* 174, 1841–1857. doi: 10.1534/genetics.106.061044

Reddi, A. R., Jensen, L. T., and Culotta, V. C. (2009). Manganese homeostasis in *Saccharomyces cerevisiae*. *Chem. Rev.* 109, 4722–4732. doi: 10.1021/cr900031u

Rengel, Z. (2000). Manganese uptake and transport in plants. *Met. Ions Biol. Syst.* 37, 57–87.

Ricachenevsky, F. K., Menguer, P. K., Sperotto, R. A., Williams, L. E., and Fett, J. P. (2013). Roles of plant metal tolerance proteins (MTP) in metal storage and potential use in biofortification strategies. *Front. Plant Sci.* 4:144. doi: 10.3389/fpls.2013.00144

Roberts, L. A., Pierson, A. J., Panaviene, Z., and Walker, E. L. (2004). Yellow stripe1. Expanded roles for the maize iron-phytosiderophore transporter. *Plant Physiol.* 135, 112–120. doi: 10.1104/pp.103.037572

Rogers, E. E., Eide, D. J., and Guerinot, M. L. (2000). Altered selectivity in an Arabidopsis metal transporter. *Proc. Natl. Acad. Sci. U.S.A.* 97, 12356–12360. doi: 10.1073/pnas.210214197

Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., Levitre, J., et al. (1989). The yeast secretory pathway is perturbed by mutations in PMR1, a member of a Ca^{2+} ATPase family. *Cell* 58, 133–145. doi: 10.1016/0092-8674(89)90410-8

Salt, D. E., Smith, R. D., and Raskin, I. (1998). Phytoremediation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 643–668. doi: 10.1146/annurev.arplant.49.1.643

Salvador, V. H., Lima, R. B., Dos Santos, W. D., Soares, A. R., Bohm, P. A., Marchiosi, R., et al. (2013). Cinnamic acid increases lignin production and inhibits soybean root growth. *PLoS ONE* 8:e69105. doi: 10.1371/journal.pone.0069105

Sasaki, A., Yamaji, N., Xia, J., and Ma, J. F. (2011). OsYSL6 is involved in the detoxification of excess manganese in rice. *Plant Physiol.* 157, 1832–1840. doi: 10.1104/pp.111.186031

Sasaki, A., Yamaji, N., Yokosho, K., and Ma, J. F. (2012). Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell* 24, 2155–2167. doi: 10.1105/tpc.112.096925

Schaaf, G., Catoni, E., Fitz, M., Schwacke, R., Von Wiren, N., and Frommer, W. B. (2002). A putative role for the vacuolar calcium/manganese proton antiporter AtCAX2 in heavy metal detoxification. *Plant Biol.* 4, 612–618. doi: 10.1055/s-2002-35432

Schaaf, G., Ludewig, U., Erenoglu, B. E., Mori, S., Kitahara, T., and Von Wiren, N. (2004). ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *J. Biol. Chem.* 279, 9091–9096. doi: 10.1074/jbc.M311799200

Schnell Ramos, M., Khodja, H., Mary, V., and Thomine, S. (2013). Using muPIXE for quantitative mapping of metal concentration in *Arabidopsis thaliana* seeds. *Front. Plant Sci.* 4:168. doi: 10.3389/fpls.2013.00168

Schroeder, J. I., Delhaize, E., Frommer, W. B., Guerinot, M. L., Harrison, M. J., Herrera-Estrella, L., et al. (2013). Using membrane transporters to improve crops for sustainable food production. *Nature* 497, 60–66. doi: 10.1038/nature11909

Shigaki, T., and Hirschi, K. D. (2006). Diverse functions and molecular properties emerging for CAX cation/H^+ exchangers in plants. *Plant Biol.* 8, 419–429. doi: 10.1055/s-2006-923950

Shigaki, T., Pittman, J. K., and Hirschi, K. D. (2003). Manganese specificity determinants in the Arabidopsis metal/H^+ antiporter CAX2. *J. Biol. Chem.* 278, 6610–6617. doi: 10.1074/jbc.M209952200

Shigaki, T., Rees, I., Nakhleh, L., and Hirschi, K. D. (2006). Identification of three distinct phylogenetic groups of CAX cation/proton antiporters. *J. Mol. Evol.* 63, 815–825. doi: 10.1007/s00239-006-0048-4

Supek, F., Supekova, L., Nelson, H., and Nelson, N. (1996). A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5105–5110. doi: 10.1073/pnas.93.10.5105

Thomine, S., Lelievre, F., Debarbieux, E., Schroeder, J. I., and Barbier-Brygoo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant J.* 34, 685–695. doi: 10.1046/j.1365-313X.2003.01760.x

Thomine, S., Wang, R., Ward, J. M., Crawford, N. M., and Schroeder, J. I. (2000). Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to *Nramp* genes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4991–4996. doi: 10.1073/pnas.97.9.4991

Ueno, D., Yamaji, N., and Ma, J. F. (2009). Further characterization of ferric-phytosiderophore transporters ZmYS1 and HvYS1 in maize and barley. *J. Exp. Bot.* 60, 3513–3520. doi: 10.1093/jxb/erp191

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., et al. (2002). IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

Very, A. A., and Davies, J. M. (2000). Hyperpolarization-activated calcium channels at the tip of Arabidopsis root hairs. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9801–9806. doi: 10.1073/pnas.160250397

Von Wiren, N., Mori, S., Marschner, H., and Romheld, V. (1994). Iron inefficiency in maize mutant *ys1* (*Zea mays* L. cv Yellow-Stripe) is caused by a defect in uptake of iron phytosiderophores. *Plant Physiol.* 106, 71–77.

Waters, B. M., Chu, H. H., Didonato, R. J., Roberts, L. A., Eisley, R. B., Lahner, B., et al. (2006). Mutations in Arabidopsis yellow stripe-like1 and yellow stripe-like3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds. *Plant Physiol.* 141, 1446–1458. doi: 10.1104/pp.106.082586

White, P. J., Bowen, H. C., Demidchik, V., Nichols, C., and Davies, J. M. (2002). Genes for calcium-permeable channels in the plasma membrane of plant root cells. *Biochim. Biophys. Acta* 1564, 299–309. doi: 10.1016/S0005-2736(02)00509-6

Wu, Z., Liang, F., Hong, B., Young, J. C., Sussman, M. R., Harper, J. F., et al. (2002). An endoplasmic reticulum-bound $Ca^{(2+)}/Mn^{(2+)}$ pump, ECA1, supports plant growth and confers tolerance to $Mn^{(2+)}$ stress. *Plant Physiol.* 130, 128–137. doi: 10.1104/pp.004440

Wymer, C. L., Bibikova, T. N., and Gilroy, S. (1997). Cytoplasmic free calcium distributions during the development of root hairs of *Arabidopsis thaliana*. *Plant J.* 12, 427–439. doi: 10.1046/j.1365-313X.1997.12020427.x

Xia, J., Yamaji, N., Kasai, T., and Ma, J. F. (2010). Plasma membrane-localized transporter for aluminum in rice. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18381–18385. doi: 10.1073/pnas.1004949107

Xiao, H., Yin, L., Xu, X., Li, T., and Han, Z. (2008). The iron-regulated transporter, MbNRAMP1, isolated from *Malus baccata* is involved in Fe, Mn and Cd trafficking. *Ann. Bot.* 102, 881–889. doi: 10.1093/aob/mcn178

Yamaji, N., Sasaki, A., Xia, J. X., Yokosho, K., and Ma, J. F. (2013). A node-based switch for preferential distribution of manganese in rice. *Nat. Commun.* 4:2442. doi: 10.1038/ncomms3442

Yang, T. J., Perry, P. J., Ciani, S., Pandian, S., and Schmidt, W. (2008). Manganese deficiency alters the patterning and development of root hairs in Arabidopsis. *J. Exp. Bot.* 59, 3453–3464. doi: 10.1093/jxb/ern195

Yanykin, D. V., Khorobrykh, A. A., Khorobrykh, S. A., and Klimov, V. V. (2010). Photoconsumption of molecular oxygen on both donor and acceptor sides of photosystem II in Mn-depleted subchloroplast membrane fragments. *Biochim. Biophys. Acta* 1797, 516–523. doi: 10.1016/j.bbabio.2010.01.014

Yen, M. R., Tseng, Y. H., and Saier, M. H. Jr. (2001). Maize Yellow Stripe1, an iron-phytosiderophore uptake transporter, is a member of the oligopeptide transporter (OPT) family. *Microbiology* 147, 2881–2883.

Yoon, S., Miller, E. W., He, Q., Do, P. H., and Chang, C. J. (2007). A bright and specific fluorescent sensor for mercury in water, cells, and tissue. *Angew. Chem. Int. Ed. Engl.* 46, 6658–6661. doi: 10.1002/anie.200701785

Zhang, Y., Xu, Y. H., Yi, H. Y., and Gong, J. M. (2012). Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. *Plant J.* 72, 400–410. doi: 10.1111/j.1365-313X.2012.05088.x

Zhao, H., and Eide, D. (1996a). The yeast *ZRT1* gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. *Proc. Natl. Acad. Sci. U.S.A.* 93, 2454–2458. doi: 10.1073/pnas.93.6.2454

Zhao, H., and Eide, D. (1996b). The *ZRT2* gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 271, 23203–23210. doi: 10.1074/jbc.271.38.23203

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Evaluation of constitutive iron reductase (*AtFRO2*) expression on mineral accumulation and distribution in soybean (*Glycine max*. L)

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Iron is an important micronutrient in human and plant nutrition. Adequate iron nutrition during crop production is central for assuring appropriate iron concentrations in the harvestable organs, for human food or animal feed. The whole-plant movement of iron involves several processes, including the reduction of ferric to ferrous iron at several locations throughout the plant, prior to transmembrane trafficking of ferrous iron. In this study, soybean plants that constitutively expressed the AtFRO2 iron reductase gene were analyzed for leaf iron reductase activity, as well as the effect of this transgene's expression on root, leaf, pod wall, and seed mineral concentrations. High Fe supply, in combination with the constitutive expression of AtFRO2, resulted in significantly higher concentrations of different minerals in roots (K, P, Zn, Ca, Ni, Mg, and Mo), pod walls (Fe, K, P, Cu, and Ni), leaves (Fe, P, Cu, Ca, Ni, and Mg) and seeds (Fe, Zn, Cu, and Ni). Leaf and pod wall iron concentrations increased as much as 500% in transgenic plants, while seed iron concentrations only increased by 10%, suggesting that factors other than leaf and pod wall reductase activity were limiting the translocation of iron to seeds. Protoplasts isolated from transgenic leaves had three-fold higher reductase activity than controls. Expression levels of the iron storage protein, ferritin, were higher in the transgenic leaves than in wild-type, suggesting that the excess iron may be stored as ferritin in the leaves and therefore unavailable for phloem loading and delivery to the seeds. Also, citrate and malate levels in the roots and leaves of transgenic plants were significantly higher than in wild-type, suggesting that organic acid production could be related to the increased accumulation of minerals in roots, leaves, and pod walls, but not in the seeds. All together, these results suggest a more ubiquitous role for the iron reductase in whole-plant mineral accumulation and distribution.

Keywords: FRO2, iron, mineral nutrition, soybean, transgenic

INTRODUCTION

Iron is one of the most important micronutrients in human and plant nutrition, and a suitable level of iron nutrition in plants is vital to providing adequate concentrations of this mineral in the harvestable plant organs for human food or animal feed. Researchers have been interested in creating plant foods that are nutrient-dense (to guarantee "nutrient security") in iron and other minerals (Carvalho and Vasconcelos, 2013), using an approach referred to as biofortification. Soybean, being an important plant food in several parts of the world, would be a suitable target for biofortification programs. However, in soybean, most biofortification strategies have aimed at increasing sulfur amino acids (Dinkins et al., 2001) and vitamins, such as α-tocopherol (Dwiyanti et al., 2011), and not at increasing mineral concentrations.

To create crop plants with more minerals, researchers must boost their mobilization and uptake from the soil, improve their movement to the edible portions of the plant and ultimately, enhance their storage in those tissues. For this, it is essential to understand the relevant contributions of the mineral transport system throughout the plant as well as the regulatory system that controls it.

One possible strategy is to utilize a "bottom up" approach, where scientists enhance the mechanism of iron uptake from the roots, and "hope" that the additional iron will be mobilized and stored in the edible parts of the plant. Mineral elements traverse the root via apoplastic and/or symplastic pathways to the stele, where they are loaded into the xylem for transport to the shoot (White and Broadley, 2009). In the plant kingdom there are two main strategies for iron uptake. Dicots, such as soybean, and non-graminaceous monocots, respond to iron limiting environments by induction of the Strategy I mechanism (Römheld and Marschner, 1986; Marschner, 1995), which involves several processes at the root membrane such as the expression of active proton pumps (AHA2) to increase solubility of ferric (Fe^{+3}) iron, a ferric reductase (FRO2) to generate ferrous (Fe^{+2}) iron, and an iron transporter (IRT1) to take up this reduced, available iron

from the rhizosphere (Hell and Stephan, 2003). This uptake system depends on the activity of specific transcription factors, such as FIT and bHLH proteins. Recent work in *Arabidopsis* and *Medicago* has shown that Strategy I plants also produce species-specific iron deficiency-elicited compounds, namely phenolics and flavins (Rodríguez-Celma et al., 2013) such as scopoletins (Fourcroy et al., 2013). Monocots utilize the Strategy II mechanism, in which there is extrusion of phytosiderophores that chelate iron and then are taken up as an iron-chelate complex by root specific transporters. Several comprehensive reviews are currently available on this topic (Curie and Briat, 2003; Walker and Waters, 2011; Hindt and Guerinot, 2012; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012).

The *Arabidopsis* FRO2 gene is a member of the FRO gene family and encodes an iron-deficiency inducible iron reductase responsible for reducing iron at the root surface (Yi and Guerinot, 1996; Robinson et al., 1999; Connolly et al., 2003). Once inside the roots, iron is transported via the xylem to the vegetative tissues in response to transpirational activity from various organs. It is thought that iron is transported in the xylem as a complex with organic acids (OA) such as citrate (Tiffin, 1966; Rellán-Álvarez et al., 2010), and that nicotianamine (NA) also may play an important role in this process (Curie et al., 2009; Ivanov et al., 2012).

The role of iron reduction in the roots is well documented (Robinson et al., 1999; Connolly et al., 2003; Mukherjee et al., 2006); however, the role of the reductase in the leaf, fruit, and grain is still unclear. It is thought that iron reduction is necessary to reduce ferric iron in the aerial parts of the plant before being transported into the leaf cells (Larbi et al., 2001; Feng et al., 2006). It was found that AtFRO7 localizes to the chloroplast and is required for efficient photosynthesis in young seedlings and for survival under iron-limiting conditions (Jeong et al., 2008). Iron reductase activity has been detected in leaves of different plant species such as sunflower (de la Guardia and Alcántara, 1996), *Vigna unguiculata* (Brüggemann et al., 1993) and sugar beet (Gonzalez-Vallejo et al., 2000; Larbi et al., 2001).

Once in the leaves, iron is used in diverse biochemical processes, or it can be stored for future use. The storage form of iron and the organelles where it is accumulated is only partially defined. It is thought that most of the iron will be stored as ferritin in the plastids (chloroplasts contain up to 90% of the leaf cell iron), with about half in the stroma and half in the thylakoid membranes. The remaining iron pool will probably be found in the vacuole (Thomine et al., 2003). There also seems to be a role for the reductase in the transport to and eventual accumulation of iron in seeds (Grusak, 1995). A pea mutant (*dgl*) was identified that has uncontrolled hyperaccumulation of Fe in vegetative tissues, and that has the ability to accumulate up to 3-fold higher concentrations of Fe in the seeds (Marentes and Grusak, 1998). Another pea mutant, *brz* (bronze leaves) also has been shown to have hyperaccumulation of iron in the leaves, but it does not move excess iron into the seeds (Grusak, 1995). Unfortunately, there are no data on the possible role of leaf iron reduction in the transport of iron to the developing seeds.

There is also very limited information on the common regulatory mechanisms that co-regulate the uptake, transport and accumulation of iron and other minerals in the plant. Several common transporters have been identified, but only limited evidence is available on the effects of higher Fe intake on the dynamics of other minerals, such as zinc (Zn), copper (Cu), manganese (Mn), magnesium (Mg), phosphorous (P), potassium (K), nickel (Ni), molybdenum (Mo), or calcium (Ca). All of these also play important roles in human and animal nutrition, and enhanced accumulation of more than one mineral could be a cost effective strategy in biofortification programs.

Ectopic expression of the *Arabidopsis thaliana* ferric chelate reductase, *AtFRO2* (Robinson et al., 1999) in transgenic soybean conferred increased iron reduction in the roots and enhanced tolerance towards iron deficiency chlorosis (Vasconcelos et al., 2006). To determine if the reductase activity is a rate-limiting step for seed mineral acquisition, and to establish *AtFRO2* as a possible molecular tool to be used in biofortification programs, we functionally analyzed the transgenic soybean line 392-3 carrying the *Arabidopsis FRO2* gene under the 35S constitutive promoter for leaf iron reductase activity and general whole-plant mineral distribution.

MATERIALS AND METHODS

PLANT MATERIAL

Soybean (*Glycine max* L.) genotypes "Thorne" wild-type (WT) and transgenic homozygous line 392-3 carrying the *FRO2* gene from *Arabidopsis thaliana* under the constitutive 35S promoter (Vasconcelos et al., 2006) were used in this study. Plants were grown in a controlled environment chamber with 16-h, 20°C-day and 8-h, 15°C-night. Relative humidity was maintained at 50% and photon flux density during the day was 350 μmol m^{-2} s^{-1}, supplied by a mixture of incandescent bulbs and fluorescent lamps. Seeds of control and homozygous transgenic plants in T_3 generation were first germinated in beakers with wet filter paper for 4 days before being transferred to hydroponics solution (four plants per 4.5 l) with different Fe treatments. The standard solution for hydroponically grown plants contained: 0.8 mM $Ca(NO_3)_2$, 1.2 mM KNO_3, 0.2 mM $MgSO_4$, 0.3 mM $NH_4H_2PO_4$, 25 μM $CaCl_2$, 25 μM H_3BO_3, 0.5 μM $MnSO_4$, 2.0 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4, and 0.1 μM $NiSO_4$. For various studies, plants were grown either at 0, 10, 32, or 100 μM Fe(III)-EDDHA [ethylenediamine-N,N'bis(o-hydroxyphenyl)acetic acid]. All nutrients were buffered with 1 mM MES (2,4-morpholino-ethane sulfonic acid), pH 5.5 and growth solutions were changed weekly until full maturity (FM) of the plants.

For mineral analysis, tissues were collected at three developmental stages: 40 (Grain-Fill I), 80 (Grain-Fill II), and 120 (Grain-Fill III) days after the initiation of flowering.

LEAF PROTOPLAST ISOLATION AND REDUCTION ACTIVITY

Trifoliate leaves of Thorne and 392-3 were collected at two weeks of age from plants grown with 0, 10, 32, or 100 μM Fe(III)-EDDHA. Protoplasts were isolated using a method modified from Larbi et al. (2001). Specifically, leaf tissue of a known surface area was cut and placed in Solution A (500 mM D-sorbitol, 1 mM $CaCl_2$, 2% [w/v] cellulase, 0.3% macerozyme, 0.1% pectolyase, 5 mM MES, pH 5.5). The macerate was incubated at 27°C for 3 h on an orbital shaker at

70 rpm, in the dark. Protoplasts were filtered, centrifuged, washed with Solution B (500 mM D-sorbitol, 1 mM $CaCl_2$ and 5 mM MES, pH 6.0), isolated after sucrose layering and centrifugation, sized, and counted for viability. Protoplasts were sized by using a calibrated eyepiece fitted to a microscope (Nikon, Japan, 40× objective). The number of protoplasts per unit volume was determined with a hemacytometer (Hausser Scientific, Horsham, PA, USA), and viability of the protoplasts was calculated by incubation of the protoplast suspension in 0.04% (w/v) Evans Blue for 5 min, and determining the number of protoplasts excluding the dye. A 500 µl volume of leaf protoplast solution was added to 500 µl of Solution B (described above) supplemented with 800 µM BPDS and 800 µM Fe(III)-EDTA. Tubes were agitated for 45 min at 27°C, centrifuged (2 min at 12,000 *g*) and absorbances read at 535 nm. The extinction coefficient of Fe(II)-$BPDS_3$ (22.14 mM^{-1} cm^{-1}) was used to calculate the Fe reduction rates; values are presented on protoplast surface area basis (nmol of Fe reduced μm^{-2} s^{-1}).

TISSUE ELEMENTAL ANALYSIS

All tissues were harvested and dried overnight in a 60°C oven. Samples were then digested overnight in borosilicate glass tubes by adding 4 ml of redistilled 98.8% HNO_3 and 1 ml of concentrated trace metal grade $HClO_4$. Samples were heated at 100°C for 1 h, 150°C for 1 h, 180°C for 1½ h and then at 210°C to dryness. Digestions were performed using a heating block (Model 1016, Tecator, Höganäs, Sweden) with an exhaust-collecting manifold. Digests were resuspended in 10 ml of redistilled 2% HNO_3. Elemental analysis was performed using inductively coupled plasma – optical emission spectroscopy (ICP-OES; CIROS ICP Model FCE12; Spectro, Kleve, Germany). Five plants were grown for each treatment as described before. Material from each plant was ground and five independent digestions were performed prior to ICP-OES analysis.

AtFRO2 AND *GmFer* EXPRESSION ANALYSIS

RNA from roots, leaves, seeds, root tips, pod walls, and petals of control and transgenic plants grown hydroponically under various iron concentrations (see above) was isolated using the RNA-easy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA was isolated from 3 plants in each treatment. The experiments were repeated twice, and PCR reactions were performed at least in triplicate. Possible contaminating genomic DNA was removed with the TURBO DNA-*free*™ kit from Ambion (Ambion Inc., Austin, TX, USA) following the manufacturer's instructions. Total RNA (0.5 µg) were subjected to reverse transcription (RT) with an anchored oligo (dT) primer and 200 units superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a volume of 20 µl according to the manufacturer's instructions. PCR reactions were carried out with 1.5 µl of the RT reaction solutions. Additional reaction components were: 10 mM polymerase buffer, 1 mM dNTP's, 0.1 units Taq polymerase (Clontech, Palo Alto, CA, USA) and 1 µM specific primers. All primers surrounded an intron so that genomic DNA was clearly distinguished from cDNA-derived products. The following primer sets were designed: *ferritin* (*GmFer*), 5′-ACTTGCTCTGTTTCTCTGAGC-3′ (forward), 5′–CGCTAGACGGTGTGACACGT-3′ (reverse); *ubiquitin* (*GmUbq*), 5′-GGGTTTTAAGCTCGTTGT-3′ (forward) and 5′-GGACACATTGAGTTCAAC-3′ (reverse). The number of cycles in each PCR reaction was 28, with 58°C annealing temperature. Amplified products from 10 µl of PCR reaction were visualized on a 1% TAE agarose gel containing ethidium bromide. Bands were photographed using the Quantity One 4.5.1 Chemidoc EQ™ Software System (Biorad, CA, USA).

Quantitative RT-PCR (Q-RT PCR) was performed for *AtFRO2* transcript quantification in different plant tissues. Reactions were carried out in an ABI PRISM® 7700 sequence detector using TaqMan One Step PCR master Mix reagents Kit and an ABI PRISM® 96-well optical reaction plate (all from Applied Biosystems, Foster City, CA, USA). Validation of the Q-RT PCR methodology was performed in order to find the appropriate RNA concentration at which there is a linear correlation between ribosomal RNA control (*18S rRNA)* and *AtFRO2* transcription. Reactions were carried out with 0.2 ng/µl RNA to a final volume of reaction of 25 µl. Specific primers designed for *AtFRO2* were: 5′-CGTATCAAGTTTGGAACATCCACTT-3′ (forward) and 5′-CCATCATTGGGAACATATACATGAA-3′ (reverse), amplifying the TaqMan Probe *AtFRO2* sequence of 5′-AAGTTTGGAACATCCACTTATTTTGGTGCCA-3′. For signal detection and quantification, Applied Biosystems-Sequence Detection Systems 1.9.1 was used (Applied Biosystems, Foster City, CA, USA). The following standard thermal profile was used for all PCRs: 40 cycles starting at 48°C 30 min, 95°C 10 min, 95°C 15 s and 60°C for 1 min. The $\Delta Ct \pm SD$ and mean transcript level were calculated between four technical replicates from two experiments. To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 15. All amplifications were analyzed with an R_n threshold of 0.02 to obtain the C_T (threshold cycle) values.

ORGANIC ACID ANALYSIS

Leaf and root tissue of control (Thorne) and transgenic (392-3) soybean plants grown hydroponically at 0, 10, 32, or 100 µM Fe (III)-EDDHA for 2 weeks were used for citrate and malate analysis. Five samples of each tissue were frozen in liquid nitrogen and ground in a ceramic mortar and pestle with 8 mM sulfuric acid. Homogenates were boiled for 30 min, filtered with a 0.2 µm filter (Falcon, USA), taken to a final volume of 2 ml with 8 mM sulfuric acid and kept at −80°C until HPLC analysis.

Organic anions were analyzed with an Acclaim OA 5 µm ion-exchange column (250 × 4 mm, DIONEX, TX, USA) with an HPLC system (ICS 3000 Ion Chromatography System, DIONEX, Houston, TX, USA), and Chromeleon software. Samples were manually injected (10-µl loop). Mobile phase (100 mM Na_2SO_4, pH 2.65) was pumped with an isocratic 0.5 ml min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to citrate and malate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma (St. Louis). Quantification was made with known amounts of each organic anion using peak areas.

STATISTICAL ANALYSIS

Welch's *t*-test and the Dunnett-*C* test to compare the means were used to compare the leaf, pod wall, root, stem and seed nutrient levels between transgenic and WT plants. A Pearson's correlation analysis with four significance levels ($P < 0.05$; $P < 0.01$; $P < 0.001$, and $P < 0.0001$) was performed to determine the correlation between 10 different minerals in different tissue types. The mineral data used for the correlation study integrated the ICP results from WT and 392-3 plants grown to maturity at the three iron concentrations, and at the three harvest dates. All statistical analyses were performed using GraphPad Prism 6, version 6.1 (La Jolla, CA, USA).

RESULTS

PLANT ORGAN WEIGHT

Fourteen days after transfer to the hydroponics solution, WT and 392-3 plants appeared similar to each other, in terms of plant size and leaf color, particularly at 100 μM Fe(III)-EDDHA (**Figure 1**). However, both WT and 392-3 plants grown in the complete absence of iron were already showing severe signs of chlorosis (**Figure 1**); therefore, this treatment had to be discontinued for the analysis that involved growing the plants to FM.

To test if the constitutive expression of *AtFRO2* and a combination of different Fe supplies would influence dry mass accumulation in soybean organs, we cultivated WT and 392-3 plants with 10, 32, or 100 μM Fe(III)-EDDHA until FM. The lowest Fe supplies (10 and 32 μM) resulted in significant differences in root dry weight (DW) between the WT and 392-3 line, while no differences were seen between genotypes in the roots at the highest Fe supply of 100 μM Fe (III)-EDDHA (**Figure 2**). The transgenic plants also had significantly heavier shoot DW ($P < 0.001$) than the WT, regardless of the Fe concentration in the growth solution. At 10 and 32 μM Fe (III)-EDDHA the transgenic soybean line 392-3 had significantly higher pod wall DW. High Fe supply caused a decrease of about 30% in root DW in the transgenic line compared to the lower concentrations (10 or 32 μM). In the WT plants, this reduction in root DW was not observed. In fact, WT plants exhibited higher root DW (12.5 ± 0.8 g and 12.2 ± 0.1g) when grown in higher Fe concentrations than when grown at 10 μM concentration (9.4 ± 0.3g).

FIGURE 1 | Wild-type (Panel A) and transgenic 392-3 (Panel B) soybean plants at 14 days of hydroponic growth supplemented with 0, 10, 32, or 100 μM Fe(III) EDDHA (left to right).

AtFRO2 AND *GmFer* EXPRESSION

Quantitative RT-PCR revealed that the CaMV 35S promoter drove *AtFRO2* expression in different organs of the transgenic plants such as root tips, basal regions of roots (without tips), leaves, petals, pod walls, and seeds, and that *AtFRO2* transcript levels were relatively similar amongst the different organs (**Figure 3**). However, when looking at different root regions, it was found that *AtFRO2* was less expressed in root tips than in the remaining regions of the root. No expression of *AtFRO2* was found in the non-transformed control.

In order to determine if the constitutive expression of *AtFRO2* is influenced by the external supply of iron, quantitative RT-PCR was performed in roots and shoots of transgenic soybean line 392-3 grown at 0, 10, 32, or 100 μM Fe(III)-EDDHA for 14 days. Similar transcript levels were observed in both roots and shoots, regardless of iron concentration in the hydroponics solution (**Figure 4**).

Ferritin expression was assessed in roots and shoots of control and transgenic plants (**Figure 5**). Higher *ferritin* levels were found in the shoots than in the roots in both control and transgenic plants, and an increase in *ferritin* expression was found when plants were grown at higher iron concentrations. Moreover, 392-3 plants appeared to have higher expression of the *ferritin* gene when compared to control.

TISSUE MINERAL CONCENTRATIONS

At an initial stage of our experiments, 392-3 soybeans were grown along with the WT plants for 14 days at 32 μM Fe(III)-EDDHA in hydroponic conditions and plants were screened for possible modulatory effects on mineral concentrations in roots, stems, and leaves (**Table 1**). It was observed that the transgenic line had significantly higher concentrations of Fe, Zn, P, K, and Mn in leaves, roots and stems, with concentrations increasing more than 50% when compared to the WT.

The 392-3 soybeans were also grown along with the WT plants at 10, 32, or 100 μM Fe (III)-EDDHA in hydroponic conditions and plants were screened up to FM. Iron and Zn determinations of the leaves, pod walls and seeds were performed at three different developmental seed filling stages: Grain-Fill I (GF I), Grain-Fill II (GF II), and FM (**Figure 6**). Significant differences in Fe accumulation in the different tissues was found when comparing WT and 392-3 plants, and differences were particularly significant at the last collection date (FM), and at the highest iron concentration (100 μM). For plants that were grown at 100 μM Fe, at the last collection date (**Table 2**; **Figure 6**) 392-3 plants had up to 1142 ± 69 $\mu g\ g^{-1}$ Fe in the leaves, whereas the WT plants had 435 ± 18 $\mu g\ g^{-1}$. Nonetheless, the increase in Fe concentration in the leaves of plants grown at 100 μM Fe (III)-EDDHA was already significant at the GF I and GF II stages. The pod wall Fe levels also were significantly higher in the 392-3 plants, relative to WT, at 32 and 100 μM Fe growth conditions at FM (**Figure 6**), with values reaching up to 120 ± 4 $\mu g\ g^{-1}$. At FM, an increase of up to 100% was found in iron levels in the leaves of plants grown at 100 μM Fe(III)-EDDHA, a higher than 60% increase

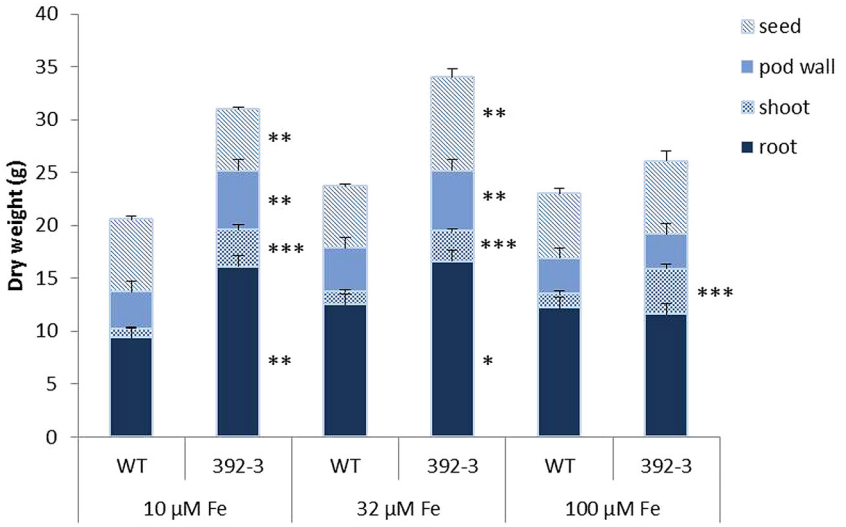

FIGURE 2 | Dry weight of seeds, pod walls, shoot (leaf and stem), and roots collected at full maturity (FM) stages of WT and transgenic 392-3 soybean plants supplied with 10, 32, or 100 µM of Fe(III)-EDDHA. Values are the averages of at least five samples ± SE. Asterisks indicate that the means (between WT and 392-3 within tissue type) are different by the Tukey HSD test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

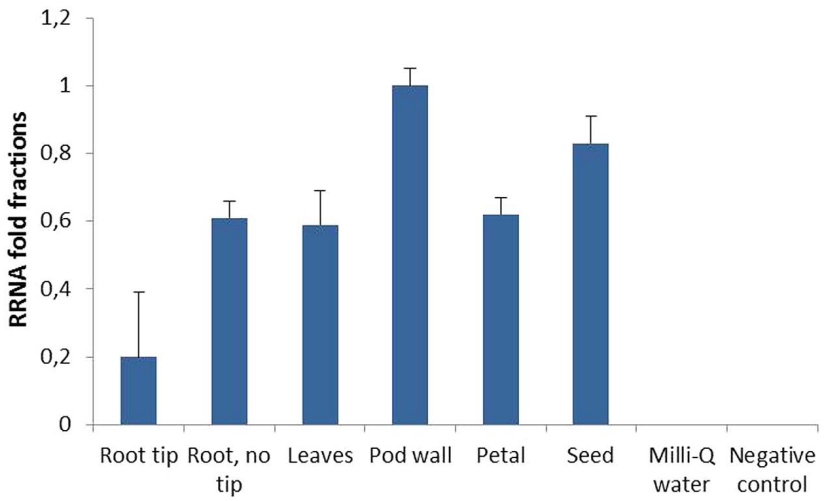

FIGURE 3 | Relative *AtFRO2* RNA expression in different soybean plant tissues measured by Quantitative RT-PCR (Biorad). Amount of transcript was calculated according to internal 18S RNA expression for each tissue. RNA was extracted from two plants for each tissue and Quantitative RT-PCR reaction was repeated three times.

was found in the levels of iron in the pod walls of these same plants, and a 10% increase was detected in the corresponding seed iron levels. Plants that had been grown in soil, in greenhouse conditions, showed a similar increase in seed iron levels (data not shown).

Zinc concentrations were also higher in the transgenic leaves and seeds but not in the pod walls. The increments were varied with the concentration of iron in the nutrient solution and with the harvest date. In the leaves, increases in Zn concentration were significantly higher at GF I and GF II, but not at FM. In the seeds, Zn concentration in 392-3 was always significantly higher than in WT plants grown at the highest Fe concentration.

Because Fe and Zn differences were more pronounced in plants grown at 100 µM Fe and at FM, we decided to determine whether other minerals were also affected, looking at this particular stage of development and treatment. **Table 2** shows that other minerals besides Fe and Zn were also modulated in the transgenic plants, with differences noted in mineral composition in the roots, pod walls, leaves, and seeds (**Table 2**). In roots, several obvious mineral concentration differences could be detected. High Fe supply, in combination with the constitutive expression of *AtFRO2*, was associated with higher concentrations of K, P, Zn, Ca, Ni, Mg, and Mo. In pod walls, transgenic plants had significantly higher Fe, K, P, Cu, and Ni concentrations

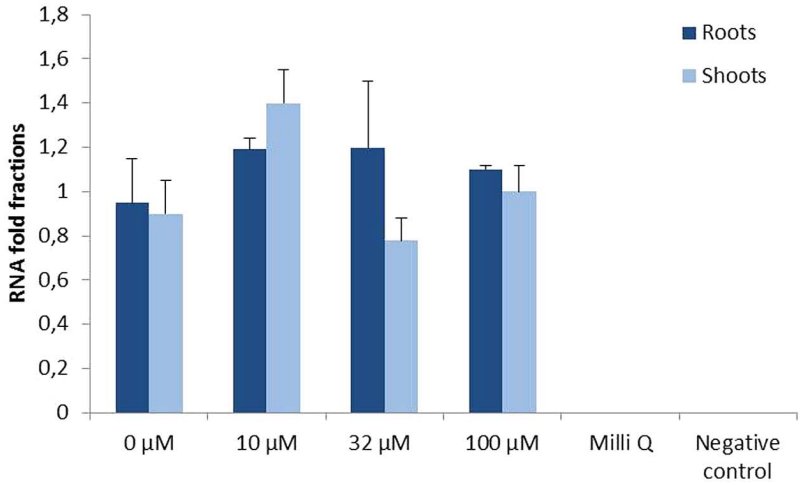

FIGURE 4 | Relative *AtFRO2* RNA expression in roots and shoots of transgenic 392-3 soybean plants supplied with 0, 10, 32, or 100 µM Fe(III) EDDHA detected by Quantitative RT-PCR (Biorad). Amount of transcript was calculated according to internal 18S RNA expression for each tissue. RNA was extracted from 2 plants for each tissue and Quantitative RT-PCR reaction was repeated three times.

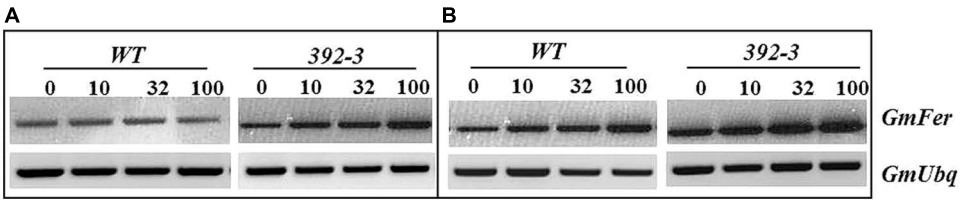

FIGURE 5 | Semi-quantitative RT-PCR analysis of the soybean ferritin (*GmFer*) expression in roots (Panel A) and shoots (Panel B) of WT and 392-3 *G. max* plants grown under different iron concentrations [0, 10, 32, or 100 µM Fe (III)-EDDHA]. Soybean ubiquitin gene (*GmUbq*) was used as template loading control.

Table 1 | Average root, stem, and leaf mineral concentration of wild-type (WT) and 392-3 transgenic soybean plants grown in hydroponic conditions with 32 µM Fe(III)-EDDHA for 2 weeks.

Mineral ($\mu g\ g^{-1}$)	Roots		Stems		Leaves	
	WT	392-3	WT	392-3	WT	392-3
Fe	201 ± 11	582 ± 25**	41 ± 3	94 ± 6**	196 ± 8	766 ± 31**
Mn	122 ± 10	258 ± 13**	122 ± 10	258 ± 13**	65 ± 2	188 ± 8**
K	46430 ± 7979	51266 ± 2875*	33692 ± 2556	46004 ± 2467*	27244 ± 389	30575 ± 1306*
P	12284 ± 738	17490 ± 880*	4558 ± 195	6106 ± 393*	4658 ± 34	6912 ± 105*
Zn	100 ± 4	346 ± 11**	56 ± 3	149 ± 9**	136 ± 3	283 ± 6**

*Values are shown as mean ± SE and represent an average of five plants. Significance within tissue types: *P < 0.05; **P < 0.01.*

and significantly lower concentrations of Ca and Mg. In leaves there was a significant increase in leaf Fe, but also in P, Cu, Ca, Ni, and Mg. In seeds, the concentrations of Fe, Zn, Cu, and Ni were significantly increased in the transgenic line, relative to WT.

MINERAL CORRELATION ANALYSIS

Pearson's correlation analysis was performed in order to find relationships among the ten minerals' concentrations in three different plant tissues. The organ with the fewest number of correlations was the seed; however, it was also the organ where the most significant correlations occurred (**Figure 7**). In general, Fe seems to be very tightly linked to several other minerals, with particular emphasis in the leaves and pod walls. As can be observed in **Figure 7**, the only positive correlations that are common for the three tissue types are the pairs Fe-Zn, Fe-Cu, and Ni-Cu. Other correlations are common in two of the three tissues.

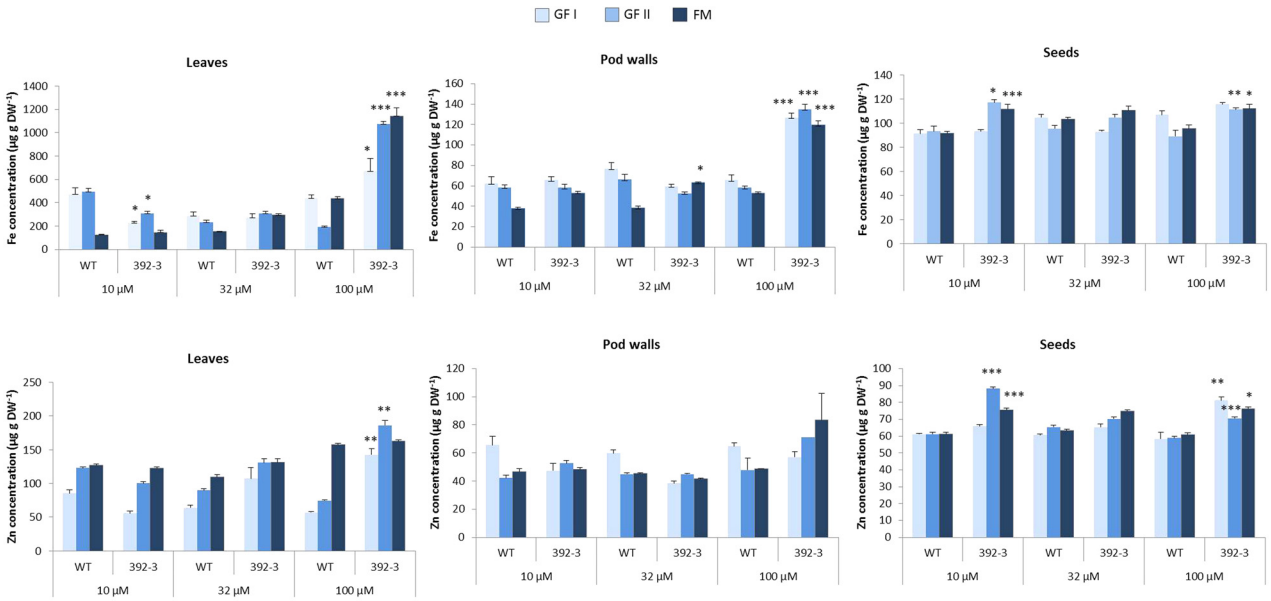

FIGURE 6 | Fe and Zn concentration ($\mu g\ g^{-1}$) in leaves, pod walls and seeds of WT and transgenic 392-3 soybean plants grown in hydroponic conditions with 10, 32, or 100 µM Fe (III)-EDDHA. Tissues were harvested at two grain filling stages (GF I and GF II) and at full maturity (FM). Asterisks indicate that the means (between WT and 392-3 within Fe supply) are different by the Tukey HSD test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Error bars may be too small to be visible in the figure. Data are means ± SE of five plants per treatment.

Table 2 | Mineral concentration ($\mu g\ g^{-1}$) in roots, pod walls, leaves, and seeds of wild-type (WT) and transgenic 392-3 soybean plants grown in hydroponic conditions with 100 µM Fe(III)-EDDHA until full maturity (FM).

Mineral ($\mu g\ g^{-1}$)	Roots		Pod walls		Leaves		Seeds	
	WT	392-3	WT	392-3	WT	392-3	WT	392-3
Fe	2904 ± 109	2592 ± 177	49 ± 1	120 ± 4***	435 ± 18	1142 ± 69***	96 ± 2	112 ± 3**
Mn	10 ± 0.6	9 ± 0.3	22 ± 2	17 ± 1	49 ± 6	47 ± 1	20 ± 1	24 ± 1*
K	13256 ± 83	41661 ± 2644*	26722 ± 436	32064 ± 620**	15385 ± 200	13336 ± 977	18920 ± 415	19341 ± 78
P	4079 ± 97	7991 ± 529*	2572 ± 37	4244 ± 89**	1427 ± 53	2414 ± 68**	6389 ± 164	6509 ± 132
Zn	45 ± 1	137 ± 50*	49 ± 1	84 ± 19**	157 ± 3	162 ± 2	61 ± 1	76 ± 1**
Cu	27 ± 1	25 ± 2	8 ± 0.1	13 ± 0.3**	3 ± 0.1	8 ± 0.4**	15 ± 0.1	17 ± 0.1***
Ca	9788 ± 72	7553 ± 200*	23605 ± 395	19275 ± 124**	21861 ± 330	30398 ± 591**	3519 ± 57	3674 ± 61
Ni	2 ± 0.4	3 ± 0.2*	3 ± 0.1	5 ± 0.1**	1 ± 0.1	2 ± 0.2**	8 ± 0.1	9 ± 0.1*
Mg	435 ± 0.5	534 ± 12*	4886 ± 61	4310 ± 59*	1648 ± 28	2346 ± 120*	2190 ± 21	2103 ± 16
Mo	5 ± 0.3	3 ± 0.3*	39 ± 2	20 ± 1	23 ± 2	18 ± 1	55 ± 0.9	55 ± 0.3

*Values are shown as mean ± SE and represent an average of five plants. Significance within tissue types: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.*

LEAF IRON REDUCTION ACTIVITY

The small differences in the seed iron concentration between WT and 392-3 transgenic plants led to the hypothesis that perhaps the higher reductase activity in the roots may not be paralleled by a higher reductase activity in the leaves or in other plant organs, which could be sources of transportable Fe. If the increased iron concentration in the leaves of the 392-3 plants is being stored in a non-transportable form, then there will not be a concomitant transport to the developing seeds. Therefore, an optimized protocol was used to measure ferric chelate reductase activity of soybean plants grown in hydroponics for two weeks at 10, 32, or 100 µM Fe (III)-EDDHA. Protoplasts isolated from leaf cells of 392-3 and WT plants showed that expression of *AtFRO2* increased leaf iron reduction capacity up to 3-fold when compared to the WT, regardless of the plant iron treatment (**Figure 8**).

CHANGES IN LEAF AND ROOT ORGANIC ACIDS

Malate and citrate have been described as important compounds in the translocation of Fe in the plant's vascular system. Both OA were

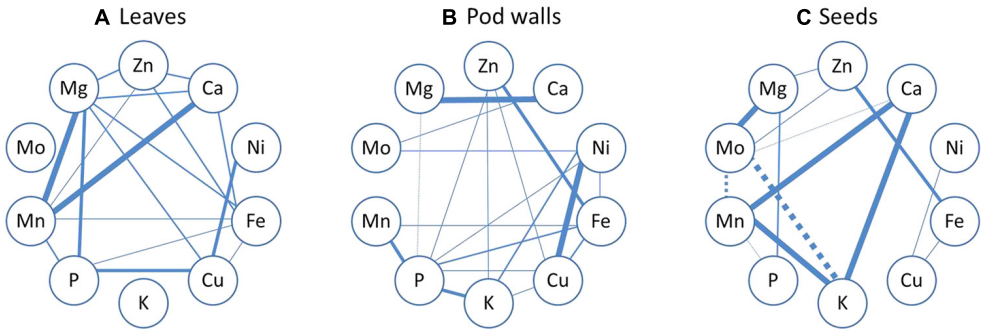

FIGURE 7 | Mineral correlation analysis. Pearson's correlation analysis of ten mineral concentrations in leaves **(A)**, pod walls **(B)**, and seeds **(C)** of Thorne and 392-3 soybean plants cultivated with 10, 32, or 100 μM of Fe (III)-EDDHA and measured at two grain filling stages (GF I and GF II) or at full maturity (FM). Solid lines represent a significant positive correlation and dashed lines represent a significant negative correlation. Thinner lines indicate significance at the $P < 0.05$, semi-thin indicate significance at the $P < 0.01$, semi-thick indicate significance at the $P < 0.001$ level and the thicker lines indicate significance at the $P < 0.0001$ level.

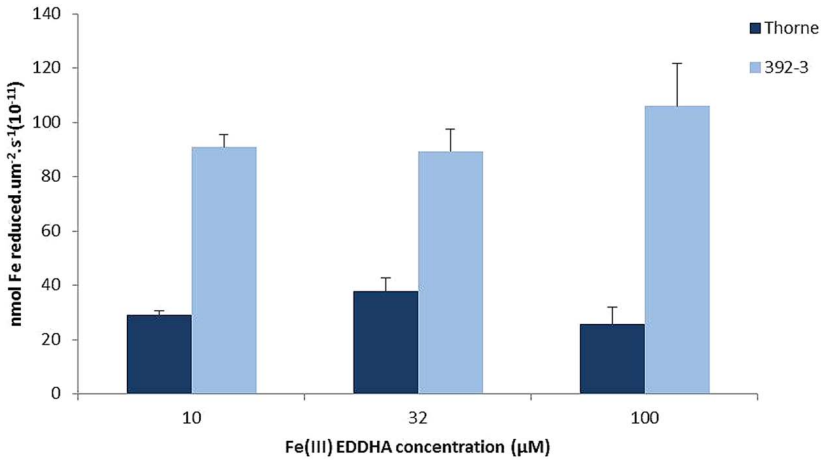

FIGURE 8 | Iron reductase activity measured *in vivo* in protoplasts isolated from WT and transgenic 392-3 soybean leaves of plants grown with 10, 32, or 100 μM Fe (III)-EDDHA. Values are shown per surface area. Measurements were made with 400 μM Fe (III)-EDTA as a substrate. Data are means ± SE of three replications each.

detected in transgenic and control leaves and roots. In general, differences between 392-3 and WT plants were more pronounced in the leaves than in the roots, and transgenic plants accumulated significantly higher amounts of both OA than WT. In the leaves of 392-3 plants, citrate and malate were higher when plants were grown at 10, 32, or 100 μM Fe (**Figures 9A,B**). A different pattern was observed in the WT plants: malate concentrations were highest in leaves of iron-starved plants, and were lower when iron was available to the plants. Leaf citrate concentrations were only elevated at 32 μM Fe.

For plants grown in the absence of Fe, citrate, and malate were not detectable in the roots of WT and transgenic plants. However, when plants were grown in Fe sufficiency, both OA were observed (**Figures 9A,B**).

DISCUSSION

There is currently a strong interest in developing strategies to increase the level of minerals, such as iron, in edible plant organs. One such strategy is the over-expression of genes that are necessary for proper plant iron status. Previous work has shown that by enhancing the reductase activity in transgenic soybean, plants are able to cope better in iron limiting soils, having higher chlorophyll values and improved agronomic performance (Vasconcelos et al., 2006). Also, a possible strategy to enhance the mineral content of plant foods is to enhance the uptake of minerals from the roots during the period of seed development (Waters and Grusak, 2008; Sperotto et al., 2012).

MINERAL CONCENTRATIONS AND CORRELATIONS

In the current study, soybean plants overexpressing *AtFRO2* were analyzed for mineral concentrations (Fe, Zn, Cu, Mn, Mg, Ca, Mo, Ni, K, and P) in source and sink tissues at different seed-filling stages. Significant differences were found in the mineral concentration of transgenic plants when compared to the WT, and differences were particularly prominent when tissues were collected at the final developmental stage (**Table 1**).

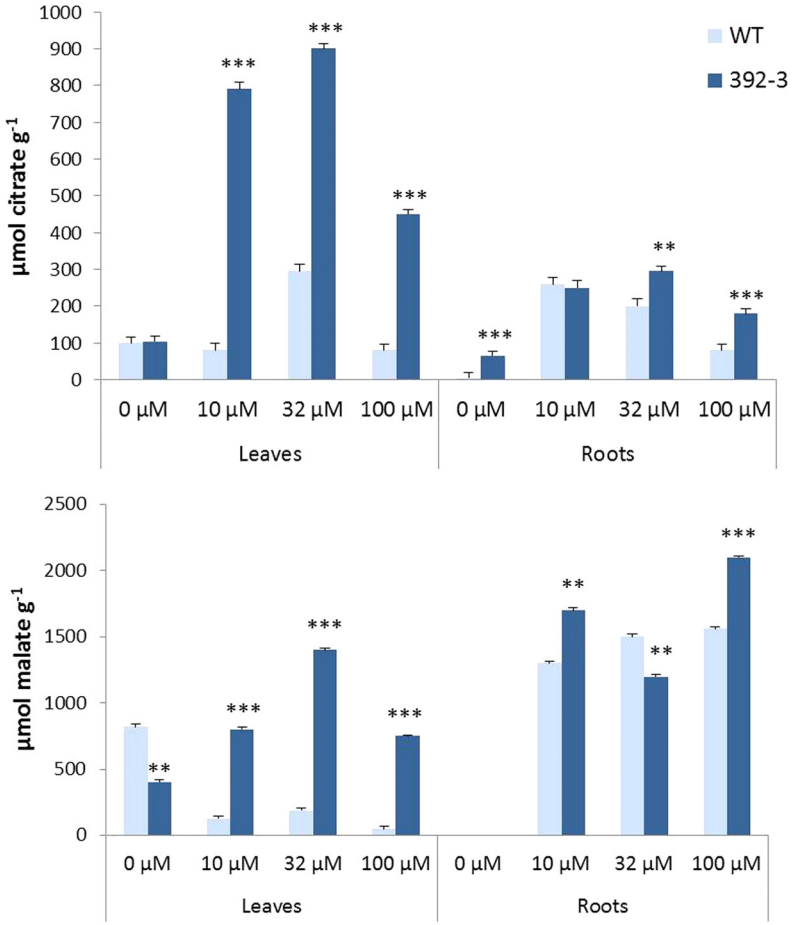

FIGURE 9 | Effects of Fe concentration on organic acid (citrate and malate) accumulation in WT and 392-3 soybean leaves and roots. Data are means ± SE of eight replicates. Asterisks represent significant differences between WT and 392-3 (**$P < 0.01$; ***$P < 0.001$).

This suggests that the iron reductase is not only involved in iron acquisition, but it plays a more general role in the regulation of ion absorption by root cells. This hypothesis has been supported by others (Marschner et al., 1982; Stephan and Grün, 1989; Welch and LaRue, 1990; Yi and Guerinot, 1996). Furthermore, after two weeks in hydroponic conditions supplemented with 0, 10, 32, or 100 µM Fe (III)-EDDHA, the transgenic line 392-3 had significantly higher concentrations of Fe, Zn, and Mn in leaves, roots, and stems, with concentrations 50% higher when compared to the WT (**Table 1**). Increased Mn uptake and subsequent translocation to the aerial parts of the plant is reasonable, because Mn moves easily from roots to shoot tissues in the xylem-sap transpiration stream (Ramani and Kannan, 1987; Marschner, 1995).

After observing that 392-3 had altered concentrations of different minerals after 14 days of growth, we grew plants to maturity and analyzed mineral accumulation in roots, leaves, pod walls, and seeds at different developmental seed filling stages (**Figure 6**). At the last harvest date (FM), it was observed that when grown with high Fe supply, in combination with the constitutive expression of *AtFRO2*, there were several significant mineral changes. In pod walls, transgenic plants had significantly higher Fe, K, P, Cu, and Ni; however, significantly lower concentrations of Ca and Mg were found (**Table 2**), suggesting an antagonistic effect of the higher Fe concentrations in these tissues, or that pod transpiration rates were depressed. Finally, besides Fe, seeds had also significantly higher concentrations of Zn, Cu, and Ni.

There were two minerals which were significantly higher across all tissues of 392-3 when compared to WT (roots, leaves, pod walls, and seeds): Ni and P. In fact, Ni rapidly re-translocates from leaves to young tissues in the phloem, particularly during reproductive growth. Up to 70% of Ni in the shoots is transported to the seeds of soybean (Tiffin, 1971).

In the case of P, more P becomes available for uptake when there is an increase of OA exudation (Remy et al., 2012). Thus, our observations of significantly higher OA production by 392-3 roots and leaves (**Figure 9**) could explain the increased uptake of P. If this hypothesis is true, then it suggests that the most important source of P for the seeds is the P taken up during seed fill, and not remobilized P. In fact, in previous work where P was either remobilized or not from the leaves, no changes in P occurred in soybean seeds (Crafts-Brandner, 1992).

When looking at mineral correlations (**Figure 7**), the positive correlations which were common in leaves, pod walls and seeds were the pairs Fe-Zn, Fe-Cu, and Ni-Cu. Ni-Cu share a common uptake system (Kochian, 1991), and Fe-Zn as well, because the IRT1 Fe transporter also mediates Zn transport (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002). The link between Fe-Cu cannot be explained by common transporters, as it has been shown that IRT1 (at least in *Arabidopsis*) cannot transport Cu. However, *35S-FRO2* transgenic *Arabidopsis* plants have elevated Cu reductase activity (Connolly et al., 2003; Zimmermann et al., 2009), and at 14d 392-3 plants also have enhanced Cu reductase activity (data not shown), which may have increased the amount of available Cu for uptake, transport and accumulation.

In leaves and pod walls, there was a positive correlation between Fe-Mn, contrary to what was found in rice (Sperotto et al., 2012). In seeds, there was a positive correlation between Ca and Mn, which is in agreement with Zeng et al. (2005) and Majumder et al. (1990). Also in the seeds, there was a positive correlation between Ca and K, confirming what was seen before in rice (Sperotto et al., 2012).

The mineral correlations in the different plant organs could also be related to differential mobilities within the plant, and several mineral elements, such as Ca and Mo, are not very phloem mobile (Marschner, 1995; Lucas et al., 2013). Once deposited in the leaves, minerals must be remobilized to the seeds via phloem transport, and K, Na, Mg, P, are transported readily, but Fe, Zn, Cu, and Mo are less mobile, and Mn and Ca are poorly mobile in the phloem of most plant species (Marschner, 1995). Mineral elements that have low phloem mobilities generally accumulate in tissues with high transpiration rates (White and Broadley, 2009).

THE ROLE OF SHOOT Fe REDUCTASE ACTIVITY

An interesting question that arises from this study is why 392-3 acquired only 10% more iron in the seeds when leaves and pod walls were more highly enriched in iron. Why did this excess iron not move to the seeds? It has been shown previously that in soybean (Lazlo, 1990), the developing ovules exhibit a gradual increase in seed Fe accretion as long as seed DW is increasing. In the current study, the source tissues of the transgenic plants showed more than double the iron concentration when compared to control plants (**Table 1**), and in certain instances, a 5-fold increase was observed in iron concentrations in the leaves. The small (yet significant) differences in the seed iron concentration between WT and 392-3 plants suggested that perhaps the higher reductase activity in the roots (Vasconcelos et al., 2006) may not have been paralleled by a higher reductase activity in the leaves. However, protoplasts isolated from leaf cells of 392-3 and WT plants showed that *AtFRO2* expression increased leaf iron reduction capacity up to 3-fold, relative to WT (**Figure 1**). However, it appears that having higher iron reduction capacity in the leaves (and presumably other vegetative tissues) may confer only a modest benefit in the availability and/or loading of iron to the phloem and subsequent transport to the seeds. Fe remobilization from leaves to seeds has been seen in legumes (Hocking and Pate, 1977; Grusak, 1994), and remobilization from leaves to seeds increases during senescence in common bean (*Phaseolus vulgaris* L.; Zhang et al., 1995). At FM, in the current study, we already had several senescing leaves, which should be working as sources of Fe to the seeds.

Chelation is a required factor for phloem transport of iron. Using the *brz* and *dgl* Fe-hyperaccumulating pea mutants, it was suggested that Fe must be chelated prior to phloem loading, since transition metal ions precipitate at alkaline pH values characteristic of phloem saps (Grusak, 1994). It is possible that our transgenic 392-3 soybean plants did not produce enough chelators to transport the excess leaf iron to the seeds. Once iron has entered the plant, both NA and citrate have been proposed to serve as iron chelators; mutants that do not properly synthesize or transport these chelators have lower Fe accumulation in the seeds (Jeong et al., 2008). We have shown that the 392-3 plants have significantly higher citrate and malate concentrations in leaves and roots. Several authors have reported an increase in xylem sap OA concentrations with Fe deficiency (Abadía et al., 2002; López-Millán et al., 2009; Larbi et al., 2010), a condition where there is up-regulation of the Fe reductase. Also, FRO2 belongs to a superfamily of flavocytochrome oxidoreductases, containing a NADPH sequence motif on the inside of the membrane (Schagerlof et al., 2006). It is possible that elevated levels of AtFRO2 lead to increased NADPH consumption, which consequently induces metabolic pathways that lead to a higher production of OA (López-Millán et al., 2000). Because the higher accumulation of malate and citrate did not enable increased seed iron levels, it seems that citrate and malate may not be limiting factors contributing to the transport of Fe to seeds.

Perhaps other factors need to be turned on for higher phloem Fe translocation from shoots to seeds. In rice, it is known that OsYSL2 expression is a necessary component for correct translocation of Fe to young shoots and developing seeds (Ishimaru et al., 2010; Masuda et al., 2012), as it is thought to transport Fe(II)-NA in the phloem (Koike et al., 2004). If there is a similar need in soybean, it is possible that the expression of this ortholog in our transgenic plants may not have been up-regulated.

IRON STORED AS FERRITIN

Another factor that could have prevented the remobilization of Fe to the seeds is if Fe was stored in a non-translocatable form, e.g., complexed within ferritin. Ferritin is one of the principal forms of iron storage in plants, and it provides a means of rapidly sequestering iron ions that might otherwise promote the formation of reactive oxygen species (Truty et al., 2001). *Ferritin* expression was assessed in roots and shoots of control and transgenic plants in order to see if the extra iron could be stored in the form of ferritin (**Figure 3**). Higher *ferritin* transcript levels were found in the shoots than in the roots in both control and transgenic plants, and an increase in *ferritin* expression was found when plants were grown at higher iron concentrations (100 μM Fe). Moreover, 392-3 plants seemed to have higher expression of the *ferritin* gene, indicating that the excess iron concentration found in the roots and shoots of the transgenic plants is at least partly stored in the form of ferritin, and thus may not be readily available for export.

CONCLUSION

Accumulation of iron in the various plant tissues during growth and development is a dynamic process resulting from an integrated regulation of genes encoding proteins for mineral uptake, transport and storage. These processes depend on the plant genotype and are greatly influenced by environmental cues. Can an improved soybean be developed that is fortified with essential minerals? The results presented herein demonstrate that constitutive expression of an iron reductase gene led to a 10% increase in seed Fe, Zn, and Cu, and a 20% increase in Mn levels, despite the fact that mineral concentration in the leaves and pod walls (two of the most important mineral sources for the seeds) reached much higher relative levels in the transgenic plants. This indicates that manipulation of the iron reductase could be an effective biofortification strategy for several minerals, especially when targeting leafy food sources.

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REFERENCES

Abadía, J., López-Millán, A. F., Rombolà, A., and Abadía, A. (2002). Organic acids and Fe deficiency: a review. *Plant Soil* 241, 75–86. doi: 10.1023/A:1016093317898

Brüggemann, W., Maas-Kantel, K., and Moog, P. R. (1993). Iron uptake by leaf mesophyll cells: the role of the plasma membrane-bound ferric-chelate reductase. *Planta* 190, 151–155. doi: 10.1007/BF00196606

Connolly, E. L., Campbell, N. H., Grotz, N., Prichard, C. L., and Guerinot, M. L. (2003). Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. *Plant Physiol.* 133, 1102–1110. doi: 10.1104/pp.103.025122

Carvalho, S., and Vasconcelos, M. (2013). Producing more with less: 'omics' at the service of plant-based food biofortification. *Food Res. Int.* 54, 961–971. doi: 10.1016/j.foodres.2012.12.021

Crafts-Brandner, S. J. (1992). Phosphorus nutrition influence on leaf senescence in soybean. *Plant Physiol.* 98, 1128–1132. doi: 10.1104/pp.98.3.1128

Curie, C., and Briat, J.-F. (2003). Iron transport and signaling in plants. *Annu. Rev. Plant Biol.* 54, 183–206. doi: 10.1146/annurev.arplant.54.031902.135018

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

de la Guardia, M., and Alcántara, E. (1996). Ferric chelate reduction by sunflower (*Helianthus annuus* L.) leaves: influence of light, oxygen, iron-deficiency and leaf age. *J. Exp. Bot.* 47, 669–675. doi: 10.1093/jxb/47.5.669

Dinkins, R. D., Reddy, M. S. S., Meurer, C. A., Yan, B., Trick, H., Thibaud-Nissen, F., et al. (2001). Increased sulfur amino acids in soybean plants overexpressing the maize 15 kda zein protein. *In Vitro Cell. Dev. Biol. Plant* 37, 742–747. doi: 10.1007/s11627-001-0123-x

Dwiyanti, M. S., Yamada, T., Sato, M., Abe, J., and Kitamura, K. (2011). Genetic variation of γ-tocopherol methyltransferase gene contributes to elevated α-tocopherol content in soybean seeds. *BMC Plant Biol.* 11:152. doi: 10.1186/1471-2229-11-152

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5624–5628. doi: 10.1073/pnas.93.11.5624

Feng, H., An, F., Zhang, S., Ji, Z., Ling, H.-Q., and Zuo, J. (2006). Light-regulated, tissue-specific, and cell differentiation-specific expression of the *Arabidopsis* Fe(III)-chelate reductase gene AtFRO6. *Plant Physiol.* 140, 1345–1354. doi: 10.1104/pp.105.074138

Fourcroy, P., Sizó-Terraza, P., Sudre, D., Savirón, M., Reyt, G., Gaymard, F., et al. (2013). *New Phytol.* 201, 155–167. doi: 10.1111/nph.12471

Gonzalez-Vallejo, E. B., Morales, F., Cistue, L., Abadia, A., and Abadia, J. (2000). Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. *Plant Physiol.* 122, 337–344. doi: 10.1104/pp.122.2.337

Grusak, M. A. (1994). Iron transport to developing ovules of *Pisum sativum*. I. Seed import characteristics and phloem iron-loading capacity of source regions. *Plant Physiol.* 104, 649–655. doi: 10.1104/pp.104.2.649

Grusak, M. A. (1995). Whole-root iron(III)-reductase activity throughout the life cycle of iron-grown *Pisum sativum* L. (Fabaceae): relevance to the iron nutrition of developing seeds. *Planta* 197, 111–117. doi: 10.1007/BF00239946

Hell, R., and Stephan, U. W. (2003). Iron uptake, trafficking and homeostasis in plants. *Planta* 216, 541–551.

Hocking, P. J., and Pate, J. S. (1977). Mobilization of minerals to developing seeds of legumes. *Ann. Bot.* 41, 1259–1278.

Hindt, M. N., and Guerinot, M. L. (2012). Getting a sense for signals: regulation of the plant iron deficiency response. *Biochim. Biophys. Acta* 1823, 1521–1530. doi: 10.1016/j.bbamcr.2012.03.010

Ishimaru, Y., Masuda, H., Bashir, K., Inoue, H., Tsukamoto, T., Takahashi, M., et al. (2010). Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. *Plant J.* 62, 379–390. doi: 10.1111/j.1365-313X.2010.04158.x

Ivanov, R., Brumbarova, T., and Bauer, P. (2012). Fitting into the harsh reality: regulation of iron-deficiency responses in dicotyledonous plants. *Mol. Plant* 5, 27–42. doi: 10.1093/mp/ssr065

Jeong, J., Cohu, C., Kerkeb, L., Pilon, M., Connolly, E. L., and Guerinot, M. L. (2008). Chloroplast Fe(III) chelate reductase activity is essential for seedling viability under iron limiting conditions. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10619–10624. doi: 10.1073/pnas.0708367105

Kobayashi, T., and Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. *Annu. Rev. Plant Biol.* 63, 131–152. doi: 10.1146/annurev-arplant-042811-105522

Kochian, L. V. (1991). "Mechanisms of micronutrient uptake and translocation in plants," in *Micronutrients in Agriculture*, 2nd Edn, ed. J. J. Mortvedt, SSSA book series # 4 (Madison, WI: Soil Science Society of America), 229–296.

Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori, S., et al. (2004). OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J.* 39, 415–424. doi: 10.1111/j.1365-313X.2004.02146.x

Korshunova, Y. O., Eide, D., Clark, W. G., Guerinot, M. L., and Pakrasi, H. B. (1999). The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Mol. Biol.* 40, 37–44. doi: 10.1023/A:1026438615520

Larbi, A., Morales, F., Abadía, A., and Abadía, J. (2010). Changes in iron and organic acid concentrations in xylem sap and apoplastic fluid of iron-deficient Beta vulgaris plants in response to iron resupply. *J. Plant Physiol.* 167, 255–260. doi: 10.1016/j.jplph.2009.09.007

Larbi, A., Morales, F., López-Millán, A.-F., Gogorcena, Y., Abadía, A., Moog, P. R., et al. (2001). Technical advance: reduction of Fe(III)-chelates by mesophyll leaf discs of sugar beet. Multi-component origin and effects of Fe deficiency. *Plant Cell Physiol.* 42, 94–105. doi: 10.1093/pcp/pce012

Lazlo, J. A. (1990). Mineral contents of soybean seed coats and embryos during development. *J. Plant Nutr.* 13, 231–248. doi: 10.1080/01904169009364070

López-Millán, A.-F., Ellis, D. R., and Grusak, M. A. (2004). Identification and characterization of several new members of the ZIP family of metal transporters in *Medicago truncatula*. *Plant Mol. Biol.* 54, 583–596. doi: 10.1023/B:PLAN.0000038271.96019.aa

López-Millán, A. F., Morales, F., Andaluz, S., Gogorcena, Y., Abadía, A., De las Rivas J., et al. (2000). Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. *Plant Physiol.* 124, 885–897. doi: 10.1104/pp.124.2.885

López-Millán, A.-F., Morales, F., Gogorcena, Y., Abadía, A., and Abadía, J. (2009). Metabolic responses in iron deficient tomato plants. *J. Plant Physiol.* 66, 375–384. doi: 10.1016/j.jplph.2008.06.011

Lucas, W. J., Groover, A., Lichtenberger, R., Furuta, K., Yadav, S.-R., Helariutta, Y., et al. (2013). The plant vascular system: evolution, development and functions. *J. Integr. Plant Biol.* 55, 294–388. doi: 10.1111/jipb.12041

Majumder, N. D., Rakshit, S. C., and Borthakur, D. N. (1990). Genetic effect on uptake of selected nutrients in some rice (*O. sativa* L.) varieties in phosphorus deficient soils. *Plant Soil* 123, 117–120.

Marentes, E., and Grusak, M. A. (1998). Iron transport and storage within the seed coat and embryo of developing seeds of pea (*Pisum sativum* L.). *Seed Sci. Res.* 8, 367–375. doi: 10.1017/S0960258500004293

Marschner, H. (1995). *Mineral Nutrition of Higher Plants*, 2nd Edn. San Diego, CA: Academic Press Inc.

Marschner, H., Römheld, V., and Ossenberg-Neuhaus, H. (1982). Rapid method for measuring changes in pH and reducing processes along roots of intact plants. *Z. Pflanzenphysiol.* 105, 407–416. doi: 10.1016/S0044-328X(82)80038-X

Masuda, H., Ishimaru, Y., Aung, M. S., Kobayashi, T., Kakei, Y., Takahashi, M., et al. (2012). Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci. Rep.* 2, 543. doi: 10.1038/srep00543

Mukherjee, I., Campbell, N. H., Ash, J. S., and Connolly, E. L. (2006). Expression profiling of the *Arabidopsis* ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper. *Planta* 223, 1178–1190. doi: 10.1007/s00425-005-0165-0

Ramani, S., and Kannan, S. (1987). Manganese absorption and transport in rice. *Physiol. Plant.* 33, 133–137. doi: 10.1111/j.1399-3054.1975.tb03780.x

Rellán-Álvarez, R., Giner-Martínez-Sierra, J., Orduna, J., Orera, I., Rodríguez-Castrillón, J. A., García-Alonso, J. I., et al. (2010). Identification of a tri-Iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol.* 51, 91–102. doi: 10.1093/pcp/pcp170

Remy, E., Cabrito, T. R., Batista, R. A., Teixeira, M. C., Sá-Correia, I., and Duque, P. (2012). The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. *New Phytol.* 195, 356–371. doi: 10.1111/j.1469-8137.2012.04167.x

Robinson, N. J., Procter, C. M., Connolly, E. L., and Guerinot, M. L. (1999). A ferric-chelate reductase for iron uptake from soils. *Nature* 397, 694–697. doi: 10.1038/17800

Rodríguez-Celma, J., Lin, W.-D., Fu, G.-M., Abadía, J., López-Millán, A.-F., and Schmidt, W. (2013). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by *Arabidopsis* and *Medicago truncatula*. *Plant Physiol.* 162, 1473–1485. doi: 10.1104/pp.113.220426

Römheld, V., and Marschner, H. (1986). Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. *Plant Physiol.* 80, 175–180. doi: 10.1104/pp.80.1.175

Schagerlof, U., Wilson, G., Hebert, H., Al-Karadaghi, S., and Hagerhall, C. (2006). Transmembrane topology of FRO2, a ferric chelate reductase from *Arabidopsis thaliana*. *Plant Mol. Biol.* 62, 215–221. doi: 10.1007/s11103-006-9015-0

Sperotto, A., Vasconcelos, M. W., Grusak, M. A., and Fett, J. P. (2012). Effects of different Fe supplies on mineral partitioning and remobilization during the reproductive development of rice (*Oryza sativa* L.). *Rice* 5, 27. doi: 10.1186/1939-8433-5-27

Stephan, U. W., and Grün, M. (1989). Physiological disorders of the nicotianamine-auxotroph tomato mutant chloronerva at different levels of iron nutrition. II. Iron deficiency response and heavy metal metabolism. *Biochem. Physiol. Pflanzen* 185, 189–200. doi: 10.1016/S0015-3796(89)80080-0

Thomine, S., Lelièvre, F., Debarbieux, E., Schroeder, J. I., and Barbier-Brygo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant J.* 34, 685–695. doi: 10.1046/j.1365-313X.2003.01760.x

Tiffin, L. O. (1966). Iron translocation. I. Plant culture, exudate sampling, iron-citrate analysis. *Plant Physiol.* 41, 510–514. doi: 10.1104/pp.41.3.510

Tiffin, L. O. (1971). Translocation of nickel in xylem exudates of plants. *Plant Physiol.* 48, 273–277. doi: 10.1104/pp.48.3.273

Truty, J., Malpe, R., and Linder, M. C. (2001). Iron prevents ferritin turnover in hepatic cells. *J. Biol. Chem.* 276, 48775–48780. doi: 10.1074/jbc.M105392200

Vasconcelos, M. W., Eckert, H., Arahana, V., Graef, G., Grusak, M. A., and Clemente, T. (2006). Molecular and phenotypic characterization of transgenic soybean expressing the *Arabidopsis* ferric chelate reductase gene, FRO2. *Planta* 224, 1116–1128. doi: 10.1007/s00425-006-0293-1

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M. L., Briat, J.-F., et al. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

Walker, E. L., and Waters, B. M. (2011). The role of transition metal homeostasis in plant seed development. *Curr. Opin. Plant Biol.* 14, 318–324. doi: 10.1016/j.pbi.2011.03.025

Waters, B. M., and Grusak, M. A. (2008). Whole-plant mineral partitioning throughout the life cycle in *Arabidopsis thaliana* ecotypes Columbia, Landsberg erecta, Cape Verde Islands, and the mutant line ysl1ysl3. *New Phytol.* 177, 389–405.

Welch, R. M., and LaRue, T. A. (1990). Physiological characteristics of Fe accumulation in the 'bronze' mutant of *Pisum sativum* L., cv 'sparkle' E107 (brz brz). *Plant Physiol.* 93, 723–729. doi: 10.1104/pp.93.2.723

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

Yi, Y., and Guerinot, M. L. (1996). Genetic evidence that induction of root Fe3+ chelate reductase activity is necessary for iron uptake under iron deficiency. *Plant J.* 10, 835–844. doi: 10.1046/j.1365-313X.1996.10050835.x

Zhang, C., Römheld, V., and Marschner, H. (1995). Retranslocation of iron from primary leaves of bean plants grown under iron deficiency. *J. Plant Physiol.* 146, 268–272. doi: 10.1016/S0176-1617(11)82052-3

Zeng, Y. W., Shen, S. Q., Wang, L. X., Liu, J. F., Pu, X. Y., Du, J., et al. (2005). Correlation of plant morphological and grain quality traits with mineral element contents in Yunnan rice. *Rice Sci.* 12, 101–106.

Zimmermann, M., Clarke, O., Gulbis, J. M., Keizer, D. W., Jarvis, R. S., Cobbett, C. S., et al. (2009). Metal binding affinities of *Arabidopsis* zinc and copper transporters: selectivities match the relative, but not the absolute, affinities of their amino-terminal domains. *Biochemistry* 48, 11640–11654. doi: 10.1021/bi901573b

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Whole shoot mineral partitioning and accumulation in pea (*Pisum sativum*)

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Several grain legumes are staple food crops that are important sources of minerals for humans; unfortunately, our knowledge is incomplete with respect to the mechanisms of translocation of these minerals to the vegetative tissues and loading into seeds. Understanding the mechanism and partitioning of minerals in pea could help in developing cultivars with high mineral density. A mineral partitioning study was conducted in pea to assess whole-plant growth and mineral content and the potential source-sink remobilization of different minerals, especially during seed development. Shoot and root mineral content increased for all the minerals, although tissue-specific partitioning differed between the minerals. Net remobilization was observed for P, S, Cu, and Fe from both the vegetative tissues and pod wall, but the amounts remobilized were much below the total accumulation in the seeds. Within the mature pod, more minerals were partitioned to the seed fraction (>75%) at maturity than to the pod wall for all the minerals except Ca, where only 21% was partitioned to the seed fraction. Although there was evidence for net remobilization of some minerals from different tissues into seeds, continued uptake and translocation of minerals to source tissues during seed fill is as important, if not more important, than remobilization of previously stored minerals.

Keywords: PEA, mineral nutrition, remobilization, continuous uptake, seeds

INTRODUCTION

Plant foods are the principal source of dietary minerals for humans and animals. In particular, cereals such as rice, wheat, and maize, and grain legumes such as beans are the staple food in many populations. Moreover, seed quality is important because it may determine seedling vigor and help to increase crop yield (Pearson et al., 1995). The amount of minerals in the seeds depends on uptake from the soil into the roots, translocation into the shoots via the xylem, transfer into the leaves and other structures and translocation into the seeds via the phloem. Plant mineral concentrations also vary, depending on the species/cultivars and on the plant tissues, which emphasizes that genetic differences exist in the plants' ability to acquire and accumulate the minerals in different tissues. We have been interested in improving the mineral concentration of pea (*Pisum sativum*), an important grain legume that serves as an important source of nutrients for humans, especially in parts of the developing world (Cousin, 1997). Thus, we have conducted studies to develop a baseline understanding of whole-plant mineral content and dynamics in pea, as a first step toward subsequent efforts to improve seed mineral concentrations in this crop.

One of the main problems in trying to increase the nutrient content in plants is the lack of understanding of different pathways and gene products involved in transporting the minerals to the seeds. Several studies have identified genes/proteins involved in uptake of the minerals from the rhizosphere, some for translocation to vegetative tissues and ultimately accumulation in seeds (Grotz et al., 1998; Eren and Arguello, 2004; Green and Rogers, 2004; Hussain et al., 2004; Verret et al., 2004; Pittman, 2005; Andrés-Colás et al., 2006; Grotz and Guerinot, 2006; Colangelo and Guerinot, 2006; Durrett et al., 2007). Iron uptake has been shown to improve when ferric chelate reductase genes are overexpressed (Connolly et al., 2003; Vasconcelos et al., 2006). Pea (*brz* and *dgl*) and Arabidopsis (*frd3*) mutants with constitutive ferric reductase activity have been shown to accumulate various minerals such as Fe, Zn, Ca, Mg, Cu, and Mn in shoot tissues (Grusak, 2000; Rogers and Guerinot, 2002; Wang et al., 2003). Expression of the ferritin gene in rice has also resulted in an increased concentration of Fe in the seeds, but only to a small extent (10%) (Goto et al., 1999; Vasconcelos et al., 2003). Even though these studies have shown increased net mineral uptake in the plants, studies are still required to understand the spatial and temporal dynamics related to the movement of these minerals from vegetative tissues to the seeds, in order to achieve large increases in mineral concentrations in the seeds. There have been several studies addressing the partitioning of nitrogen in seeds and it has been established that remobilized N is a major source of seed protein components (Hortensteiner and Feller, 2002; Schiltz et al., 2005). There have also been some studies on partitioning and accumulation of other minerals in seeds which have shown that minerals may be remobilized from the vegetative tissues (Hocking and Pate, 1977; Himelblau and Amasino, 2001), but the data available are less abundant. Nonetheless, some studies have shown that continued mineral uptake and transport during seed fill is more important than remobilization, with respect to final mineral accumulation in seeds (Pate and Hocking, 1978; Waters and Grusak, 2008).

Studies have also shown that nutrients can be translocated to the seeds via different tissues. However, information on these mineral dymanics is lacking, especially in important legume crops such as pea. In this paper, we describe the partitioning of nine different minerals (Ca, Mg, K, Cu, Fe, Mn, P, S, and Zn) prior to and during seed development in different tissues in pea (*Pisum sativum*), in order to assess the partitioning and the extent of remobilization of these minerals to the seeds.

MATERIALS AND METHODS

Seeds of pea (*Pisum sativum* L., cv. Sparkle) were imbibed overnight in deionized water and grown hydroponically in aerated, buffered nutrient solution containing the following mineral nutrients: KNO_3, 1.2 mM; $Ca(NO_3)_2$, 0.8 mM; $NH_4H_2PO_4$, 0.3 mM; $MgSO_4$, 0.2 mM; $CaCl_2$, 25 μM; H_3BO_3, 25 μM; $MnSO_4$, 2 μM; $ZnSO_4$, 2 μM; $CuSO_4$, 0.5 μM; H_2MoO_4, 0.5 μM; and $NiSO_4$, 0.1 μM and were buffered with 1 mM MES [2-(N-morpholino) ethanesulfonic acid] buffer to maintain a pH between 5.5 and 6.0. Iron was added as Fe(III)-EDDHA (Grusak, 1994). Plants were grown in 3.5 L pots and the nutrient solutions were changed biweekly until reproductive maturity (10 weeks). Solution levels in the pots were checked daily and were adjusted to 3.5 L with nutrient solution as needed. All side shoots were excised at first appearance so as to yield a plant with only one main shoot. All plants were grown in a controlled environmental chamber with a 16 h, 20°C, and 8 h, 15°C day-night regime with a mixture of fluorescent and incandescent lights.

TISSUE ANALYSIS

Individual plants were harvested at weekly intervals, beginning at 2 weeks after planting, and were separated into seeds, pod walls, peduncles, flowers, and shoot remainder (main stem, stipules, and leaflets). Seeds from pods younger than 9 days after flowering were not separated from the pod walls. For flowers at a pre-anthesis state, or for post-anthesis flowers with non-emergent pod structures, the flower or flower bud was retained with the peduncle fraction. Note that because vegetative growth occurs throughout much of the plant's life cycle, and because flowers arise at sequential nodes up the stem, tissues of different developmental ages were combined at each harvest time point. Roots from weeks 4, 7, and 10 were included for mineral analysis. Roots from the other time points were not included for mineral analysis because they were used to measure the iron (III) reductase activity in a separate study (Grusak, 1995). Roots were rinsed in two changes of aerated deionized water, with each rinse lasting 2.5 min. They were then blotted dry and placed in paper bags for oven drying and subsequent dry weight determination. A total of 3–6 plants (replicates) were analyzed per time point.

All tissue samples were dried at 60°C to constant mass. Bulk tissues were homogenized with stainless steel grinders. For each sample, aliquots of 0.25 g of dried tissue sample were digested using 4 mL of concentrated nitric acid and 2 mL of perchloric acid at temperatures up to 200°C and then taken to dryness. Digests were resuspended in 1 mL 2 M HNO_3 and, after 1 h, were brought to 10 mL with deionized water. The acids used were trace metal grade (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and the water was deionized via a MilliQ system (Millipore, Billerica, Massachusetts, USA). Samples were analyzed for nine different minerals, Ca, Mg, K, Cu, Fe, Zn, Mn, S, and P using inductively coupled plasma-optical emission spectroscopy (CIROS ICP Model FCE12; Spectro, Kleve, Germany) to detect mineral concentrations, as previously described (Farnham et al., 2011).

Mineral content for each tissue was calculated for all nine minerals by multiplying each tissue's average mineral concentration by the average total tissue weight at a given time point. Net loss of minerals from the vegetative tissues during the reproductive phase was estimated by subtracting the final (week 10) mineral content in the non-seed tissues (leaves, stem, peduncles and pod wall) from the mineral content in the non-seed tissues during the beginning of the reproductive phase (week 5). To calculate the contribution of individual organs to seed mineral content (due to net remobilization), the mineral content of each tissue (at their peak level) was divided by the peak seed mineral content (week 10). For example, for Fe, the contribution of the vegetative tissues were based on the peak level (week 6) relative to peak seed mineral content at week 10.

STATISTICS

Analyses of variance were performed using the JMP 10.0 program (SAS Institute, Cary, NC, USA). One Way ANOVA with Tukeys HSD was used to analyze the significance of differences in tissue mineral content across different time points and differences in mineral content between tissues. One Way ANOVA with Tukeys HSD was also used to calculate the difference between the different minerals in the percent of total minerals in seed fraction and the seed mineral content as percent of the total pod content (**Figures 5**, **6**). Values after the "±" sign represent standard errors throughout the text.

RESULTS

Organ-specific growth dynamics are presented in **Figure 1**. Plant DW was separated into the following components: seeds, pod walls, peduncles and flowers (referred to here as peduncles), and shoot remainder (main stem, stipules and leaflets). Seeds were the largest proportion of total shoot weight from weeks 8 through 10.

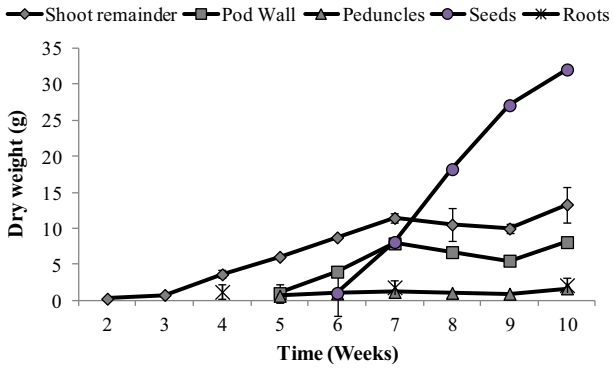

FIGURE 1 | Dry weight (g) of the different tissues (leaves + stem, pod, peduncle, and seeds) over time. Values ± SE (*n* varies from 3 to 6). Some error bars do not extend beyond the symbols.

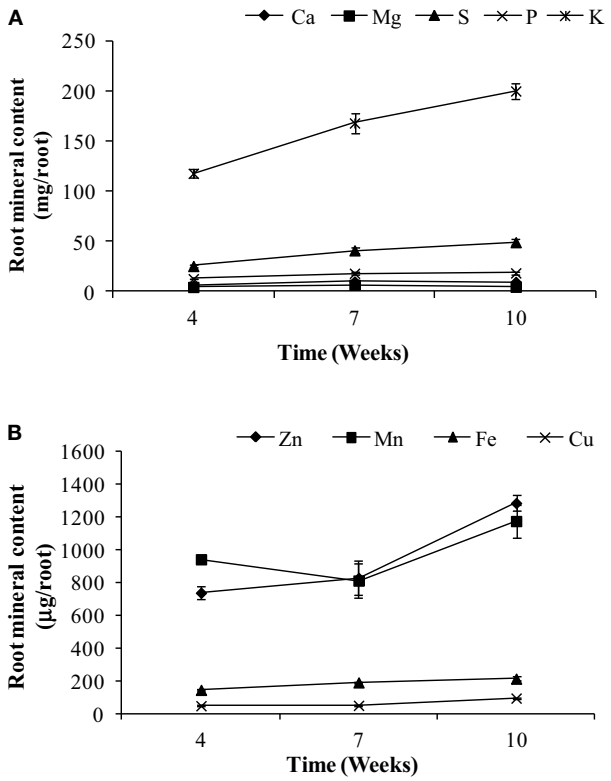

FIGURE 2 | Root content of (A) macronutrients and (B) micronutrients for weeks 4, 7, and 10 in pea. Values ± SE (*n* varies from 3 to 6). Some error bars do not extend beyond the symbols.

Reproductive tissues (pod wall + seeds) represent 73% of the total shoot weight, while seeds represent 80% of the total reproductive tissue, at the final time point (**Figure 1**).

The main objective of this paper was to assess the whole-plant partitioning of different minerals to the seeds, especially the potential of the vegetative tissues to remobilize these minerals to the seeds. For this purpose, mineral content was calculated thoughout the plant's life cycle. Mineral content for different tissues are presented in **Figures 2–4**. For roots, we could only assess the mineral content at weeks 4, 7, and 10 due to the use of root tissues for a separate study, as mentioned above. Root weights are presented in **Figure 1**; average root weights increased from 1.19 ± 0.06 g DW (week 4), to 1.82 ± 0.09 g DW (week 7), to 2.12 ± 0.10 g DW (week 10). The root mineral content for most minerals increased or stayed constant over the time course (**Figure 2**), indicating that there was no net remobilization of minerals from the roots during the period of seed fill.

To gauge the loss of minerals from the different tissues due to remobilization, we calculated the contribution of the different tissues based on their peak levels relative to the final seed mineral content at week 10. Overall, the total accumulation in the shoot tissue increased after the start of flowering (week 5). Net mineral content loss for some of the minerals and tissues was not evident and therefore could not be calculated. Mineral content of Ca, Mn, and Zn increased significantly in the vegetative tissues (leaves+stem) over the time course (**Supplementary Table 1**). For all other minerals, there was a significant decrease in mineral content in the vegetative tissues (between weeks 6 and 9) suggesting some amount of net remobilization from the tissues ($p \leq 0.05$). There was also a significant decrease in the mineral content in the pod wall fraction over the time course for all the minerals except Ca, which suggests remobilization from the pod wall fraction ($p \leq 0.05$) (**Figures 3**, **4**). Total seed content of different minerals varied significantly between 4 and 82% of the total shoot mineral content at maturity (**Figure 5**). Among the macronutrients, only 4.6% of the shoot Ca was partitioned to the seeds which was significantly lower than all the other minerals, while 50% of the total shoot Mg and K was fractioned to the seeds and 65 and 82% of the total shoot S and P was fractioned to the seed tissues, respectively. Among the micronutrients, 21% of shoot Mn and 32% of shoot Zn was fractioned to the seeds, while significantly higher amounts of Fe and Cu (70–73%) were fractioned to the seeds ($p \leq 0.05$) (**Figure 5**).

To assess the contribution of net remobilization from individual organs to seed mineral content, we have calculated the contribution of each of these individual tissues' peak levels relative to the peak seed mineral levels. For seed Ca, remobilization from the vegetative or pod tissue does not seem to be the source of seed Ca. For seed Mg, K, S, and Cu, remobilization from the vegetative tissues accounts for 50–60% of the total seed content while only 20% of the P from the vegetative tissues is fractioned into the seeds. For Fe, remobilization from the vegetative tissues is the highest among the minerals, accounting for 70% of the total seed Fe. There was no evidence of net remobilization from the vegetative tissues for Mn or Zn. Although starting with small pool sizes, all the minerals except Ca seem to remobilize from the pod wall and peduncles to the seeds. Remobilization from the pod wall fraction was the highest for Mn (40%) followed by K and Mg (30 and 34% respectively) while P, Cu, Fe, and Zn mobilized 20% or less of the total seed minerals. Remobilization from the pod wall fraction was the lowest for S at 9%. Remobilization from the peduncles accounted for less than 10% for all the minerals.

To understand the mineral distribution within the mature pod (pod wall + seed), the percentage of the total pod mineral content contained in seeds was calculated for each of the minerals (**Figure 6**). Except for Ca, the distribution pattern for all the minerals were similar, where more minerals were partitioned to the seed fraction than the pod wall (>75% of the total pod content). The seed mineral content for all the minerals was 75–95% of the total pod mineral content except for Ca, which was significantly lower where only 21% of the total fraction was partitioned to the seeds ($p \leq 0.05$) (**Figure 6**). Also, the percent of minerals in the seeds that was taken up by the plant during the reproductive phase could be estimated only for Fe (90%). This might be because some of the incoming minerals will be partitioned to new leaves, stems, and roots.

DISCUSSION

Although there are a number of groups interested in increasing seed mineral concentrations in important plant foods such as

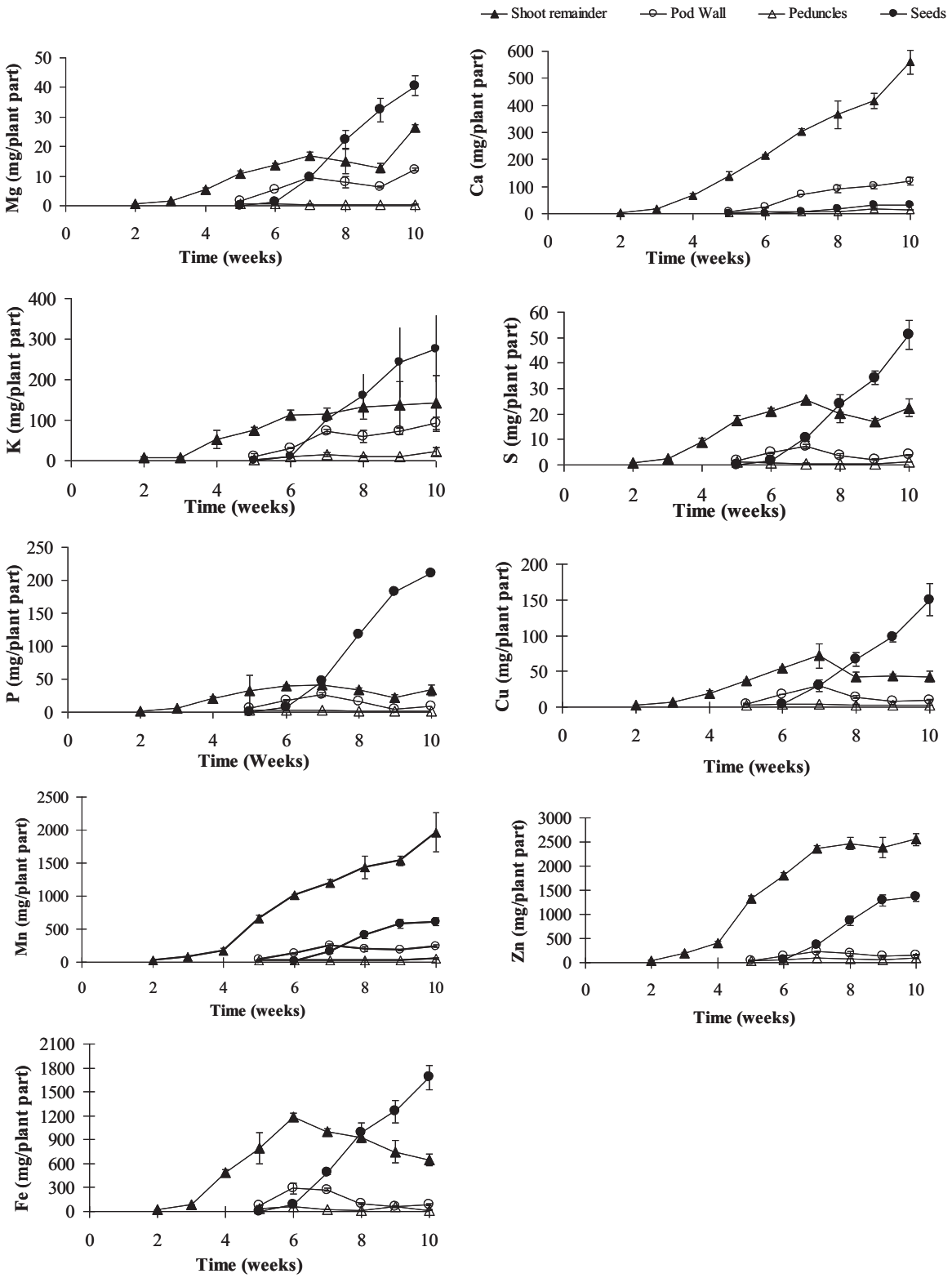

FIGURE 3 | Mineral contents of calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulfur (S), and zinc (Zn) in pea shoot tissues (leaves + stem, pod, peduncle, and seeds) over time. Values ± SE (n varies from 3 to 6). Some error bars do not extend beyond the symbols ($p \leq 0.05$).

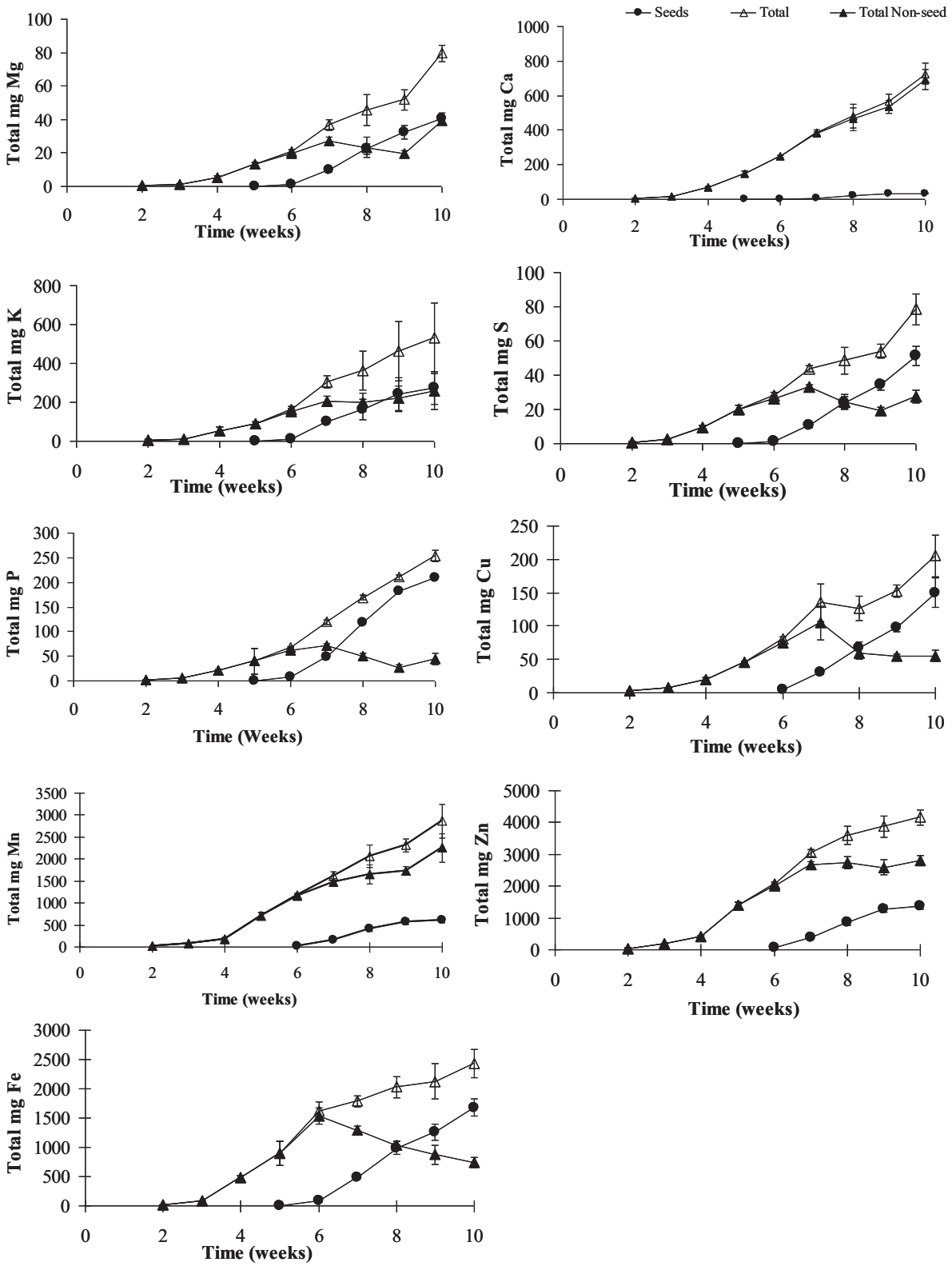

FIGURE 4 | Total mineral contents of calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulfur (S), and zinc (Zn) in pea shoots over time. Values ± SE (*n* varies from 3 to 6). Some error bars do not extend beyond the symbols.

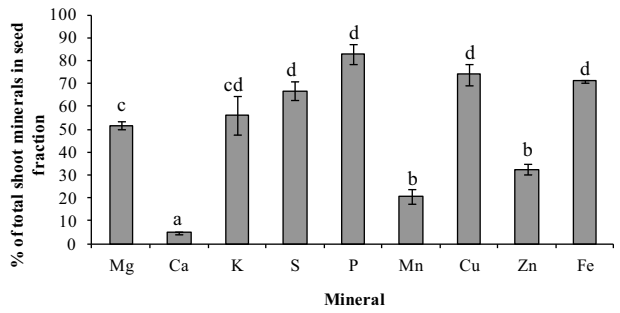

FIGURE 5 | Total mineral content in seed fraction as a percent of total shoot mineral content for the different minerals at seed maturity (week 10). Values ± SE (*n* varies from 3 to 6). Some error bars do not extend beyond the bars. Significant differences between the minerals is indicated by letters a–d ($p \leq 0.05$).

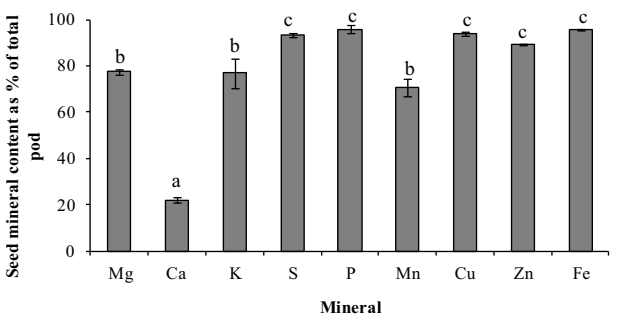

FIGURE 6 | Seed mineral content as percent of total pod mineral content for the different minerals in pea. Values ± SE (*n* varies from 3 to 6). Some error bars do not extend beyond the bars. Significant differences between the minerals is indicated by letters a–c ($p \leq 0.05$).

rice, wheat, maize, and beans (Pfeiffer and McClafferty, 2007), our understanding of transport and remobilization of minerals to seeds is still incomplete. The goal of our study was to determine the main source of seed minerals in pea, and to analyze the potential of remobilization of previously stored minerals from various source tissues versus the continued uptake and translocation of minerals from the roots. Therefore, an increase in mineral content in one tissue can be either due to uptake and translocation from the soil into the plants, to the remobilization of minerals from one tissue to another, or both of these processes. In this study, we have measured changes in mineral content in different tissues in order to monitor the uptake, translocation and remobilization of the different minerals from one tissue to another, especially to seeds. We have also excised all side shoots to the point that we had only one main shoot.

Although remobilization from previously stored minerals in the vegetative tissues, such as leaves and roots has been studied previously (Hocking, 1994; Uauy et al., 2006), the contribution of remobilized minerals to seed mineral content is still unclear. The amounts of minerals that are being remobilized from the vegetative tissues will depend on the ability of the tissues to accumulate an excess of what is required locally in that tissue and also on the phloem mobility of each of the minerals. Remobilization from leaves has been observed for Cu, K, P, S, and Zn in two different studies in wheat (Hocking, 1994; Miller et al., 1994), while in another study there was very little remobilization of Zn from the vegetative tissues suggesting continuous uptake from the roots during seed fill (Garnett and Graham, 2005). In our study, among the macronutrients, mineral content of Mg, S, and P decreased in the vegetative tissues over time and could account for 20–65% of the total seed Mg, S, and P, suggesting remobilization from these tissues. Our study showed no net loss of Ca and K, (**Figures 2**, **3**) consistent with studies in lupin, Arabidopsis and wheat (Hocking and Pate, 1977; Hocking, 1994; Himelblau and Amasino, 2001). Among the micronutrients, mineral content of Fe and Cu decreased in the vegetative tissues during the reproductive phase indicating remobilization of the minerals to the seeds. Remobilization of Fe and Cu from the pod wall fraction accounts for 48–70% of the total seed Fe and Cu. Previous studies have also shown remobilization of both Fe and Cu in wheat, beans, peas, and Arabidopsis (Grusak, 1995; Zhang et al., 1995; Himelblau and Amasino, 2001; Garnett and Graham, 2005; Waters and Grusak, 2008). In our study, there was no net remobilization observed from the vegetative tissues for Mn and Zn. Mn and Zn are known to have limited remobilization from the vegetative tissues. Consistent with our results, previous studies in Arabidopsis (Waters and Grusak, 2008), wheat (Pearson and Rengel, 1994) and clover (Nable and Loneragan, 1984; Himelblau and Amasino, 2001) for Mn and in wheat (Garnett and Graham, 2005) for Zn have also shown that they have limited mobility from the vegetative tissues, indicating continuous uptake from the root as the major source of these minerals in the seeds.

In addition to vegetative tissues, mineral loss from pod wall and peduncles could also account for the minerals being remobilized to the seeds. In a study with three legume species, Hocking and Pate (1977) showed that mobilization of minerals (N P, K, Ca, Mg, Fe, Cu, Mn, and Zn) from pods accounted for 5–39% of the total accumulation of specific seed minerals. In Arabidopsis, silique hulls have been shown to be a source of seed minerals (Waters and Grusak, 2008). In wheat, the Zn that was detected in different parts of the florets was detected in the grain (Pearson and Rengel, 1994). Although the pod wall pool is small to begin with, our results indicated that pod wall tissues are significant sources of seed minerals. Our results showed that 75–95% of the total pod (pod wall + seed) mineral content was partitioned to the seed tissue. The pod wall tissue contribution to the total seed mineral content varied between 9–40% for the different minerals, except for Ca where very little remobilization was observed from the pod wall tissues (**Figure 6**). Remobilization from the peduncles was also observed for all the minerals, although they contributed less than 10% of the total seed mineral content for most minerals. Although remobilization from the maternal fruit tissues is effective to increase the total seed mineral content, the plant would still require more total minerals coming in to the reproductive tissues to be partitioned to the seeds and increase the total seed mineral content.

In the present study, root mineral content was measured on weeks 4, 7, and 10. For all minerals, there was no evidence of a

decline in mineral content during seed fill, demonstrating no net remobilization of any mineral to the seeds. Previous studies in wheat and Arabidopsis have shown that when minerals are readily available to the roots during the period of seed fill, the plants continue to take up those minerals and use them for seed mineral accumultion, suggesting that when sufficient nutrients are available in the soil, continuous uptake from the soil supersedes remobilization. However, in nutrient deficient conditions, minerals are remobilized from both root and shoot tissues (Peng and Li, 2005; Waters and Grusak, 2008; Waters et al., 2009).

In all of the studies mentioned above and in our present study, remobilization could not account for all of the seed minerals, indicating that plants continuously take up and translocate minerals to the seeds during the seed filling period. Although targeting enhanced remobilization of minerals from vegetative pools to seeds is a reasonable approach to increase seed mineral density, enhancing continuous uptake and translocation of minerals within the plant during seed fill is equally, if not more important, for increasing seed mineral density. Biofortification of pea will thus require a simultaneous enhancement of remobilization of minerals from different source tissues and more importantly will require targeting the processes involved in continuous uptake and translocation of minerals into seeds during seed development. Thus, understanding the genes responsible for both root uptake and for translocation may lead to strategies to produce biofortified seeds.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00149/abstract

Supplementary Table 1 | Mineral contents of pea tissues over time.

Means ± SE. Significant differences over time for each tissue is indicated by letters (a–f) ($P \leq 0.05$).

REFERENCES

Andrés-Colás, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D. J., Ecker, J. R., et al. (2006). The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. *Plant J.* 45, 225–236. doi: 10.1111/j.1365-313X.2005.02601.x

Colangelo, E. P., and Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Curr. Opin. Plant. Biol.* 9, 322–330. doi: 10.1016/j.pbi.2006.03.015

Connolly, E. L., Campbell, N. H., Grotz, N., Prichard, C. L., and Guerinot, M. L. (2003). Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. *Plant Physiol.* 133, 1102–1110. doi: 10.1104/pp.103.025122

Cousin, R. (1997). Peas (*Pisum sativum* L.). *Field Crops Res.* 53, 111–130. doi: 10.1016/S0378-4290(97)00026-9

Durrett, T. P., Gassmann, W., and Rogers, E. E. (2007). The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiol.* 144, 197–205. doi: 10.1104/pp.107.097162

Eren, E., and Arguello, J. M. (2004). Arabidopsis HMA2, a divalent heavy metal-transporting P-IB-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. *Plant Physiol.* 136, 3712–3723. doi: 10.1104/pp.104.046292

Farnham, M. W., Keinath, A. P., and Grusak, M. A. (2011). Mineral concentration of broccoli florets in relation to year of cultivar release. *Crop Sci.* 51, 2721–2727. doi: 10.2135/cropsci2010.09.0556

Garnett, T. P., and Graham, R. D. (2005). Distribution and remobilization of iron and copper in wheat. *Ann. Bot.* 95, 817–826. doi: 10.1093/aob/mci085

Goto, F., Yoshihara, T., Shigemoto, N., Toki, S., and Takaiwa, F. (1999). Iron fortification of rice seed by the soybean ferritin gene. *Nat. Biotechnol.* 17, 282–286. doi: 10.1038/7029

Green, L. S., and Rogers, E. E. (2004). FRD3 controls iron localization in Arabidopsis. *Plant Physiol.* 136, 2523–2531. doi: 10.1104/pp.104.045633

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., and Eide, D. (1998). Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7220–7224. doi: 10.1073/pnas.95.12.7220

Grotz, N., and Guerinot, M. L. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochim. Biophys. Acta.* 1763, 595–608. doi: 10.1016/j.bbamcr.2006.05.014

Grusak, M. A. (1994). Iron transport to developing ovules of *Pisum sativum* L. Seed import characteristics and phloem iron-loading capacity of source regions. *Plant Physiol.* 104, 649–655.

Grusak, M. A. (1995). Whole-root iron(iii)-reductase activity throughout the life-cycle of iron-grown *Pisum sativum* L. (fabaceae) - relevance to the iron nutrition of developing seeds. *Planta* 197, 111–117. doi: 10.1007/BF00239946

Grusak, M. A. (2000). Strategies for improving the iron nutritional quality of seed crops: lessons learned from the study of unique iron-hyperaccumulating pea mutants. *Pisum Genet.* 32, 1–5.

Himelblau, E., and Amasino, R. M. (2001). Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. *J. Plant Physiol.* 158, 1317–1323. doi: 10.1078/0176-1617-00608

Hocking, P. J. (1994). Dry-matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat. *J. Plant Nutr.* 17, 1289–1308. doi: 10.1080/01904169409364807

Hocking, P. J., and Pate, J. S. (1977). Mobilization of minerals to developing seeds of legumes. *Ann. Bot.* 41, 1259–1278.

Hortensteiner, S., and Feller, U. (2002). Nitrogen metabolism and remobilization during senescence. *J. Exp. Bot.* 53, 927–937. doi: 10.1093/jexbot/53.370.927

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., et al. (2004). P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* 16, 1327–1339. doi: 10.1105/tpc.020487

Miller, R. O., Jacobsen, J. S., and Skogley, E. O. (1994). Aerial accumulation and partitioning of nutrients by hard red spring wheat. *Commun. Soil Sci. Plant Anal.* 25, 1891–1911. doi: 10.1080/00103629409369162

Nable, R. O., and Loneragan, J. F. (1984). Translocation of manganese in subterranean clover (*Trifolium subterraneum* L CV seaton park) 1. Redistribution during vegetative growth. *Aust. J. Plant Physiol.* 11, 101–111. doi: 10.1071/PP9840101

Pate, J. S., and Hocking, P. J. (1978). Phloem and xylem transport in supply of minerals to a developing legume (*Lupinus albus* L.) fruit. *Ann. Bot.* 42, 911–921.

Pearson, J. N., and Rengel, Z. (1994). Distribution and remobilization of Zn and Mn during grain development in wheat. *J. Exp. Bot.* 45, 1829–1835. doi: 10.1093/jxb/45.12.1829

Pearson, J. N., Rengel, Z., Jenner, C. F., and Graham, R. D. (1995). Transport of zinc and manganese to developing wheat grains. *Physiol. Plant.* 95, 449–455. doi: 10.1111/j.1399-3054.1995.tb00862.x

Peng, Z. P., and Li, C. J. (2005). Transport and partitioning of phosphorus in wheat as affected by P withdrawal during flag-leaf expansion. *Plant and Soil* 268, 1–11. doi: 10.1007/s11104-004-0297-1

Pfeiffer, W. H., and McClafferty, B. (2007). HarvestPlus: breeding crops for better nutrition. *Crop Sci.* 47, S88–S105. doi: 10.2135/cropsci2007.09.0020IPBS

Pittman, J. K. (2005). Managing the manganese: molecular mechanisms of manganese transport and homeostasis. *New Phytol.* 167, 733–742. doi: 10.1111/j.1469-8137.2005.01453.x

Rogers, E. E., and Guerinot, M. L. (2002). FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis. *Plant Cell* 14, 1787–1799. doi: 10.1105/tpc.001495

Schiltz, S., Munier-Jolain, N., Jeudy, C., Burstin, J., and Salon, C. (2005). Dynamics of exogenous nitrogen partitioning and nitrogen remobilization from vegetative organs in pea revealed by N-15 *in vivo* labeling throughout seed filling. *Plant Physiol.* 137, 1463–1473. doi: 10.1104/pp.104.056713

Uauy, C., Distelfeld, A., Fahima, T., Blechl, A., and Dubcovsky, J. (2006). A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* 314, 1298–1301. doi: 10.1126/science.1133649

Vasconcelos, M., Datta, K., Oliva, N., Khalekuzzaman, M., Torrizo, L., Krishnan, S., et al. (2003). Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. *Plant Sci.* 164, 371–378. doi: 10.1016/S0168-9452(02)00421-1

Vasconcelos, M., Eckert, H., Arahana, V., Graef, G., Grusak, M. A., and Clemente, T. (2006). Molecular and phenotypic characterization of transgenic soybean expressing the Arabidopsis ferric chelate reductase gene, FRO2. *Planta* 224, 1116–1128. doi: 10.1007/s00425-006-0293-1

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., et al. (2004). Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Lett.* 576, 306–312. doi: 10.1016/j.febslet.2004.09.023

Wang, Y. H., Kochian, L. V., Doyle, J. J., and Garvin, D. F. (2003). Two tomato non-symbiotic haemoglobin genes are differentially expressed in response to diverse changes in mineral nutrient status. *Plant Cell Environ.* 26, 673–680. doi: 10.1046/j.1365-3040.2003.01002.x

Waters, B. M., and Grusak, M. A. (2008). Whole-plant mineral partitioning throughout the life cycle in *Arabidopsis thaliana* ecotypes Columbia, Landsberg erecta, Cape Verde Islands, and the mutant line ysl1ysl3. *New Phytol.* 177, 389–405. doi: 10.1111/j.1469-8137.2007.02288.x

Waters, B. M., Uauy, C., Dubcovsky, J., and Grusak, M. A. (2009). Wheat (Triticum aestivum) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain. *J. Exp. Bot.* 60, 4263–4274. doi: 10.1093/jxb/erp257

Zhang, C. D., Romheld, V., and Marschner, H. (1995). Retranslocation of iron from primary leaves of bean-plants grown under iron-deficiency. *J. Plant Physiol.* 146, 268–272. doi: 10.1016/S0176-1617(11)82052-3

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There and back again, or always there? The evolution of rice combined strategy for Fe uptake

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Fe UPTAKE MECHANISMS AND TRANSCRIPTIONAL CONTROL

Iron (Fe) is an essential micronutrient for almost all living organisms and represents one of the most versatile metals in biology, being involved in many ubiquitous metabolic processes such as respiration and photosynthesis, and required as a co-factor for numerous enzymes (Sperotto et al., 2010; Grillet et al., 2014a). In plants, Fe deficiency can cause severe chlorosis, growth arrest, and even plant death. Although highly abundant in the earth's crust, Fe phytoavailability is usually low, mainly because Fe^{3+} forms insoluble Fe oxides (Lemanceau et al., 2009). To circumvent this problem, plants developed mechanisms to acquire Fe from the rhizosphere (Sperotto et al., 2012; Grillet et al., 2014a).

Fe uptake mechanisms were classically separate into two strategies. Strategy I, or reduction strategy, is carried out by all plants except those from Poaceae family, and consists of: (a) H^+ extrusion by P-type ATPases to acidify the rhizosphere and increase Fe^{3+} solubility (e.g., AtAHA2, Arabidopsis H^+-pump ATPase); (b) reduction of Fe^{3+} by a plasma membrane (PM)-bound ferric chelate reductase to more soluble Fe^{2+} (e.g., AtFRO2, Ferric Reductase Oxidase); and (c) Fe^{2+} absorption into root epidermal cells by transmembrane transporters (e.g., AtIRT1, Iron-Regulated Transporter) (Hindt and Guerinot, 2012; Ivanov et al., 2012). All three components of this strategy increase their activities during Fe deficiency. Strategy II, or chelation strategy, is used by plants from Poaceae family, and involves: (a) synthesis and release of small molecular weight compounds of the mugineic acid family called phytosiderophores (PS) into the rhizosphere, which bind Fe^{3+} with high affinity, via TOM1/OsZIFL4 (Nozoye et al., 2011; Ricachenevsky et al., 2011); and (b) Fe(III)-PS complex uptake into root cells by a Yellow Stripe/Yellow Stripe-Like (YSL) transporters. Both processes (PS excretion and Fe(III)-PS transport) are increased in response to Fe deficiency.

Since both maize (*Zea mays*) *ys1* mutant (defective for Fe(III)-PS transport) and *Arabidopsis thaliana irt1* mutant (defective for Fe^{2+} transport) cannot survive under Fe deficiency conditions, it was first widely accepted that these two Fe uptake strategies were the main mechanisms for Fe acquisition in each plant group. However, later work on rice (*Oryza sativa*) showed that two functional Fe^{2+} transporters, OsIRT1 and OsIRT2, were expressed in roots upon Fe deficiency (Ishimaru et al., 2006; Walker and Connolly, 2008). It was proposed that rice uses a combined strategy, which has all features of a strategy II plant (PS release through TOM1/OsZIFL4 and Fe(III)-PS uptake through OsYSL15, the YS1 ortholog—Inoue et al., 2009; Lee et al., 2009) and some features of a strategy I plant (Fe^{2+} uptake using IRT transporters). The other two components of strategy I plants, proton extrusion, and Fe(III)-chelate reductase activity, were not detected in Fe-deficient rice roots (Ishimaru et al., 2006). Further evidence for combined strategy was provided by rice plants carrying a mutation in the NICOTIANAMINE AMINOTRANSFERASE (NAAT) gene, a key enzyme in PS synthesis. This mutant, which lacks PS, is able to grow if Fe^{2+} is supplied as Fe source (Cheng et al., 2007). Based on these findings, it was proposed that the ability to absorb Fe^{2+} evolved in rice as an adaptation to the soil conditions in flooded paddies, where Fe^{2+} is more abundant than Fe^{3+} (Ishimaru et al., 2006; Walker and Connolly, 2008; Hindt and Guerinot, 2012). So far, rice is the only plant described to use the combined strategy mechanism.

A number of studies described key players and major transcriptional networks that control Fe homeostasis in both grasses and non-grasses (Hindt and Guerinot, 2012; Ivanov et al., 2012). Interestingly, orthologous genes have been described in rice and *Arabidopsis thaliana*, showing similar roles. The bHLH transcription factor FIT (FER-like iron-deficiency-induced transcription factor) from *A. thaliana* interacts with bHLH038 and bHLH039 to regulate IRT1 and FRO2 under Fe deficiency (Yuan et al., 2008). FIT has no ortholog in rice, but bHLH38/39 are highly similar to OsIRO2 (Hindt and Guerinot, 2012), a known downstream regulator of Fe deficiency-responsive genes. OsIRO2 regulates the Fe(III)-PS transport-related genes, but not OsIRT1 (Ogo et al., 2007). OsIDEF1, acting upstream of OsIRO2, and OsIDEF2, are transcriptional regulators of distinct but partially overlapping branches of Fe deficiency response in rice (Ogo et al., 2008; Kobayashi et al., 2009) However,

no ortholog for OsIDEF1 or OsIDEF2 was described in *A. thaliana*, although similar genes are found in the genomic sequence (Kobayashi and Nishizawa, 2012).

In *A. thaliana*, a second regulatory network is controlled by bHLH transcription factor named POPEYE (PYE), which targets distinct metal homeostasis genes. PYE seems to be regulated by interacting partners such as BRUTUS (BTS), an E3 ubiquitin ligase with metal and DNA binding domains that negatively regulates the response to Fe deficiency (Long et al., 2010; Kobayashi et al., 2013). In rice, Zheng et al. (2011) identified a negative regulator of the Fe deficiency response, OsIRO3, which could be the ortholog of PYE (Hindt and Guerinot, 2012). Interestingly, BTS orthologs OsHRZ1 and OsHRZ2 were recently characterized as negative regulators of Fe uptake and Fe utilization genes (Kobayashi et al., 2013). Thus, it seems that control of Fe deficiency response is partly conserved between *A. thaliana* and rice. Moreover, Urzica et al. (2012) performed a trans-system analysis looking for genes responsive to low Fe supply in *Chlamydomonas reinhardtii*, *A. thaliana* and rice, and observed that BTS/HRZs, IRT1, and IRT2 are conserved throughout the plant lineage.

EVOLUTION OF THE Fe DEFICIENCY RESPONSE

Currently there is no model for the evolution of Fe deficiency response in plants, especially in Poaceae, and few studies have focused on testing the hypothesis that rice is the only combined strategy species. Considering the available evidence, two models are possible (**Figure 1A**). In the first, named "recent combined strategy," combined strategy is an evolutionary novelty restricted to rice or close ancestral species, not shared with other extant species from Poaceae, which all use strategy II, and rice has acquired ability to induce an IRT1-like transporter under Fe deficiency, partially resembling strategy I, as an adaptation to flooding. It implies that Poaceae last common ancestor (LCA) has lost strategy I-based Fe acquisition capacity and gained strategy II-based Fe uptake mechanism before diversification within the family (**Figure 1A**).

In the second model, named "ancient combined strategy," combined strategy is considered an evolutionary ancient trait (**Figure 1A**). Poaceae LCA has gained the ability to use strategy II for Fe uptake, but maintained strategy I. During Poaceae diversification and speciation, both strategy I and strategy II-specific genes were available for natural selection, and thus distinct groups could have adapted differently to respond to low Fe condition. As an example, the rice lineage has maintained IRT1-like up-regulation from the original strategy I response, while rhizosphere acidification and Fe reduction traits were lost. While all Poaceae would use the more efficient Fe acquisition mechanism strategy II (Curie and Briat, 2003), the model predicts that extant species could also show partial strategy I as rice does (i.e., IRT1-like Fe^{2+} uptake).

Indirect evidence favors the ancient combined strategy model. *OsIRT1* ortholog in barley (*Hordeum vulgare*), *HvIRT1*, is also up-regulated by Fe deficiency and transports Fe, Mn, Zn, and Cd (Pedas et al., 2008). The maize ortholog, *ZmIRT1*, was both described as not Fe regulated by Nozoye et al. (2013) and as Fe regulated by Li et al. (2013). Either way, Li et al. (2013) observed strong up-regulation of ZmIRT1 under Fe deficiency and showed ZmIRT1 ability to complement Fe and Zn uptake-defective yeast strains. Interestingly, *IRT1*-like genes described in Poaceae clustered together with *AtIRT1* in a phylogenetic analysis (Li et al., 2013). *AtIRT2* and *OsIRT2*, two genes similar to *AtIRT1* and *OsIRT1* that code for Fe transporters (Ishimaru et al., 2006; Vert et al., 2009), are part of the same cluster. Strikingly, *AtIRT1*/*AtIRT2* and *OsIRT1*/*OsIRT2* gene pairs are localized in tandem in their respective genomic regions and, despite monocot/dicot divergence dates 120 to 200 million years ago (Salse et al., 2002), they still show some degree of microsynteny (**Figure 1B**). These data indicate that OsIRT1 (and probably OsIRT2) shares not only functional similarity but also common evolutionary origin with AtIRT1, as the ancient combined strategy model predicts. Moreover, phenolics were described as important for Fe deficiency response in *A. thaliana* and rice, indicating that less understood aspects of Fe deficiency response are conserved between strategy I and strategy II plants (Bashir et al., 2011; Rodríguez-Celma et al., 2013; Fourcroy et al., 2014; Schmid et al., 2014).

We should also consider the likeliness of each model. In recent combined strategy model, the LCA *IRT1* gene would have lost Fe deficiency responsiveness, presumably through deleterious mutations related to promoter activity (i.e., hampering transcription factor binding, interaction with enhancers, etc.). For *OsIRT1* to be able to respond again to low Fe concentration in combined strategy, such mutations (or any changes that rendered IRT1 non-regulated) would need to be reversed. That implies re-activation of non-functional regulatory sequences on the promoter of the same gene, which is part of the *ZIP* gene family of around ten members in Poaceae genomes, several of them encoding Fe transporters (Li et al., 2013), and re-insertion into intricate regulatory circuits (Kobayashi and Nishizawa, 2012). Although possible, the ancient combined strategy model is more parsimonious, predicting that IRT1 function was conserved through plant lineage evolution, and that preference for combined strategy or strategy II in Poaceae was a late adaptation (**Figure 1A**).

CONCLUSIONS AND FUTURE PERSPECTIVES

It has long been established that Poaceae species rely on strategy II mechanism, while all other plant groups use strategy I. Rice has been considered an exception to the Poaceae-strategy II rule (Ishimaru et al., 2006; Sperotto et al., 2012); however, although it is clear that strategy II is the main mechanism for Fe uptake in graminaceous plants, the presence of IRT1-based Fe^{2+} transport in roots might play a non-overlapping role, not only in rice but in other species (Ishimaru et al., 2006; Pedas et al., 2008; Li et al., 2013). The ancient combined strategy model proposed here states that AtIRT1 ortolog kept their ancient function, observed early in the plant lineage (Urzica et al., 2012), all the way from monocot/eudicot split to extant *Oryza*

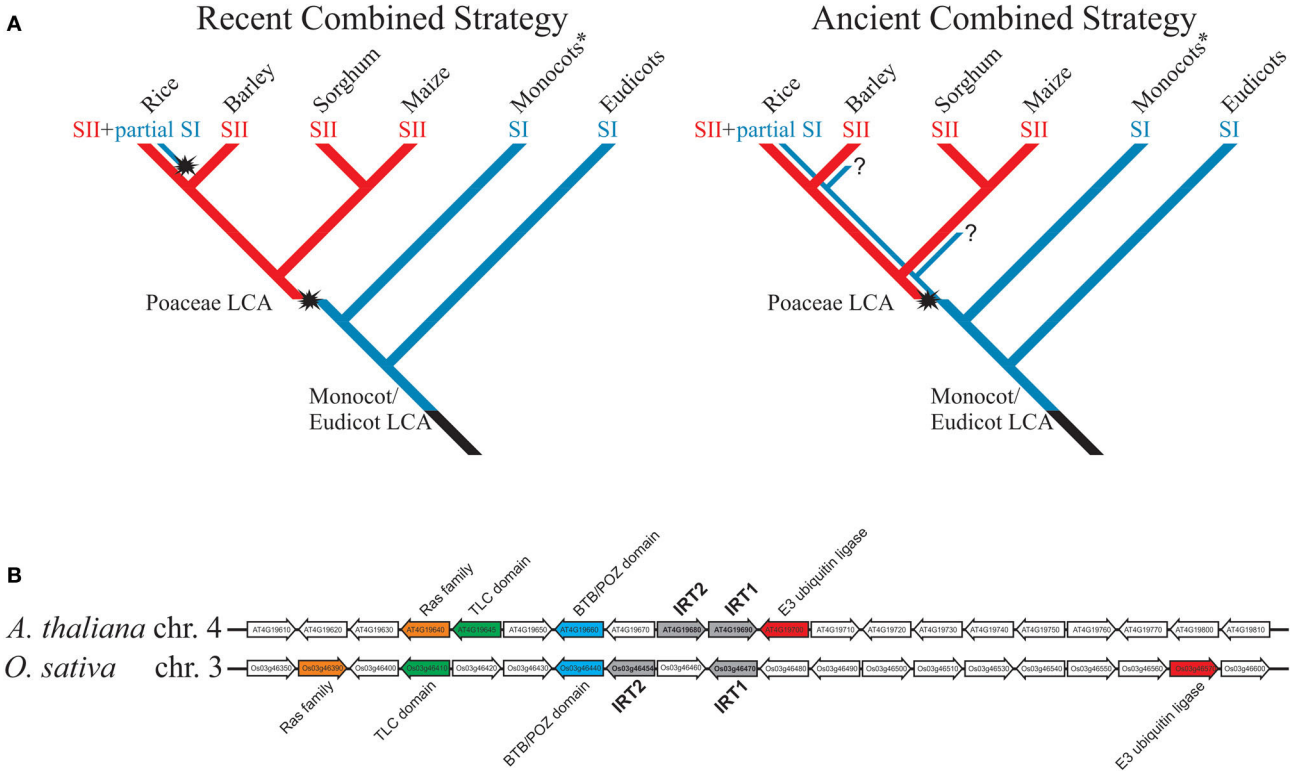

FIGURE 1 | (A) Models for evolution of rice combined strategy for Fe uptake. Blue lines represent plants using strategy I for Fe uptake; red lines represent plants using strategy II. Evolutionary novelties are highlighted, as the appearance of strategy II in Poaceae group and the putative re-appearance of IRT1-mediated Fe^{2+} uptake in rice, also known as the combined strategy (strategy II + partial strategy I). Question marks denote unclear points. Monocots* are all non-Poaceae monocotyledonous plants. SI = strategy I; SII = strategy II; LCA = last common ancestor. The selected monocot species are only to illustrate the divergence of Poaceae species, representing the subfamilies Ehrhartoideae (rice), Pooideae (barley), and Panicoideae (maize and sorghum). **(B)** Microsynteny between *AtIRT1*/*AtIRT2* and *OsIRT1*/*OsIRT2* loci. Conserved genes between rice and Arabidopsis were found using CoGe tool GEVo (http://genomevolution.org/CoGe/) and the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/). Homologs are shown with same colors; *IRT1* and *IRT2* in each genome are in gray.

sativa species, rather than re-emerged with the same function in rice (**Figure 1**). Then, it is possible that distinct Poaceae subfamilies and species evolved independently to strategy II-exclusive or combined strategy-like strategies.

Many studies have focused on Fe acquisition genes and underlying signaling pathway controlling these strategies, but most available data is on model species *A. thaliana* and rice (Hindt and Guerinot, 2012; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012). Besides these, studies in maize, barley and *Brachypodium distachyon* have indicated the role of YSL transporters in Fe(III)-PS acquisition (Curie et al., 2001; Murata et al., 2006; Yordem et al., 2011). However, the role of IRT1-like transporters is still poorly understood. Studies on the role of IRT1, as well as other strategy I-related genes such as Fe^{3+}-reductase, in Poaceae species, should shed light into how exclusive rice combined strategy is.

We should also consider that other species might use variants of strategy I and strategy II, or even rely on distinct mechanisms. Recently, it was demonstrated that peanut (*Arachis hypogaea*), an eudicot, is able to absorb Fe(III)-PS complexes through AhYSL1 transporter. The complexes, however, are only present after intercropping with maize, which secretes PS in the soil, increasing Fe efficiency of peanut (Xiong et al., 2013). An exciting new study described a previously unknown Fe uptake mechanism, where Fe is delivered to embryos of pea (*Pisum sativum*) and *A. thaliana* in the form of Fe(III)-malate/citrate complexes, and is then chemically reduced to Fe^{2+} by ascorbate, which is effluxed from embryos, for subsequent uptake (Grillet et al., 2014b).

With lowering costs and increased access to technologies such as next-generation sequencing (Mardis, 2013) and genome editing (Gaj et al., 2013), it is becoming feasible to perform comparative genomics and transcriptomic studies with plants species for which genetic resources are not available, and eventually test key genes identified. These comparisons will allow testing the models discussed here, as well as uncovering new genes and strategies for Fe acquisition.

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REFERENCES

Bashir, K., Ishimaru, Y., Shimo, H., Kakei, Y., Senoura, T., Takahashi, R., et al. (2011). Rice phenolics efflux transporter 2 (PEZ2) plays an important role in solubilizing apoplasmic iron. *Soil Sci. Plant Nutr.* 57, 803–812. doi: 10.1080/00380768.2011.637305

Cheng, L., Wang, F., Shou, H., Huang, F., Zheng, L., He, F., et al. (2007). Mutation in nicotianamine aminotransferase stimulated the Fe(II) acquisition system and led to iron accumulation in rice. *Plant Physiol.* 145, 1647–1657. doi: 10.1104/pp.107.107912

Curie, C., and Briat, J. F. (2003). Iron transport and signaling in plants. *Ann. Rev. Plant Biol.* 54, 183–206. doi: 10.1146/annurev.arplant.54.031902.135018

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S. L., Briat, J. F., and Walker, E. L. (2001). Maize *yellow stripe1* encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409, 346–349. doi: 10.1038/35053080

Gaj, T., Gersbach, C. A., and Barbas, C. F. 3rd. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31, 397–405. doi: 10.1016/j.tibtech.2013.04.004

Fourcroy, P., Sisó-Terraza, P., Sudre, D., Savirón, M., Reyt, G., Gaymard, F., et al. (2014). Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency. *New Phytol.* 201, 155–167. doi: 10.1111/nph.12471

Grillet, L., Mari, S., and Schmidt, W. (2014a). Iron in seeds - loading pathways and subcellular localization. *Front. Plant Sci.* 4:535. doi: 10.3389/fpls.2013.00535

Grillet, L., Ouerdane, L., Flis, P., Hoang, M. T., Isaure, M. P., Lobinski, R., et al. (2014b). Ascorbate efflux as a new strategy for iron reduction and transport in plants. *J. Biol. Chem.* 289, 2515–2525. doi: 10.1074/jbc.M113.514828

Hindt, M. N., and Guerinot, M. L. (2012). Getting a sense for signals: regulation of the plant iron deficiency response. *Biochim. Biophys. Acta* 1823, 1521–1530. doi: 10.1016/j.bbamcr.2012.03.010

Inoue, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., et al. (2009). Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J. Biol. Chem.* 284, 3470–3479. doi: 10.1074/jbc.M806042200

Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., et al. (2006). Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *Plant J.* 45, 335–346. doi: 10.1111/j.1365-313X.2005.02624.x

Ivanov, R., Brumbarova, T., and Bauer, P. (2012). Fitting into the harsh reality: regulation of iron deficiency responses in dicotyledonous plants. *Mol. Plant* 5, 27–42. doi: 10.1093/mp/ssr065

Kobayashi, T., Itai, R. N., Ogo, Y., Kakei, Y., Nakanishi, H., Takahashi, M., et al. (2009). The rice transcription factor IDEF1 is essential for the early response to iron deficiency, and induces vegetative expression of late embryogenesis abundant genes. *Plant J.* 60, 948–961. doi: 10.1111/j.1365-313X.2009.04015.x

Kobayashi, T., Nagasaka, S., Senoura, T., Itai, R. N., Nakanishi, H., and Nishizawa, N. K. (2013). Iron-binding haemerythrin RING ubiquitin ligases regulate plant iron responses and accumulation. *Nat. Commun.* 4, 2792. doi: 10.1038/ncomms3792

Kobayashi, T., and Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. *Annu. Rev. Plant Biol.* 63, 131–152. doi: 10.1146/annurev-arplant-042811-105522

Lee, S., Chiecko, J. C., Kim, S. A., Walker, E. L., Lee, Y., Guerinot, M. L., et al. (2009). Disruption of *OsYSL15* leads to iron inefficiency in rice plants. *Plant Physiol.* 150, 786–800. doi: 10.1104/pp.109.135418

Lemanceau, P., Bauer, P., Kraemer, S., and Briat, J. F. (2009). Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. *Plant Soil* 321, 513–535. doi: 10.1007/s11104-009-0039-5

Li, S., Zhou, X., Huang, Y., Zhu, L., Zhang, S., Zhao, Y., et al. (2013). Identification and characterization of the zinc-regulated transporters, iron-regulated transporter-like protein (ZIP) gene family in maize. *BMC Plant Biol.* 13:114. doi: 10.1186/1471-2229-13-114

Long, T. A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D. E., and Benfey, P. N. (2010). The bHLH transcription factor POPEYE regulates response to iron deficiency in Arabidopsis roots. *Plant Cell* 22, 2219–2236. doi: 10.1105/tpc.110.074096

Mardis, E. R. (2013). Next-generation sequencing platforms. *Annu. Rev. Anal. Chem.* 6, 287–303. doi: 10.1146/annurev-anchem-062012-092628

Murata, Y., Ma, J. F., Yamaji, N., Ueno, D., Nomoto, K., and Iwashita, T. (2006). A specific transporter for iron(III)-phytosiderophore in barley roots. *Plant J.* 46, 563–572. doi: 10.1111/j.1365-313X.2006.02714.x

Nozoye, T., Nagasaka, S., Kobayashi, T., Takahashi, M., Sato, Y., Uozumi, N., et al. (2011). Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. *J. Biol. Chem.* 286, 5446–5454. doi: 10.1074/jbc.M110.180026

Nozoye, T., Nakanishi, H., and Nishizawa, N. K. (2013). Characterizing the crucial components of iron homeostasis in the maize mutants *ys1* and *ys3*. *PLoS ONE* 8:e62567. doi: 10.1371/journal.pone.0062567

Ogo, Y., Itai, R. N., Nakanishi, H., Kobayashi, T., Takahashi, M., Mori, S., et al. (2007). The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. *Plant J.* 51, 366–377. doi: 10.1111/j.1365-313X.2007.03149.x

Ogo, Y., Kobayashi, T., Nakanishi, I. R., Nakanishi, H., Kakei, Y., Takahashi, M., et al. (2008). A novel NAC transcription factor, IDEF2, that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. *J. Biol. Chem.* 283, 13407–13417. doi: 10.1074/jbc.M708732200

Pedas, P., Ytting, C. K., Fuglsang, A. T., Jahn, T. P., Schjoerring, J. K., and Husted, S. (2008). Manganese efficiency in barley: identification and characterization of the metal ion transporter HvIRT1. *Plant Physiol.* 148, 455–466. doi: 10.1104/pp.108.118851

Ricachenevsky, F. K., Sperotto, R. A., Menguer, P. K., Sperb, E. R., Lopes, K. L., and Fett, J. P. (2011). ZINC-INDUCED FACILITATOR-LIKE family in plants: lineage-specific expansion in monocotyledons and conserved genomic and expression features among rice (*Oryza sativa*) paralogs. *BMC Plant Biol.* 11:20. doi: 10.1186/1471-2229-11-20

Rodríguez-Celma, J., Lin, W. D., Fu, G. M., Abadía, J., López-Millán, A. F., and Schmidt, W. (2013). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by Arabidopsis and Medicago truncatula. *Plant Physiol.* 162, 1473–1485. doi: 10.1104/pp.113.220426

Salse, J., Piégu, B., Cooke, R., and Delseny, M. (2002). Synteny between *Arabidopsis thaliana* and rice at the genome level: a tool to identify conservation in the ongoing rice genome sequencing project. *Nucleic Acids Res.* 30, 2316–2328. doi: 10.1093/nar/30.11.2316

Schmid, N. B., Giehl, R. F., Döll, S., Mock, H. P., Strehmel, N., Scheel, D., et al. (2014). Feruloyl-CoA 6′-Hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in arabidopsis. *Plant Physiol.* 164, 160–172. doi: 10.1104/pp.113.228544

Sperotto, R. A., Ricachenevsky, F. K., Stein, R. J., Waldow, V. A., and Fett, J. P. (2010). Iron stress in plants: dealing with deprivation and overload. *Plant Stress* 4, 57–69.

Sperotto, R. A., Ricachenevsky, F. K., Waldow, V. A., and Fett, J. P. (2012). Iron biofortification in rice: it's a long way to the top. *Plant Sci.* 190, 24–39. doi: 10.1016/j.plantsci.2012.03.004

Urzica, E. I., Casero, D., Yamasaki, H., Hsieh, S. I., Adler, L. N., Karpowicz, S. J., et al. (2012). Systems and *trans*-system level analysis identifies conserved iron deficiency responses in the plant lineage. *Plant Cell* 24, 3921–3948. doi: 10.1105/tpc.112.102491

Vert, G., Barberon, M., Zelazny, E., Séguéla, M., Briat, J. F., and Curie, C. (2009). Arabidopsis IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta* 229, 1171–1179. doi: 10.1007/s00425-009-0904-8

Walker, E. L., and Connolly, E. L. (2008). Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. *Curr. Opin. Plant Biol.* 11, 530–535. doi: 10.1016/j.pbi.2008.06.013

Xiong, H., Kakei, Y., Kobayashi, T., Guo, X., Nakazono, M., Takahashi, H., et al. (2013). Molecular evidence for phytosiderophore-induced improvement of iron nutrition of peanut intercropped with maize in calcareous soil. *Plant Cell Environ.* 36, 1888–1902. doi: 10.1111/pce.12097

Yordem, B. K., Conte, S. S., Ma, J. F., Yokosho, K., Vasques, K. A., Gopalsamy, S. N., et al. (2011). *Brachypodium distachyon* as a new model system for understanding iron homeostasis in grasses: phylogenetic and expression analysis of Yellow Stripe-Like (YSL) transporters. *Ann. Bot.* 108, 821–833. doi: 10.1093/aob/mcr200

Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., et al. (2008). FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. *Cell Res.* 18, 385–397. doi: 10.1038/cr.2008.26

Zheng, L., Ying, Y., Wang, L., Wang, F., Whelan, J., and Shou, H. (2011). Identification of a novel iron regulated basic helix-loop-helix protein involved in Fe homeostasis in *Oryza sativa*. *BMC Plant Biol.* 10:166. doi: 10.1186/1471-2229-10-166

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Brachypodium distachyon as a model system for studies of copper transport in cereal crops

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Copper (Cu) is an essential micronutrient that performs a remarkable array of functions in plants including photosynthesis, cell wall remodeling, flowering, and seed set. Of the world's major cereal crops, wheat, barley, and oat are the most sensitive to Cu deficiency. Cu deficient soils include alkaline soils, which occupy approximately 30% of the world's arable lands, and organic soils that occupy an estimated 19% of arable land in Europe. We used *Brachypodium distachyon* (brachypodium) as a proxy for wheat and other grain cereals to initiate analyses of the molecular mechanisms underlying their increased susceptibility to Cu deficiency. In this report, we focus on members of the CTR/COPT family of Cu transporters because their homologs in *A. thaliana* are transcriptionally upregulated in Cu-limited conditions and are involved either in Cu uptake from soils into epidermal cells in the root, or long-distance transport and distribution of Cu in photosynthetic tissues. We found that of five COPT proteins in brachypodium, BdCOPT3, and BdCOPT4 localize to the plasma membrane and are transcriptionally upregulated in roots and leaves by Cu deficiency. We also found that BdCOPT3, BdCOPT4, and BdCOPT5 confer low affinity Cu transport, in contrast to their counterparts in *A. thaliana* that confer high affinity Cu transport. These data suggest that increased sensitivity to Cu deficiency in some grass species may arise from lower efficiency and, possibly, other properties of components of Cu uptake and tissue partitioning systems and reinforce the importance of using brachypodium as a model for the comprehensive analyses of Cu homeostasis in cereal crops.

Keywords: *Brachypodium*, CTR/COPT transporters, copper transport, copper homeostasis, wheat

INTRODUCTION

Copper (Cu) is an essential micronutrient for all organisms because it acts as a cofactor for enzymes participating in important biological processes such as respiration, photosynthesis, and scavenging of oxidative stress (Burkhead et al., 2009; Merchant, 2010; Ravet and Pilon, 2013). In addition to these functions, plants also employ Cu for the perception of ethylene, nitrogen metabolism, molybdenum cofactor synthesis, cell wall remodeling, response to pathogens, flowering, and seed set (Shorrocks and Alloway, 1988; Marschner, 1995; Burkhead et al., 2009; Mendel and Kruse, 2012; Ravet and Pilon, 2013). This remarkable array of functions is attributed to the ability of Cu to change its oxidation state ($Cu^{2+} \leftrightarrow Cu^{+}$). However, the same property imposes toxicity when free Cu ions accumulate in cells in excess because of their ability to promote oxidative stress (Valko et al., 2005). Copper availability, and thus crop productivity on agricultural soils depend on soil type and agricultural practices (Shorrocks and Alloway, 1988; Marschner, 1995; Solberg et al., 1999). For example, Cu deficiency develops on alkaline soils due to low solubility of Cu at high pH, and on organic soils due to Cu binding to organic matter (Shorrocks and Alloway, 1988; Marschner, 1995; Solberg et al., 1999). While Cu deficiency can be remedied by the application of Cu-based fertilizers, this strategy is not environmentally friendly, and the repeated use of fertilizers, as well as Cu-based pesticides, has led to the build-up of toxic levels of Cu in soils (Shorrocks and Alloway, 1988; Marschner, 1995). In this regard, organic farming has emerged as a preferred production system that relies on using natural fertilizers; however natural fertilizers increase soil organic matter and thus, further reduce Cu bioavailability. Sensitivity to Cu bioavailability in soils varies among crops species. Of the major cereal crops, wheat is regarded as the most sensitive to Cu deficiency (Shorrocks and Alloway, 1988; Solberg et al., 1999). In contrast, crops like canola have not shown Cu deficiency symptoms or responded to Cu fertilizer when grown on soils that would cause Cu deficiency in wheat or barley (Solberg et al., 1999). Higher sensitivity of some cereal crops to Cu deficiency compared to other crop species has been recognized in farm reports and fact sheets from some states in the United States and different countries in the world. Among the major cereal crops, sensitivity to Cu deficiency is reported to be in the following rank order, from higher to lower sensitivity: winter wheat > spring wheat > flax > barley > oats > triticale > rye (Shorrocks and Alloway, 1988; Solberg et al., 1999). Remarkably, the molecular mechanisms that underlie this tendency are unknown.

Plants tightly regulate Cu homeostasis to prevent deficiency while avoiding toxicity. This regulation includes transcriptional control of genes encoding proteins that are involved in Cu uptake, trafficking, tissue partitioning, and reallocation among Cu requiring enzymes. Of known Cu transporters, members of

the CTR/COPT family are among the main contributors to initial Cu uptake in plants, the green alga *Chlamydomonas reinhardtii*, yeast, *Drosophila*, and humans (Merchant, 2010; Nevitt et al., 2012; Ravet and Pilon, 2013). The plant CTR/COPT family is best characterized in *Arabidopsis thaliana* and *Oryza sativa*, where it is represented by six and seven members, respectively (Peñarrubia et al., 2010; Yuan et al., 2011). *A. thaliana* COPT1, COPT2, and COPT6 are transcriptionally regulated by Cu deficiency, localize to the plasma membrane, mediate Cu uptake, and complement the growth defect of the *S. cerevisiae* Cu uptake mutant lacking functional Cu transporters, Ctr1p, Ctr2p, and Ctr3p (*ctr1*Δ*ctr3*Δ or *ctr1*Δ*ctr2*Δ*ctr3*Δ) (Sancenon et al., 2004; Andres-Colas et al., 2010; Jung et al., 2012; Garcia-Molina et al., 2013; Gayomba et al., 2013; Perea-García et al., 2013). COPT1 and COPT2 function primarily in Cu uptake into the root, while COPT6 also contributes to Cu partitioning in photosynthetic tissues (Jung et al., 2012; Garcia-Molina et al., 2013). In contrast, COPT5 localizes to the tonoplast and the pre-vacuolar compartment and functions by remobilizing Cu from these organelles during Cu deficiency (Garcia-Molina et al., 2011; Klaumann et al., 2011). CTR/COPT proteins homotrimerize to form a pore within the membrane to transport Cu across the lipid bilayer, but can also form heterocomplexes with other CTR/COPT family members and/or other proteins (Lee et al., 2002; De Feo et al., 2009; Yuan et al., 2010, 2011). Studies in *A. thaliana* have shown that while COPT6 interacts with COPT1, this interaction is not required for the ability of COPT6 or COPT1 to complement the Cu uptake deficiency phenotype of the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ mutant strain (Jung et al., 2012). Unlike CTR/COPT transporters from *A. thaliana*, CTR/COPT transporters from *O. sativa* complement the Cu uptake mutant of yeast only when are co-expressed with another OsCOPT member, and OsCOPT7 is the only high-affinity Cu transporter in the *O. sativa* CTR/COPT family (Yuan et al., 2010, 2011). These findings suggest that some properties of CTR/COPT transporters are distinct between grass and non-grass species. These differences are not entirely surprising, given that *A. thaliana* is only distantly related to the *Poaceae* family and lacks many biological features of monocotyledonous grass crops (Brkljacic et al., 2011).

A member of the grass species, *Brachypodium distachyon* (from then on referred to as brachypodium) has emerged as a valuable experimental model for the study of small-grain cereals due to its less complex and fully sequenced genome, the continued development of numerous genetic resources, including efficient protocols for *Agrobacterium*-mediated transformation (Vogel and Hill, 2008), whole genome TILLING mutant alleles (Brkljacic et al., 2011; Thole et al., 2012), a T-DNA insertion mutant collection (http://brachypodium.pw.usda.gov/TDNA/; Bragg et al., 2012), as well as other attributes such as short life cycle (8–12 weeks), small stature (15–20 cm), diploid accessions, self-fertility, and simple growth requirements (Brkljacic et al., 2011). Because brachypodium and wheat grains have similar structure, brachypodium is an attractive model for molecular, genetic, and genomic studies of Cu homeostasis of wheat (Brkljacic et al., 2011; Mochida et al., 2011; Mur et al., 2011). Furthermore, root anatomy of brachypodium and wheat is similar and is distinct from the root anatomy of another *Poaceae* family member, *O. sativa*, which is specialized for overcoming anaerobic conditions associated with submerged roots, and thus, it is suggested that brachypodium and wheat may have similar root-related genes, including those that are involved in mineral ion uptake (Chochois et al., 2012). Hence, brachypodium becomes a preferred model for studies of ion homeostasis, as was recently demonstrated by the analyses of brachypodium Yellow Stripe-Like (YSL) proteins, which are involved in uptake and internal translocation of iron (Fe) (Yordem et al., 2011).

Members of the CTR/COPT family of Cu transporters are the best characterized in both, *A. thaliana* and rice, but corresponding members in brachypodium have not yet been characterized, and are not fully annotated, according to database collections such as the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). In contrast, a specialized database for *A. thaliana* membrane proteins, ARAMEMNON 7.0 (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al., 2003) has a more complete annotation of putative membrane proteins of *A. thaliana* and provides their homologs from other species, including brachypodium, based on amino acid similarity and motif organization. According to *in silico* predictions using ARAMEMNON 7.0, we have identified five putative CTR/COPT family members in brachypodium and provided the initial characterization of their function in Cu homeostasis. We have also developed growth conditions for studies of Cu homeostasis in brachypodium as a model for small grain cereals and established protocols for the isolation and transfection of protoplasts isolated from brachypodium mesophyll cells. We used these procedures, along with functional complementation assays in the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ mutant strain, gene expression analyses, and yeast-two-hybrid assays of protein-protein interactions to study members of the CTR/COPT family of Cu transporters in brachypodium.

METHODS AND MATERIALS

PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of the 21-3 inbred line of brachypodium (Vogel and Hill, 2008) were sterilized in 100% ethanol for 1 min and rinsed three times with sterile water. The lamella and palea were softened by further incubation in sterile water for 2 h and removed with forceps, taking care not to damage the seed. Prepared seeds were then spread onto rinsed perlite irrigated with a standard hydroponic solution, described below. After stratification at 4°C for 24 h, plants were grown for 7 days at 22°C; 12-h light/12-h dark photoperiod at photosynthetic photon flux density of 150 μmol photons $m^{-2}s^{-1}$ before transferring to a hydroponic solution prepared as described (Arteca and Arteca, 2000) except that Cu (as $CuSO_4$) was added at a higher concentration of 0.25 μM. For Cu limitation and sensitivity assays, 7-day-old seedlings were transferred from perlite to hydroponic solution supplemented with the indicated concentrations of $CuSO_4$, or without $CuSO_4$ but with the specific Cu chelator, bathocuproine disulfonate (BCS) (Rapisarda et al., 2002). Plants were grown for 18 days before subsequent analyses. For all experiments, the hydroponic solution was changed every 7 days.

RNA EXTRACTION

Root and leaf tissues were separated from 25-day-old plants grown under the indicated conditions and flash-frozen in liquid nitrogen. Samples were homogenized in liquid nitrogen using a mortar and a pestle, and total RNA was isolated using the Plant RNA Kit (Omega Bio-Tek), according to the manufacturer's instructions. Genomic DNA in RNA samples was digested with DNAse *I* (Omega Bio-Tek) prior to cDNA synthesis using the iScript cDNA Synthesis kit (BioRad).

QUANTITATIVE REAL-TIME (qRT)-PCR ANALYSIS

Prior to qRT-PCR analysis, primers (**Supplemental Table 1**) and cDNA concentrations were optimized to reach a qRT-PCR amplification efficiency of 100 ± 10%. Two microliters of 10-fold diluted cDNA was used as a template in a total reaction volume of 10 µl containing 500 nM of each PCR primer, 50 mM KCl, 20 mM Tris-HCI, pH 8.4, 0.2 mM dNTPs, and 1.25 units of iTaq DNA polymerase in iQ SYBR Green Supermix (BioRad), containing 3 mM $MgCl_2$, SYBR Green I, 20 nM fluoresceine, and stabilizers. PCR was carried out using the CFX96 Real-Time PCR system (BioRad). The thermal cycling parameters were as follows: denaturation at 95°C for 3 min, followed by 39 cycles of 95°C for 10 s then 55°C for 30 s. Amplicon dissociation curves, i.e., melting curves, were recorded after cycle 39 by heating from 60 to 95°C with 0.5°C increments and an average ramp speed of 3.3°C s^{-1}. Data were analyzed using the CFX Manager Software, version 1.5 (BioRad). A brachypodium gene encoding ACTIN2 was used as a reference for normalizing gene expression. qRT-PCR experiments were conducted using three independent biological samples, each consisting of three technical replicates, unless indicated otherwise. Statistical analysis was performed using the Relative Expression Software Tool (REST, Qiagen; Pfaffl et al., 2002).

PLASMID CONSTRUCTION

Total RNA isolated from brachypodium leaves and oligonucleotides pairs that are indicated in **Supplemental Table 1** were used for RT-PCR amplification of *BdCOPT1*, *BdCOPT3*, *BdCOPT4*, and *BdCOPT5* cDNAs with or without the stop codon. Primers were designed to contain *attB* sites on resulting PCR products for subsequent Gateway cloning (Invitrogen) into the DONR-Zeo entry vector (Invitrogen) and appropriate destination vectors described below.

FUNCTIONAL COMPLEMENTATION OF THE *S. CEREVISIAE* COPPER UPTAKE DEFICIENT *CTR1*Δ *CTR2*Δ *CTR3*Δ MUTANT STRAIN

S. cerevisiae SEY6210 (*MATa ura3-52 leu2-3,-112 his3*Δ*200 trp1*Δ*901 lys2-801 suc2*Δ*9*) wild-type and the *ctr1*Δ*ctr2*Δ*ctr3*Δ triple mutant (*MATa ura3-52 his3*Δ*200 trp1-901 ctr1::ura3::Knr ctr2::HIS3 ctr3::TRP1*) used for functional complementation assays were the generous gift of Dr. Dennis Thiele (Duke University). Yeast cells were transformed with YES3-Gate-BdCOPT1, YES3-Gate-BdCOPT3, YES3-Gate-BdCOPT4, YES3-Gate-BdCOPT5 constructs or an empty YES3-Gate vector using the Frozen-EZ yeast Transformation II Kit (Zymo Research). Transformants were selected for uracil prototrophy on YNB medium (YNB-Ura) containing 0.67% (w/v) Yeast Nitrogen Base without amino acids (Difco), 0.077% (w/v) CSM-Ura, 0.05% (w/v) NaCl, 2% dextrose, 2% (w/v) agar. Respiration competence was evaluated by testing the ability of transformants to grow on the non-fermentable carbon sources, glycerol and ethanol (YPEG) as described (Dancis et al., 1994a; Puig et al., 2002). Briefly, transformants were grown in liquid YNB-Ura to an $OD_{600\,nm} = 1.0$, serially-10-fold diluted and spotted onto YPEG medium containing 1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% (v/v) glycerol 2% (v/v) ethanol, and 2% (w/v) agar and the indicated concentration of $CuSO_4$or onto YNB-Ura for controls. Plates were incubated for 3 days at 30°C.

ISOLATION OF PROTOPLASTS FROM BRACHYPODIUM LEAVES

Protoplasts were isolated from leaves of 25-day-old brachypodium grown as described above. The protoplast isolation procedure was based on (Zhai et al., 2009). Briefly, 0.2 g of young leaf tissue was immersed in 15 ml of filter-sterilized TVL solution (**Supplemental Table 2**), finely chopped with a fresh razor blade and transferred to a 200 ml beaker containing 20 ml of Enzyme solution (**Supplemental Table 2**). The beaker was wrapped in aluminum foil to protect samples from light and samples were vacuum infiltrated for 30 min before incubation at 30°C for 60 min. The mixture was then agitated at 35 rpm at room temperature for 18–20 h. Released protoplasts were collected into 50-ml Falcon centrifuge tubes by carefully sieving the mixture through eight layers of cheesecloth, pre-wetted with W5 solution (**Supplemental Table 2**). To increase the protoplast yield, the cheesecloth was rinsed with an additional 10 ml of W5 solution. Sieved protoplasts were then carefully overlaid with 5 ml of W5 solution and left at room temperature for 1 h to allow protoplasts to float to the interface of Enzyme solution and W5 solution. Fifteen milliliters of protoplasts were collected from the interface and transferred into a new 50 ml Falcon tube containing 20 ml of W5 solution. Protoplasts were collected by centrifugation for 7 min at 100 × *g*. The residual Enzyme solution was removed by two rounds of rinsing protoplasts with 10 ml W5 solution and centrifuged for 5 min at 60 × *g*. Purified protoplasts were resuspended in 3–5 ml W5 solution and the protoplast yield was evaluated by cell counting using a hemocytometer. Protoplast viability was evaluated using the Plant Cell Viability Assay Kit (Sigma), according to manufacturer's recommendations.

TRANSFECTION OF PROTOPLASTS WITH PLASMID DNA

Protoplasts were transfected using a procedure adopted from (Jung et al., 2011). Briefly, purified protoplasts were incubated on ice for 30 min and centrifuged for 5 min at 60 × *g* and W5 solution was removed. Protoplasts were then re-suspended in MMG solution (**Supplemental Table 2**) and 100 µl aliquots were transferred into a 2-ml round-bottom microcentrifuge tube. Plasmid DNA (5–10 µg) was added to protoplasts and mixed gently. For controls, DNA was omitted and replaced with equivalent volumes of sterile water (mock transfection). Transfection was initiated by the addition of 110 µl of PEG-calcium solution (**Supplemental Table 2**). Protoplasts were gently mixed with PEG-calcium solution by tapping the tube followed by incubation for 7 min at room temperature. Transfection was terminated by diluting the mixture by an addition of 700 µl of W5 solution.

Transfected protoplasts were collected by centrifugation for 2 min at 100 × *g*, and supernatant was removed to leave 50–100 μl of protoplast suspension. Each sample was then brought up to a volume of 1 ml with W5 solution and incubated in the dark at room temperature for 18 h.

SUBCELLULAR LOCALIZATION AND FLUORESCENT MICROSCOPY

For studies of the subcellular localization in brachypodium protoplasts, *BdCOPT3*, and *BdCOPT4* cDNAs lacking the stop codon were fused at the C-terminus to the enhanced green fluorescent protein (EGFP) of the SAT6-N1-EGFP-Gate vector and expressed under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. The resulting $35S_{pro}$*-BdCOPT-EGFP* constructs or SAT6-N1-EGFP-Gate lacking cDNA inserts were transfected into brachypodium protoplasts as described above. Plasma membranes were stained with 50 μM FM4-64 as described (Ueda et al., 2001).

For studies of subcellular localization in *S. cerevisiae*, entry clones containing *BdCOPT1*, *BdCOPT3*, *BdCOPT4*, or *BdCOPT5* cDNAs without the stop codon were fused at C-terminus with EGFP in the YES3-EGFP-Gate vector (Jung et al., 2012). The resulting constructs and the empty YES3-EGFP-Gate vector were transformed into *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ triple mutant using the Frozen-EZ yeast Transformation II Kit (Zymo Research) and transformants were selected on YNB-Ura medium, as described above. Subcellular localization was assessed in cells grown overnight in liquid YNB-Ura.

EGFP- and FM4-64- mediated fluorescence, and chlorophyll autofluorescence were visualized using FITC (for EGFP) or rhodamine (FM4-64 and chlorophyll) filter sets of the Axio Imager M2 microscope equipped with the motorized Z-drive (Zeiss). Z-stack (1.3 μm-thick) images were collected with the high-resolution AxioCam MR Camera and then 3D deconvoluted using an inverse filter algorithm of the Zeiss AxioVision 4.8 software. Images were processed using the Adobe Photoshop software package, version 12.0.

SPLIT-UBIQUITIN MEMBRANE YEAST TWO-HYBRID SYSTEM (MYTH)

Vectors and *S. cerevisiae* strains THY.AP4 (*MATa leu2-3,112 ura3-52 trp1-289 lexA::HIS3 lexA::ADE2 lexA::lacZ*) and THY.AP5 (*MATα URA3 leu2-3,112 trp1-289 his3-*Δ*1 ade2*Δ*::loxP*) for MYTH were obtained from the Frommer lab (Stanford University) depository at *Arabidopsis* Biological Resource Center (ABRC) http://www.arabidopsis.org/abrc/index.jsp. AtCOPT6-Cub-PLV and AtCOPT6-NubG fusions were previously described (Jung et al., 2012). Full-length *BdCOPT1, BdCOPT3, BdCOPT4*, and *BdCOPT5* cDNAs without stop codons were introduced into the MetYC-dest and pXN-dest22-3HA destination vectors by Gateway cloning (Invitrogen) to generate bait, BdCOPT-CubPLV, and prey, BdCOPT-NubG, constructs in THY.AP4 and THY.AP5 strains, respectively. In all cases, C- and N terminal fragments of ubiquitin were placed at the C-terminus of the BdCOPT proteins. Protein interactions were selected in diploid cells after 2 days of growth on SC medium lacking adenine and histidine. Interactions were verified using β-galactosidase assays, as detailed in Kittanakom et al. (2009).

CONSTRUCTION OF PHYLOGENETIC TREE

The phylogenetic tree was built using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in units of the number of amino acid differences per sequence. The analysis involved 22 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 75 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011) with *A. thaliana* IRT1 as an outgroup.

ACCESSION NUMBERS

Accession numbers for genes used in this study were according to nomenclature from ARAMEMNON 7.0 (http://aramemnon.botanik.uni-koeln.de/) and MIPS (http://mips.helmholtz-muenchen.de/plant/brachypodium/): *Bradi1g24180* (*BdCOPT1*), *Bradi1g24190* (*BdCOPT2*), *Bradi2g51210* (*BdCOPT3*), *Bradi4g31330* (*BdCOPT4*), *Bradi5g09580* (*BdCOPT5*), *Bradi1g10630.1* (*BdACTIN*), and *At2g26975* (*AtCOPT6*).

RESULTS

THE PREDICTED COPT TRANSPORTERS OF BRACHYPODIUM SHARE CONSERVED FEATURES OF THE CTR/COPT FAMILY

To identify putative members of the CTR/COPT family in brachypodium, we used the Plant Membrane Protein database, ARAMEMNON 7.0 (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al., 2003), which has the most complete annotation of putative CTR/COPT transporters based on amino acid similarity and motif organization of CTR/COPT proteins in different species. We found that the brachypodium genome possesses five genes that encode putative CTR/COPT transporters *Bradi1g24180*, *Bradi1g24190*, *Bradi2g51210*, *Bradi4g31330*, *Bradi5g09580,* designated *COPT1* through *COPT5* (*alias BdCOPT1-BdCOPT5*) (**Table 1**). *BdCOPT1* and *BdCOPT2* are located on chromosome 1, while *BdCOPT3*, *BdCOPT4*, and *BdCOPT5* are found on chromosome 2, 4, and 5, respectively (**Table 1**). Based on the *BdCOPT* gene structures in the ARAMEMNON 7.0 database, *BdCOPT1*, *BdCOPT3, BdCOPT4*, and *BdCOPT5* lack introns, similar to *COPT* genes in rice (Yuan et al., 2011) and *A. thaliana* (http://arabidopsis.org). In contrast, *BdCOPT2* possesses two introns (**Figure 1A**).

BdCOPTs share 16–69% amino acid sequence identity and 26–73% sequence similarity to each other (**Table 2**). Phylogenetic analysis revealed that a majority of CTR/COPT proteins from brachypodium and *O. sativa* cluster together and are separate from CTR/COPTs from *A. thaliana* (**Figure 1B**). One exception is BdCOPT4, which forms a separate cluster along with AtCOPT5, OsCOPT7, and OsCOPT6 (**Figure 1B**). BdCOPT4 is

Table 1 | The proposed nomenclature and accession numbers of putative brachypodium COPT transporters annotated at different databases.

Suggested nomenclature	MIPS/ ARAMEMNON	NCBI	UniPROT	Chromosome
BdCOPT1	*Bradi1g24180*	*not annotated*	*I1GT99*	I
BdCOPT2	*Bradi1g24190*	*not annotated*	*I1GTA0*	I
BdCOPT3	*Bradi2g51210*	*XP_003569917.1*	*I1HS28*	II
BdCOPT4	*Bradi4g31330*	*XP_003578182.1*	*I1IQG8*	IV
BdCOPT5	*Bradi5g09580*	*not annotated*	*I1IXK4*	V

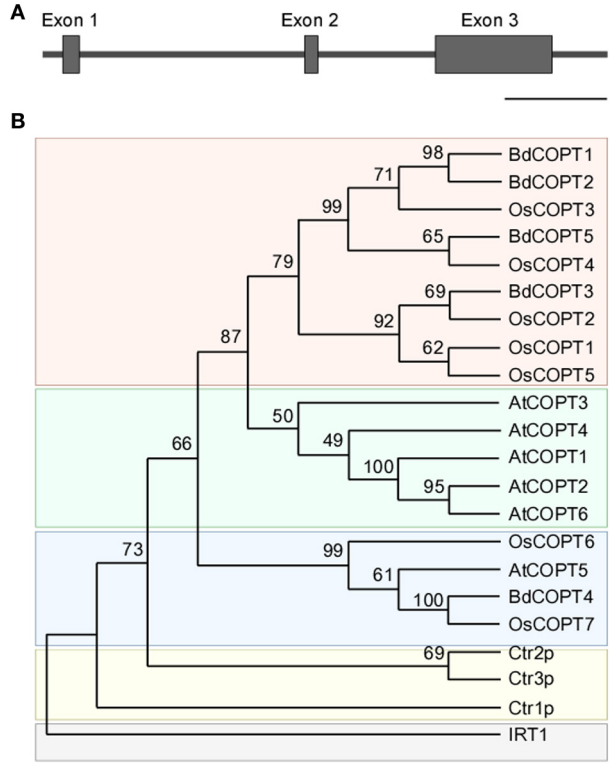

FIGURE 1 | Gene structure of *BdCOPT2* and phylogenetic analysis of the brachypodium COPT family. (A) Gene structure of *BdCOPT2*. Note the presence of introns, which are absent in other plant CTR/COPT members. Scale bar = 500 bp. **(B)** A phylogenetic tree of CTR/COPT members of *A. thaliana* (designated as AtCOPT1-6), *O. sativa* (designated as OsCOPT1-7), brachypodium (designated as BdCOPT1-5), and *S. cerevisiae* (designated as Ctr1p-CTR3p). The *A. thaliana* protein, IRT1, is included as an outgroup.

highly similar (65%) to AtCOPT5, whereas other BdCOPT proteins share 38–54% similarity to their counterparts in *A. thaliana* (**Table 3**). Similar to *OsCOPT* members (Yuan et al., 2011), the nucleotide sequence of *BdCOPT* genes is GC-rich, ranging from GC content of 67.9% (*BdCOPT2*) to 73.2% (*BdCOPT3*), in contrast to an average GC content of 50% in *A. thaliana COPT*s.

CTR/COPT family members possess conserved structural features that include three putative transmembrane helices (TMs), the N- and C-termini located toward the extracellular space and cytosol respectively, N-terminally-located methionine-rich motifs (Mets motifs), and M*XXX*M-X_{12}-G*XXX*G motifs located within TM2 and TM3, respectively (Puig et al., 2002; De Feo et al., 2007, 2009; Peñarrubia et al., 2010). Importantly, M*XXX*M motifs of TM2 in Ctr1p of *S. cerevisiae*, and COPT2 and COPT6 of *A. thaliana* contain a positionally conserved Met residue that is essential for the ability of these proteins to transport Cu (Puig et al., 2002; Jung et al., 2012; Gayomba et al., 2013). Some CTR/COPT proteins also contain the C-terminal cysteine-rich C*X*C motif, which is suggested to bind Cu ions for transfer to cytosolic Cu chaperones, or to downregulate Ctr1p activity in response to toxic Cu levels (Puig et al., 2002; Xiao et al., 2004; Wu et al., 2009). This motif is present in *A. thaliana* COPT1 and COPT2, but is absent in COPT6; nevertheless, COPT6 is a functional Cu transporter (Jung et al., 2012).

Table 2 | Percentage of amino acid identity (similarity) among putative COPT proteins in brachypodium.

	BdCOPT1	BdCOPT2	BdCOPT3	BdCOPT4	BdCOPT5
BdCOPT1	100 (100)	69 (73)	40 (48)	21 (30)	43 (51)
BdCOPT2	–	100 (100)	34 (42)	16 (26)	38 (46)
BdCOPT3	–	–	100 (100)	27 (35)	42 (50)
BdCOPT4	–	–	–	100 (100)	31 (42)
BdCOPT5	–	–	–	–	100 (100)

Computer algorithm-assisted analysis of membrane topology and motif organization in brachypodium CTR/COPT proteins revealed that all BdCOPTs, except for BdCOPT3, are predicted to contain the classical three TMs (**Figure 2**) with N-terminal domains oriented toward the extracellular space, while C-terminal domains are predicted to be located in the cytosol. In contrast, BdCOPT3 is predicted to have two transmembrane domains, with both N- and C-termini located in the cytosol. While polypeptides of all BdCOPT included the N-terminal Mets motifs, the distribution of M*XXX*M-X_{12}-G*XXX*G motifs varied within different BdCOPT polypeptides (**Figure 2**). For example, M*XXX*M motifs and thus, positionally conserved essential Met residues, were located within TM2 in BdCOPT3, BdCOPT4, and BdCOPT5, but outside of TM2 in BdCOPT1 and BdCOPT2. Although the predicted membrane topology of BdCOPT proteins has to be validated experimentally, the location of essential positionally conserved Met residues of the M*XXX*M motif outside of the predicted membrane domain suggest that BdCOPT1 and BdCOPT2 might not mediate Cu transport. The C-terminal C*X*C motif is present in BdCOPT3 as a CC motif and in BdCOPT4 as a C*X*C motif, but is absent in BdCOPT1, BdCOPT2, and BdCOPT5 (**Figure 2**).

BdCOPT3 AND BdCOPT4 RESPOND TRANSCRIPTIONALLY TO Cu STATUS

To determine whether *BdCOPT* genes respond transcriptionally to Cu status of the plant like their counterparts in *A. thaliana* and *O. sativa*, we first developed growth conditions under which Cu limitation would minimally affect the growth and development

Table 3 | Amino acid length (aa length), molecular mass (shown in kDa), and percentage of amino acid identity (similarity) of putative brachypodium COPT (BdCOPT1-5) transporters in comparison to their homologs in *A. thaliana* (AtCOPT1-6).

	aa length/kDa	AtCOPT1	AtCOPT2	AtCOPT3	AtCOPT4	AtCOPT5	AtCOPT6
BdCOPT1	183/18.8	40.0 (47.1)	36.7 (47.5)	38.4 (49.0)	34.5 (43.4)	28.1 (41.8)	42.8 (54.5)
BdCOPT2	214/22.5	35.9 (44.7)	34.2 (45.6)	36.4 (47.0)	33.1 (46.2)	26.0 (38.4)	41.4 (53.8)
BdCOPT3	162/16.6	41.4 (53.7)	40.5 (53.8)	36.4 (48.3)	34.5 (48.3)	30.8 (40.4)	43.4 (53.8)
BdCOPT4	151/15.8	31.1 (45.0)	29.8 (44.4)	29.1 (42.4)	26.9 (38.6)	57.5 (65.1)	31.0 (44.1)
BdCOPT5	159/16.3	42.1 (51.6)	38.0 (46.2)	39.7 (51.0)	34.5 (45.5)	28.1 (39.7)	40.7 (52.4)

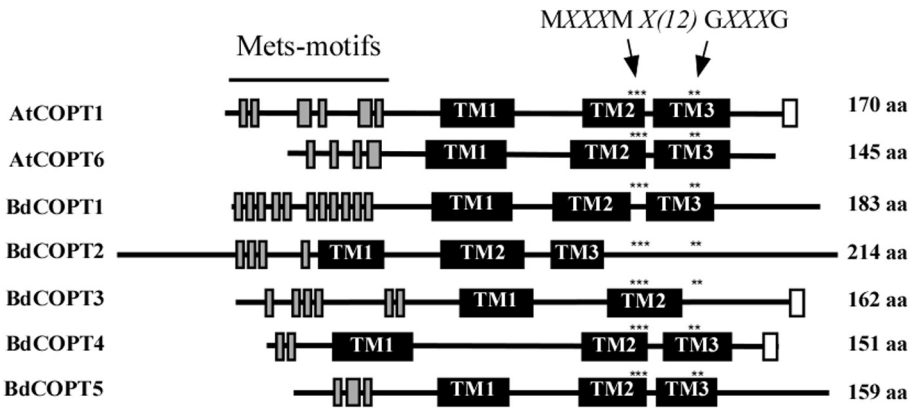

FIGURE 2 | A diagram showing conserved motifs within the primary sequence of BdCOPT proteins and their homologs in *A. thaliana*, AtCOPT1 and AtCOPT6. Topology predictions are based on the TMHMM software, version 1.0. Shown are methionine-rich motifs within the predicted N-terminal domain (Mets-motifs, gray bars), transmembrane domains (TM1, TM2, and TM3; black bars) and cysteine-rich motifs within the predicted C-terminal domain (white bars). Asterisks "***" and "**" indicate the location of conserved M*XXX*M and G*XXX*G motifs. Note that BdCOPT3 has a unique structure of two transmembrane domains and that BdCOPT3 and BdCOPT4 have cysteine-rich domains in the C-terminal domain.

of brachypodium. Seeds were germinated in perlite irrigated with a modified hydroponic medium supplemented with 0.25 μM $CuSO_4$ (**Supplemental Figure 1** and Methods and Materials) and grown for 7 days before transferring to hydroponic medium to grow further for 18 days under the following conditions: (1) control conditions (0.25 μM $CuSO_4$); (2) Cu limited conditions (0 μM $CuSO_4$); (3) Cu deficiency (0 μM $CuSO_4$ and supplemented with 500 μM of the Cu chelator, bathocuprione disulfonate (BCS); and (4) Cu excess (3 μM $CuSO_4$). While there were no signs of chlorosis in leaves of plants grown under Cu deficiency or Cu excess (**Supplemental Figure 2A**), plants grown under Cu deficiency had decreased height, and shoot and root dry weight compared to plants grown under control conditions (**Supplemental Figure 2B**). Plants grown under Cu excess had decreased shoot and root dry weight, but were of the same height as control plants. Cu limitation did not significantly alter plant growth or shoot and root biomass (**Supplemental Figure 2B**).

We then analyzed the steady-state levels of *BdCOPT* mRNAs in different plant tissues of brachypodium grown under the Cu regimes described above. Expression studies in roots (**Figure 3A**) revealed that Cu limitation increased mRNA expression of *BdCOPT3* and *BdCOPT4*. Cu deficiency increased *BdCOPT3* and *BdCOPT4* expression even further and, in addition, increased expression of *BdCOPT1*. There were no statistically significant differences in mRNA expression levels of *BdCOPT2* and *BdCOPT5* in root tissue of plants grown under these conditions (**Figure 3A**). In roots of plants grown under Cu toxicity, *BdCOPT4* was the only gene whose expression was responsive to this treatment (**Figure 3A**). In young leaves (**Figure 3B**), Cu limitation elevated the abundance of *BdCOPT3* and *BdCOPT4* transcripts, which increased even further under Cu deficiency. In young leaves of plants grown under Cu toxicity, expression of *BdCOPT4* decreased, but expression of *BdCOPT2* increased. Expression studies in old leaves (**Figure 3C**) of plants grown in Cu limited or Cu deficient conditions showed an increase of the abundance of *BdCOPT3* and *BdCOPT4* transcripts. In contrast, both, Cu toxic and Cu limited conditions, increased expression of *BdCOPT1*, while expression of *BdCOPT2* and *BdCOPT5* were downregulated under Cu deficiency.

To summarize, of five *BdCOPT* genes, *BdCOPT3*, and *BdCOPT4*, are the most responsive to fluctuations in Cu availability, and changes in their expression are most prominent in roots and young leaves. *BdCOPT2* is significantly upregulated under Cu toxicity only in young leaves, while *BdCOPT1* is regulated by Cu mainly in old leaves. Expression of *BdCOPT5* was the least responsive to Cu availability and was downregulated only by Cu deficiency and only in old leaves.

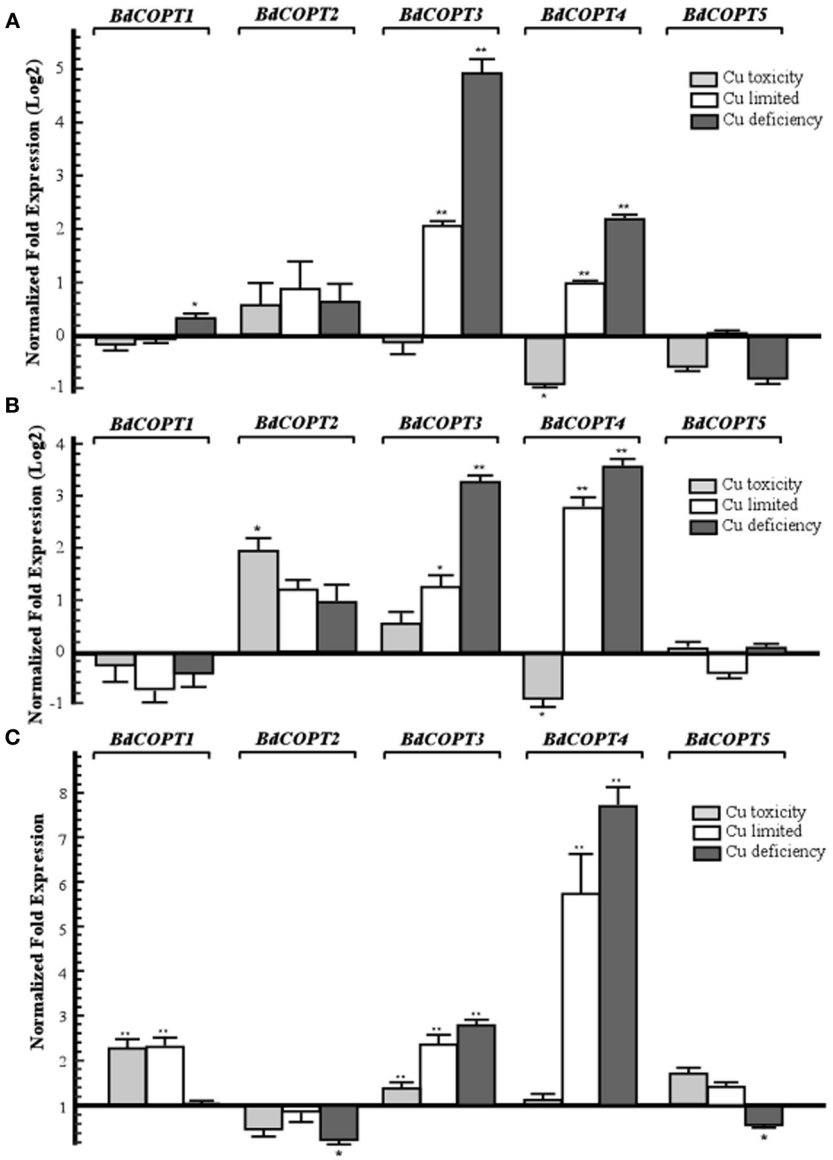

FIGURE 3 | Quantitative real-time (qRT)-PCR analysis of the effect of Cu on expression *COPT1* through *5* in roots (A), young leaves (B), and older leaves (C) of brachypodium. For all treatments, 7-day-old wild-type seedlings were transferred to hydroponic solution and were grown for 18 days in medium that, in addition to macro- and micronutrients, contained either 0.25 μM $CuSO_4$ (control conditions), 0 μM $CuSO_4$ (Cu limited), 0 μM $CuSO_4$ + 500 μM BCS (Cu deficiency), or 3 μM $CuSO_4$ (Cu toxicity). Error bars show SE ($n = 9$). Differences of the mean values between control and treated plants are indicated as $^*p \leq 0.05$ or $^{**}p \leq 0.001$. Results are presented relative to the expression of genes under control conditions.

HETEROLOGOUSLY EXPRESSED *BdCOPT3, BdCOPT4,* AND *BdCOPT5* PARTIALLY RESCUE GROWTH DEFECTS OF THE *S. CEREVISIAE CTR1*Δ *CTR2*Δ *CTR3*Δ CU UPTAKE MUTANT ON NON-FERMENTABLE GROWTH MEDIUM

We next tested whether BdCOPT proteins are involved in Cu transport. In this regard, the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ mutant, which lacks high-affinity plasma membrane-localized Cu uptake transporters Ctr1p and Ctr3p, and the vacuolar membrane-localized Ctr2p, is deficient in Cu uptake and release of Cu from the vacuole, and has been successfully used to identify Cu transport capabilities of CTR/COPT transporters from higher plants (Kampfenkel et al., 1995; Barhoom et al., 2008; Yuan et al., 2010, 2011; Garcia-Molina et al., 2011; Klaumann et al., 2011; Jung et al., 2012; Gayomba et al., 2013). Analyses of Cu transport capabilities using yeast as a heterologous system is based on the fact that cells lacking functional Cu uptake systems are unable to deliver Cu to cytochrome *c* oxidase in the mitochondrial respiratory chain, preventing cell growth on non-fermentable carbon sources such as glycerol and ethanol (YPEG medium), unless high concentrations of exogenous Cu are added to the growth medium (Dancis et al., 1994b; Glerum et al., 1996). Therefore, we expected that if BdCOPT proteins act as high-affinity Cu transporters, then their expression in the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ mutant would promote Cu uptake

and suppress growth defects of the mutant on non-fermentable medium.

Here, we focused on analyses of BdCOPT1, BdCOPT3, BdCOPT4, and BdCOPT5 due to difficulty in cloning of BdCOPT2. As a positive control, we used the previously characterized CTR/COPT transporter from *A. thaliana*, AtCOPT6 (Jung et al., 2012). We found that all yeast lines grew at the same rate on standard medium (YNB-Ura) supplied with glucose as a carbon source or on YPEG medium supplemented with 100 μM $CuSO_4$ (**Figure 4**). In contrast, only the empty vector-expressing wild-type cells and *ctr1*Δ*ctr2*Δ*ctr3*Δ cells expressing *AtCOPT6* grew well on YPEG medium (**Figure 4**), suggesting that none of BdCOPTs tested were able to rescue the growth defect of the *S. cerevisiae* mutant on YPEG medium. However, addition of a low concentration of Cu (10 μM $CuSO_4$) to YPEG medium allowed *BdCOPT3* (**Figure 4B**), *BdCOPT4* (**Figure 4C**), and *BdCOPT5* (**Figure 4D**) but not *BdCOPT1* (**Figure 4A**) to complement partially the growth defect of *ctr1*Δ*ctr2*Δ*ctr3*Δ cells. These results suggest that transport capabilities of brachypodium COPT transporters differ from their counterparts in *A. thaliana*.

BdCOPT3 AND BdCOPT4 LOCALIZE TO THE PLASMA MEMBRANE IN *S. CEREVISIAE* CELLS AND BRACHYPODIUM PROTOPLASTS

S. cerevisiae Ctr1p and Ctr3p localize to the plasma membrane and contribute to Cu uptake into the cell (Dancis et al., 1994a; Peña et al., 2000), while Ctr2p localizes to the vacuolar membrane and remobilizes Cu from this internal store upon Cu deficiency (Rees et al., 2004). To determine whether BdCOPTs rescue growth defects of the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ mutant by facilitating Cu uptake into the cell from the external medium or by vacuolar remobilization, we determined their subcellular localization in yeast cells as well as in brachypodium protoplasts.

For studies in *S. cerevisiae*, we inserted *BdCOPT3*, *BdCOPT4*, and *BdCOPT5* cDNAs without the stop codon into the YES3-EGFP-Gate vector to generate translational C-terminal EGFP fusions. We then verified whether the BdCOPT-EGFP constructs were functional by expressing them in the *ctr1*Δ*ctr2*Δ*ctr3*Δ mutant and assessing the growth of transformed cells on YPEG

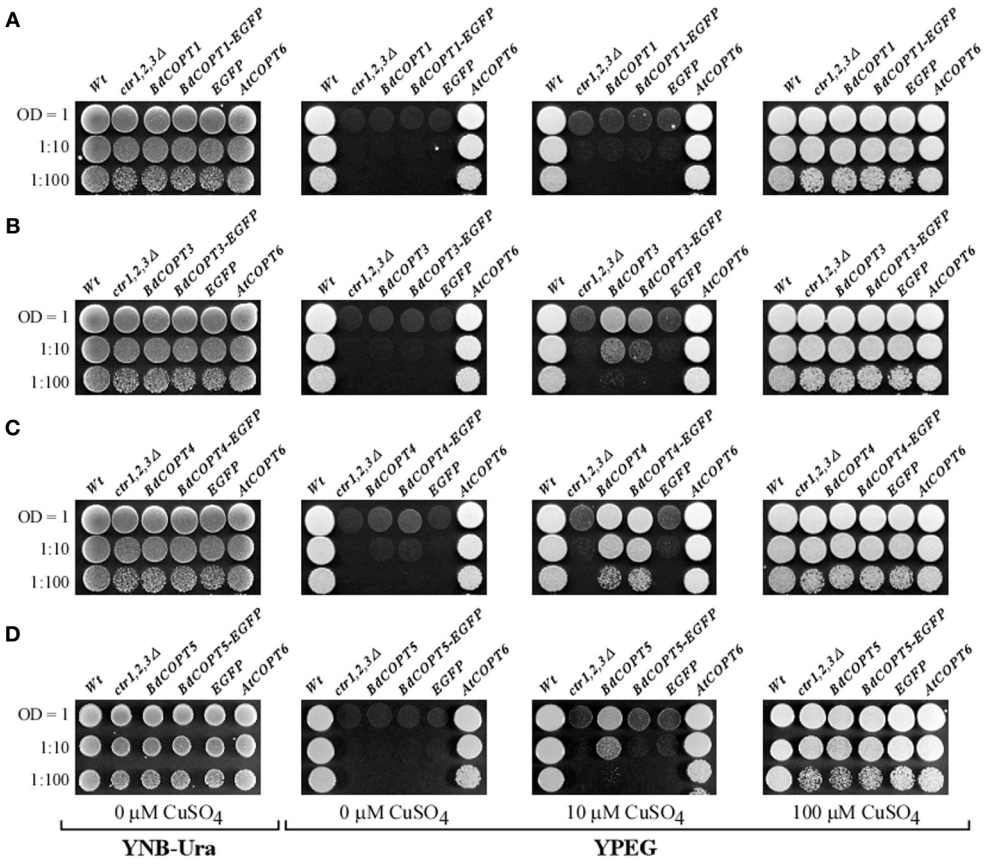

FIGURE 4 | *BdCOPT3, BdCOPT4,* and *BdCOPT5* rescue the growth defect of the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ triple mutant on ethanol/glycerol medium (YPEG). The *ctr1*Δ*ctr2*Δ*ctr3*Δ mutant was transformed with the YES3-Gate vector harboring *BdCOPT1* **(A)**, *BdCOPT3* **(B)**, *BdCOPT4* **(C)**, and *BdCOPT5* **(D)** along with corresponding EGFP-fusions and spotted onto YPEG plates supplemented with the indicated concentrations of $CuSO_4$. As negative controls, the *ctr1*Δ*ctr2*Δ*ctr3*Δ mutant strain was transformed with the empty YES3-Gate (*ctr1,2,3*Δ) or empty YES3-EGFP-Gate vector (*EGFP*). The isogenic wild-type, SEY6210, transformed with the empty YES3-Gate vector (*Wt*) and *ctr1*Δ*ctr2*Δ*ctr3*Δ cells transformed with YES3-Gate harboring the *A. thaliana COPT6* (*AtCOPT6*) cDNA insert were used as positive controls.

media. We found that expression of BdCOPT3-, and BdCOPT4-EGFP fusions in *ctr1*Δ*ctr2*Δ*ctr3*Δ cells resulted in growth phenotypes mirroring results of cells expressing un-tagged proteins (**Figures 4B,C**). However, the BdCOPT5-EGFP construct was unable to rescue growth of *ctr1*Δ*ctr2*Δ*ctr3*Δ cells, suggesting that fusing EGFP with BdCOPT5 resulted in the loss of its activity (**Figure 4D**). Since unfunctional BdCOPT5-EGFP might mislocalize in yeast cells as well as in brachypodium protoplasts, this construct was omitted from subsequent study. We did not analyze the subcellular localization of BdCOPT1 for the same reason. Fluorescent microscopy revealed that EGFP-mediated fluorescence in BdCOPT3-EGFP and BdCOPT4-EGFP expressing cells localized mainly to the cell periphery and the distribution of the EGFP signal was distinct in *S. cerevisiae* cells expressing the EGFP-only vector (**Figure 5**). These data suggest that BdCOPT3 and BdCOPT4 localize to the plasma membrane in this heterologous system.

We next analyzed the subcellular localization of BdCOPT3 and BdCOPT4 in brachypodium using transient expression in protoplasts. After establishing a procedure for the isolation of viable protoplasts from brachypodium (**Figure 6**), we transfected protoplasts with BdCOPT3-EGFP or BdCOPT4-EGFP constructs with C-terminal EGFP fusions expressed from the CaMV promoter of the SAT-N1-EGFP-Gate vector, or with the empty SAT-N1-EGFP-Gate vector. We found that EGFP-mediated fluorescence originating from BdCOPT3-EGFP or BdCOPT4-EGFP constructs was located at the periphery of transfected protoplasts and did not overlap with chlorophyll-mediated autofluorescence (**Figures 7A,B** and **Supplemental Figures 3A,B**). Furthermore, fluorescence from BdCOPT3- and BdCOPT4-EGFP constructs was distinct from the fluorescence pattern exhibited by protoplasts transfected with the empty EGFP vector (**Figure 7C** and **Supplemental Figure 3C**). We note that internally-localized EGFP-mediated fluorescence is likely an artifact of the degradation of the BdCOPT-EGFPs. We then co-stained protoplasts expressing BdCOPT3- or BdCOPT4-EGFP with the lipophylic dye, FM4-64, which selectively labels the plasma membrane under low-temperature conditions (Vida and Emr, 1995). We found strict co-localization of BdCOPT3- or BdCOPT4-EGFP and FM4-64-mediated fluorescence (**Figures 7A,B**), suggesting that BdCOPT3 and BdCOPT4 are located at the plasma membrane.

BdCOPT PROTEINS INTERACT WITH EACH OTHER IN YEAST-TWO-HYBRID ASSAYS

Homo- and heterodimerization of CTR/COPT proteins have been demonstrated in eukaryotes (Lee et al., 2002; De Feo et al.,

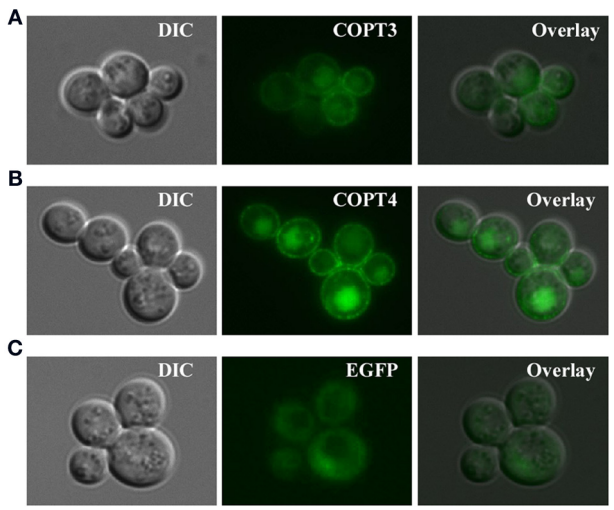

FIGURE 5 | Subcellular localization of BdCOPT3-EGFP (A) or BdCOPT4-EGFP (B) fusions or EGFP (C) in *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ cells. Superimposed images (Overlay) from differential interference contrast microscopy (DIC) and EGFP-mediated fluorescence (EGFP) show that BdCOPT3 and BdCOPT4 localize to the plasma membrane and that the pattern of fluorescence of EGFP-fused proteins is distinct from EGFP.

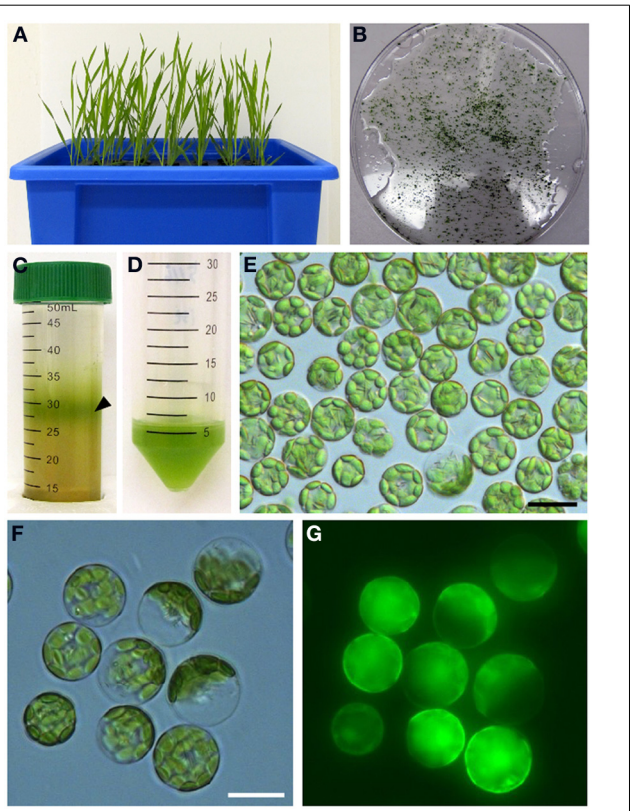

FIGURE 6 | Isolation of protoplasts from brachypodium. Hydroponically grown 25-day-old plants **(A)** were used for the isolation of protoplasts from leaf tissue. Chopped brachypodium leaves in filter-sterilized TVL solution are shown in **(B)**. Enzymatic digestion of the cell wall and fractionation by sucrose density gradient yielded protoplasts at the interface of the enzyme solution and W5 solution (**C**, black arrow). Protoplasts were collected and purified from sucrose density gradient solution **(D)** and visualized under microscopy using bright-field filter sets **(E)**. In our method, 0.2 g of leaf tissue from 25-day-old seedlings yields 5×10^6–10^7 protoplasts. Close-up of brachypodium protoplasts through bright-field **(F)** and FITC **(G)** filter sets to assess protoplast viability after staining with the membrane-permeable non-fluorescent dye, fluorescein diacetate. After diffusion into viable protoplasts fluorescein diacetate is hydrolyzed into a polar compound, causing the cytosoplasm of the cell to fluoresce under the FITC filter set. Scale bar = 20 μm.

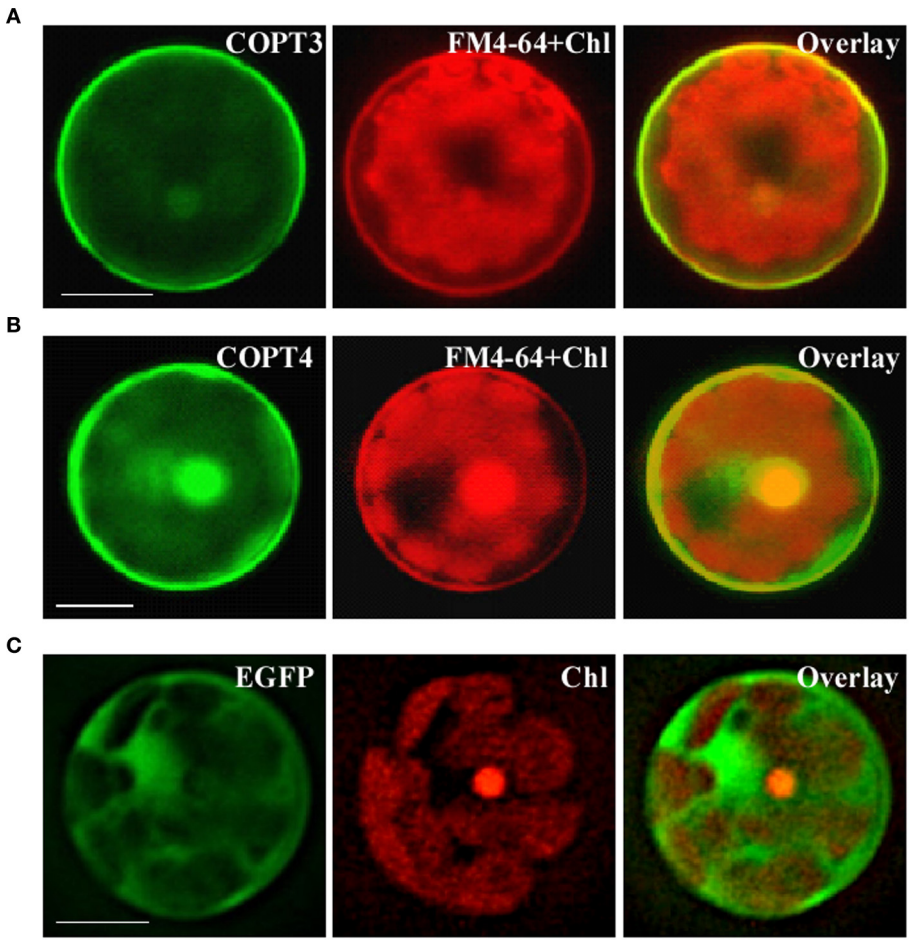

FIGURE 7 | Subcellular localization of BdCOPT3 and BdCOPT4 in brachypodium protoplasts. Protoplasts isolated from leaves of 25-day-old plants were transfected with BdCOPT3-EGFP **(A)** or BdCOPT4-EGFP **(B)** constructs or the empty SAT6-N1-EGFP vector **(C)** and co-stained with the plasma-membrane dye, FM4-64. EGFP-mediated fluorescence derived from BdCOPT3-EGFP (COPT3) or BdCOPT4-EGFP, (COPT4), or from EGFP of the SAT6-N1-EGFP vector (EGFP) was detected using the FITC filter set while FM4-64 (FM4-64) and chlorophyll autofluorescence (Chl) were visualized using the Rhodamine filter set of an Axio Imager M2 microscope equipped with a motorized Z-drive (Zeiss). Images collected from FITC and Rhodamine filter sets were overlaid (Overlay) to show the plasma membrane subcellular localization of Cu transporters. Scale bar = 10 μm.

2007, 2009; Yuan et al., 2010, 2011). Furthermore, although interaction of *A. thaliana* COPT6 with COPT1 is not required for the ability of these proteins to transport Cu (Jung et al., 2012), the activity of *O. sativa* CTR/COPT proteins seem to depend on their interactions with each other (Yuan et al., 2011). Therefore, we tested if the CTR/COPT family members in brachypodium would also interact with either themselves or/and with other family members. For this purpose we used the split-ubiquitin-based membrane yeast-two-hybrid (MYTH) approach (Kittanakom et al., 2009). In this system, a modified ubiquitin protein is split into its C- and N-terminal halves (Cub and NubG, respectively), which are fused to membrane-localized bait or prey proteins, respectively. The C-terminus of ubiquitin is attached to an artifical transcription factor, PLV (protein A-LexA-VP16). If bait and prey proteins are oriented in the cytosol and interactions occur, the modified ubiquitin is reconstituted and recognized by ubiquitin-specific proteases, which release PLV from Cub. PLV enters the nucleus to induce expression of *lexA*-controlled reporter genes *ADE2*, *HIS3,* and *lacZ* (Kittanakom et al., 2009), allowing protein interactions to be assessed by adenine and histidine prototrophy and by the β-galactosidase assay.

We fused Cub-PLV and NubG at the C-terminus of BdCOPT1, BdCOPT3, BdCOPT4, and BdCOPT5 since their predicted topology is consistent with a cytosolic orientation of their C-termini. We then co-expressed BdCOPT proteins in different combinations with themselves or with the empty Cub-PLV vector as a negative control (**Figure 8**). We included *S. cerevisiae* co-expressing AtCOPT6-Cub-PLV and AtCOPT6-NubG in our assays as a positive control since AtCOPT6 interacts with itself in the MYTH assay (Jung et al., 2012). To control for false positives, the empty Cub-PLV vector was co-expressed with BdCOPTs fused to NubG. These studies showed that all of the tested BdCOPT proteins interacted in both homo- and heterodimer combinations, regardless of whether interactions were assayed by growth on selective medium or with β-galactosidase (**Figure 8**). Whether these

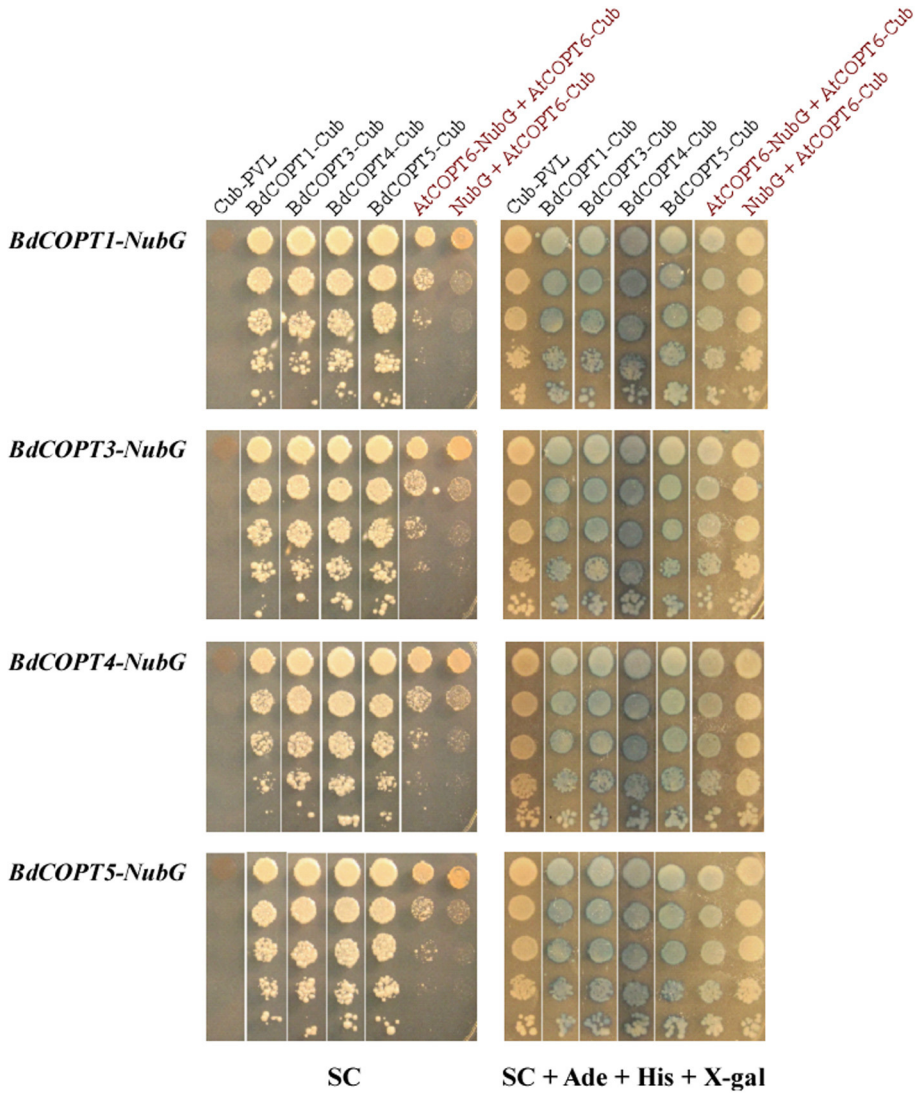

FIGURE 8 | Analyses of protein-protein interactions of brachypodium CTR/COPT transporters using the split-ubiquitin membrane yeast two-hybrid system (MYTH). Shown are yeast cells co-expressing NubG constructs fused with *BdCOPT1*, *BdCOPT3*, *BdCOPT4*, or *BdCOPT5* cDNA (*BdCOPT1-, BdCOPT3-, BdCOPT4-, BdCOPT5-NubG*) and a Cub-PLV construct lacking a cDNA insert (*Cub-PLV*) or Cub-PLV fused to *BdCOPT1-*, *BdCOPT3-*, *BdCOPT4-*, *BdCOPT5* (*BdCOPT1-, BdCOPT3-, BdCOPT4-, BdCOPT5-Cub*). Interactions were visualized by colony formation on selective media SC medium (SC) or as blue colonies in a ß-galactosidase assay (SC + Ade + His + X-gal). Growth was monitored for 2 days under conditions indicated below each panel. Interactions of AtCOPT6 with itself (*AtCOPT6-NubG* + *AtCOPT6-Cub*), or lack of interactions between *AtCOPT6-Cub-PLV* and the empty NubG vector (*NubG* + *AtCOPT6-Cub*) were used as controls and are indicated in red. Shown are representative results of at least three biological replicates. SC, synthetic complete medium; Ade, adenine; His, histidine; X-gal, bromo-chloro-indolylgalactopyranoside.

interactions are required for their ability to transport Cu remains to be elucidated.

DISCUSSION

Biological attributes of brachypodium, such as root architecture, grain structure, and the continued development of molecular and genetic resources prompted us to establish this plant as a preferred model for Cu homeostasis studies in grain cereals (e.g., wheat, barley, and oat) which are reported to be more sensitive to Cu availability in agricultural soils in comparison to other crops (Shorrocks and Alloway, 1988; Solberg et al., 1999). To begin investigations of the underlying molecular basis of this phenomenon, we initiated studies of the brachypodium CTR/COPT Cu transporters since members of this family in *A. thaliana* provide an entry point for Cu into the root and are suggested to contribute to subsequent Cu partitioning in photosynthetic tissues (Burkhead et al., 2009; Merchant, 2010; Ravet and Pilon, 2013). Toward this goal, we have identified five putative CTR/COPT family members in brachypodium based on amino acid similarity and motifs organization of CTR/COPT proteins in different species and we classified them as BdCOPT1 through 5. Phylogenetic analyses

of the predicted CTR/COPT members in brachypodium and their counterparts from a dicot, *A. thaliana*, and a monocot, *O. sativa*, show that, with the exception of BdCOPT4, brachypodium and *O. sativa* CTR/COPT proteins are more closely related to each other than to *A. thaliana* (**Figure 1B**), reflecting a closer evolutionary relationship of brachypodium to *O. sativa* than to *A. thaliana*. Furthermore, analysis of membrane topology and the motif organization in brachypodium CTR/COPT proteins revealed that while polypeptides of all BdCOPT included the N-terminal Mets motifs, the location of M*XXX*M-X_{12}-G*XXX*G motifs varies within different BdCOPT polypeptides, affecting the location of the positionally conserved Met residue, which is essential for the translocation of Cu across lipid bilayer (**Figure 2**). For example, M*XXX*M motifs are predicted to be located within TM2 only in BdCOPT3, BdCOPT4, and BdCOPT5, similar to CTR/COPT family members in other species. In contrast, M*XXX*M motifs are predicted to be located outside of TM2 in BdCOPT1 and BdCOPT2, suggesting that these proteins might not be able to mediate Cu transport due to a shift in the position of the essential Mets motifs. Although the topology predictions have to be validated experimentally, the ability of BdCOPT3, BdCOPT4, and BdCOPT5, but not BdCOPT1, to rescue partially the Cu-deficiency associated respiratory defects of the *S. cerevisiae ctr1*Δ*ctr2*Δ*ctr3*Δ mutant (**Figure 4**) was consistent with this suggestion. We also note that BdCOPT3, BdCOPT4, and BdCOPT5 conferred growth to the yeast mutant only when a low concentration of Cu was added to the growth medium (**Figure 4**). This result might be interpreted using at least two not mutually exclusive scenarios: (1) BdCOPT3, BdCOPT4, and BdCOPT5 are low affinity Cu transporters, unlike their high-affinity counterparts from *A. thaliana* and/or (2) in order to confer high affinity transport, BdCOPT3, BdCOPT4, and/or BdCOPT5 must interact with other CTR/COPTs and/or other transporters. In this regard it has been shown that most of CTR/COPTs in *O. sativa* form dimers/trimers with other CTR/COPTs or other proteins to function as high-affinity transporters (Yuan et al., 2010, 2011). In contrast, the plasma membrane-localized CTR/COPT family members from *A. thaliana* are high-affinity transporters by themselves even though can form hetero-complexes (Nakagawa et al., 2010; Jung et al., 2012; Gayomba et al., 2013). We found that brachypodium CTR/COPT transporters homo- and heterooligomerize in the MYTH system (**Figure 8**); whether these interactions are required for transport capabilities of brachypodium CTR/COPTs has yet to be determined.

We have also examined the subcellular localization of BdCOPTs by analyzing the localization pattern of functional BdCOPT3-EGFP and BdCOPT4-EGFP constructs by heterologous expression in yeast or transient expression in brachypodium protoplasts. Toward this goal we have established procedures for preparing viable protoplasts from brachypodium mesophyll cells and for protoplast transfection (**Figure 6**). These studies showed that both, BdCOPT3-EGFP and BdCOPT4-EGFP, localize to the plasma membrane, regardless of whether assays were done in yeast or protoplasts (**Figures 5**, **7**), further suggesting that these two CTR/COPT proteins may function in Cu uptake. Finally, BdCOPT3 and BdCOPT4 genes were highly expressed in roots, and old and young leaves, and their expression was tightly regulated by Cu availability (**Figure 3**), as was shown for other CTR/COPT proteins from different species, including plants.

To conclude, this manuscript shows that brachypodium can be used for analyses of the molecular mechanisms underlying the increased susceptibility of small grain cereals to Cu deficiency and of Cu homeostasis overall. Analyses of the phylogenetic relationship between CTR/COPT proteins of brachypodium, *O. sativa*, and *A. thaliana*, and the clear divergence of *A. thaliana* CTR/COPT members, suggest that differences may exist in characteristics of the CTR/COPT proteins between monocots and dicots. This is further validated by studies of Cu transport capabilities of brachypodium CTR/COPTs. Heterologous expression of brachypodium CTR/COPTs in the *S. cerevisiae* Cu uptake mutant *ctr1*Δ*ctr2*Δ*ctr3*Δ suggest that increased sensitivity to Cu deficiency in some grass species may arise from lower efficiency of Cu uptake and, possibly, other properties of components of Cu uptake and tissue partitioning systems. It is also possible that the CTR/COPT family members in brachypodium require homo- or heterooligomerization for high-affinity Cu uptake, unlike corresponding family members in *A. thaliana*, reinforcing the importance of using brachypodium as a model for the comprehensive analyses of Cu homeostasis in cereal crops.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00236/abstract

Supplemental Figure 1 | Establishing growth conditions for brachypodium. Plants were grown on standard nutrient solution at 22°C and 12-h light/12-h dark photoperiod at photosynthetic photon flux density of 150 μmol photons m^{-2} s^{-1}. The glume and lemma of seeds were removed and seeds were sown on rinsed perlite **(A)**. Seven-day-old seedlings grown on perlite irrigated with standard nutrient solution **(B)**. Seven-day-old seedlings were transferred from perlite to hydroponic medium and shown from above **(C)** and from the side view **(D)**. Scale bar = 5 cm.

Supplemental Figure 2 | Phenotypes of brachypodium grown under different copper conditions. (A) Twenty five-day-old plants grown in hydroponic solution without $CuSO_4$ and supplemented with 500 μM BCS (*500 BCS*), or indicated concentrations of $CuSO_4$. **(B)** Plants from **(A)** were used to measure height (*Plant height*), shoot dry weight (*Shoot dry weight*), and root dry weight (*Root dry weight*). Asterisks (**) indicate statistically significant differences ($p \leq 0.01$, ANOVA) of the mean values of treatments compared to control conditions (0.25 μM $CuSO_4$). Error bars show *SD* ($n = 3$).

Supplemental Figure 3 | Subcellular localization of BdCOPT3 and BdCOPT4 in brachypodium protoplasts. Protoplasts were transfected with BdCOPT3-EGFP **(A)** or BdCOPT4-EGFP **(B)** constructs or empty SAT6-N1-EGFP vector **(C)** and co-stained with the plasma-membrane dye, FM4-64. EGFP-mediated fluorescence derived from BdCOPT3-EGFP (COPT3) or BdCOPT4-EGFP (COPT4), or from EGFP of the SAT6-N1-EGFP vector (EGFP) was detected using the FITC filter set while FM4-64 (FM4-64) and chlorophyll autofluorescence (Chl) were visualized using the Rhodamine filter set of an Axio Imager M2 microscope equipped with the motorized Z-drive (Zeiss). Images collected from FITC and Rhodamine filter sets were overlaid (Overlay) to show the plasma membrane subcellular localization of the Cu transporters.

Supplemental Table 1 | Primers used in this study. Sequences in italics indicate *att* sites used for Gateway cloning and "*" indicate sequences without a stop codon.

Supplemental Table 2 | Solutions used in protoplast isolation and transfection. All solutions were filter sterilized before use.

REFERENCES

Andres-Colas, N., Perea-Garcia, A., Puig, S., and Peñarrubia, L. (2010). Deregulated copper transport affects *Arabidopsis* development especially in the absence of environmental cycles. *Plant Physiol.* 153, 170–184. doi: 10.1104/pp.110.153676

Arteca, R. N., and Arteca, J. M. (2000). A novel method for growing *Arabidopsis thaliana* plants hydroponically. *Physiol. Plantarum* 108, 188–193. doi: 10.1034/j.1399-3054.2000.108002188.x

Barhoom, S., Kupiec, M., Zhao, X., Xu, J., and Sharon, A. (2008). Functional characterization of CgCTR2, a putative vacuole copper transporter that is involved in germination and pathogenicity in *Colletotrichum gloeosporioides*. *Eukaryot. Cell* 7, 1098–1108. doi: 10.1128/EC.00109-07

Bragg, J. N., Wu, J., Gordon, S. P., Guttman, M. E., Thilmony, R., Lazo, G. R., et al. (2012). Generation and characterization of the western regional research center brachypodium T-DNA insertional mutant collection. *PLoS ONE* 7:e41916. doi: 10.1371/journal.pone.0041916

Brkljacic, J., Grotewold, E., Scholl, R., Mockler, T., Garvin, D. F., Vain, P., et al. (2011). *Brachypodium* as a model for the grasses: today and the future. *Plant Physiol.* 157, 3–13. doi: 10.1104/pp.111.179531

Burkhead, J., Reynolds, K., Abdel-Ghany, S., Cohu, C., and Pilon, M. (2009). Copper homeostasis. *New Phytol.* 182, 799–816. doi: 10.1111/j.1469-8137.2009.02846.x

Chochois, V., Vogel, J. P., and Watt, M. (2012). Application of *Brachypodium* to the genetic improvement of wheat roots. *J. Exp. Bot.* 63, 3467–3474. doi: 10.1093/jxb/ers044

Dancis, A., Haile, D., Yuan, D. S., and Klausner, R. D. (1994a). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J. Biol. Chem.* 269, 25660–25667.

Dancis, A., Yuan, D., Haile, D., Askwith, C., Eide, D., Moehle, C., et al. (1994b). Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell* 76, 393–402. doi: 10.1016/0092-8674(94)90345-X

De Feo, C., Aller, S., and Unger, V. (2007). A structural perspective on copper uptake in eukaryotes. *Biometals* 20, 705–716. doi: 10.1007/s10534-006-9054-7

De Feo, C. J., Aller, S. G., Siluvai, G. S., Blackburn, N. J., and Unger, V. M. (2009). Three-dimensional structure of the human copper transporter hCTR1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4237–4242. doi: 10.1073/pnas.0810286106

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.2307/2408678

Garcia-Molina, A., Andrés-Colás, N., Perea-García, A., del Valle-Tascón, S., Peñarrubia, L., and Puig, S. (2011). The intracellular *Arabidopsis* COPT5 transport protein is required for photosynthetic electron transport under severe copper deficiency. *Plant J.* 65, 848–860. doi: 10.1111/j.1365-313X.2010.04472.x

Garcia-Molina, A., Andres-Colas, N., Perea-Garcia, A., Neumann, U., Dodani, S. C., Huijser, P., et al. (2013). The *Arabidopsis* COPT6 transport protein functions in copper distribution under copper-deficient conditions. *Plant Cell Physiol.* 54, 1378–1390. doi: 10.1093/pcp/pct088

Gayomba, S. R., Jung, H. I., Yan, J., Danku, J., Rutzke, M. A., Bernal, M., et al. (2013). The CTR/COPT-dependent copper uptake and SPL7-dependent copper deficiency responses are required for basal cadmium tolerance in *A. thaliana*. *Metallomics* 5, 1262–1275. doi: 10.1039/c3mt00111c

Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996). Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. *J. Biol. Chem.* 271, 14504–14509. doi: 10.1074/jbc.271.24.14504

Jung, H. I., Gayomba, S. R., Rutzke, M. A., Craft, E., Kochian, L. V., and Vatamaniuk, O. K. (2012). COPT6 is a plasma membrane transporter that functions in copper homeostasis in *Arabidopsis* and is a novel target of SQUAMOSA promoter-binding protein-like 7. *J. Biol. Chem.* 287, 33252–33267. doi: 10.1074/jbc.M112.397810

Jung, H. I., Zhai, Z., and Vatamaniuk, O. K. (2011). Direct transfer of synthetic double-stranded RNA into protoplasts of *Arabidopsis thaliana*. *Methods Mol. Biol.* 744, 109–127. doi: 10.1007/978-1-61779-123-9_8

Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D., and Van Montagu, M. (1995). Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. *J. Biol. Chem.* 270, 28479–28486. doi: 10.1074/jbc.270.47.28479

Kittanakom, S., Chuk, M., Wong, V., Snyder, J., Edmonds, D., Lydakis, A., et al. (2009). Analysis of membrane protein complexes using the split-ubiquitin membrane yeast two-hybrid (MYTH) system. *Methods Mol. Biol.* 548, 247–271. doi: 10.1007/978-1-59745-540-4_14

Klaumann, S., Nickolaus, S. D., Fürst, S. H., Starck, S., Schneider, S., Ekkehard Neuhaus, H., et al. (2011). The tonoplast copper transporter COPT5 acts as an exporter and is required for interorgan allocation of copper in *Arabidopsis thaliana*. *New Phytol.* 192, 393–404. doi: 10.1111/j.1469-8137.2011.03798.x

Lee, J., Peña, M. M., Nose, Y., and Thiele, D. J. (2002). Biochemical characterization of the human copper transporter ctr1. *J. Biol. Chem.* 277, 4380–4387. doi: 10.1074/jbc.M104728200

Marschner, H. (1995). *Mineral Nutrition of Higher Plants.* London, San Diego, CA: Academic Press.

Mendel, R. R., and Kruse, T. (2012). Cell biology of molybdenum in plants and humans. *Biochim. Biophys. Acta* 1823, 1568–1579. doi: 10.1016/j.bbamcr.2012.02.007

Merchant, S. S. (2010). The elements of plant micronutrients. *Plant Physiol.* 154, 512–515. doi: 10.1104/pp.110.161810

Mochida, K., Yoshida, T., Sakurai, T., Yamaguchi-Shinozaki, K., Shinozaki, K., and Tran, L.-S. P. (2011). *In silico* analysis of transcription factor repertoires and prediction of stress-responsive transcription factors from six major Gramineae plants. *DNA Res.* 18, 321–332. doi: 10.1093/dnares/dsr019

Mur, L. A., Allainguillaume, J., Catalan, P., Hasterok, R., Jenkins, G., Lesniewska, K., et al. (2011). Exploiting the *Brachypodium* tool box in cereal and grass research. *New Phytol.* 191, 334–347. doi: 10.1111/j.1469-8137.2011.03748.x

Nakagawa, Y., Kikuchi, S., Sakamoto, Y., and Yano, A. (2010). Identification and characterization of CcCTR1, a copper uptake transporter-like gene, in *Coprinopsis cinerea*. *Microbiol. Res.* 165, 276–287. doi: 10.1016/j.micres.2009.05.004

Nei, M., and Kumar, S. (2000). *Molecular Evolution and Phylogenetics.* New York, NY: Oxford University Press.

Nevitt, T., Öhrvik, H., and Thiele, D. J. (2012). Charting the travels of copper in eukaryotes from yeast to mammals. *Biochim. Biophys. Acta* 1823, 1580–1593. doi: 10.1016/j.bbamcr.2012.02.011

Peña, M. M., Puig, S., and Thiele, D. J. (2000). Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. *J. Biol. Chem.* 275, 33244–33251. doi: 10.1074/jbc.M005392200

Peñarrubia, L., Andres-Colas, N., Moreno, J., and Puig, S. (2010). Regulation of copper transport in *Arabidopsis thaliana*: a biochemical oscillator? *J. Biol. Inorg. Chem.* 15, 29–36. doi: 10.1007/s00775-009-0591-8

Perea-García, A., Garcia-Molina, A., Andrés-Colás, N., Vera-Sirera, F., Pérez-Amador, M. A., Puig, S., et al. (2013). Arabidopsis copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. *Plant Physiol.* 162, 180–194. doi: 10.1104/pp.112.212407

Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30:e36. doi: 10.1093/nar/30.9.e36

Puig, S., Lee, J., Lau, M., and Thiele, D. (2002). Biochemical and genetic analysis of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. *J. Biol. Chem.* 277, 26021–26030. doi: 10.1074/jbc.M202547200

Rapisarda, V. A., Volentini, S. I., Farias, R. N., and Massa, E. M. (2002). Quenching of bathocuproine disulfonate fluorescence by Cu(I) as a basis for copper quantification. *Anal. Biochem.* 307, 105–109. doi: 10.1016/S0003-2697(02)00031-3

Ravet, K., and Pilon, M. (2013). Copper and iron homeostasis in plants: the challenges of oxidative stress. *Antioxid. Redox Signal.* 19, 919–932. doi: 10.1089/ars.2012.5084

Rees, E. M., Lee, J., and Thiele, D. J. (2004). Mobilization of intracellular copper stores by the Ctr2 vacuolar copper transporter. *J. Biol. Chem.* 279, 54221–54229. doi: 10.1074/jbc.M411669200

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sancenon, V., Puig, S., Mateu-Andres, I., Dorcey, E., Thiele, D., and Peñarrubia, L. (2004). The *Arabidopsis* copper transporter COPT1 functions in root elongation and pollen development. *J. Biol. Chem.* 279, 15348–15355. doi: 10.1074/jbc.M313321200

Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., et al. (2003). ARAMEMNON, a novel database for Arabidopsis integral membrane proteins. *Plant Physiol.* 131, 16–26. doi: 10.1104/pp.011577

Shorrocks, V. M., and Alloway, B. J. (1988). *Copper in Plant, Animal and Human Nutrition Potters Bar*. Hertfordshire: Copper Development Association.

Solberg, E., Evans, I., and Penny, D. (1999). Copper deficiency: diagnosis and correction. Agri-facts. Soil fertility/crop nutrition. alberta agriculture, food and rural development, *Agdex* 532–533, 1–9.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Thole, V., Peraldi, A., Worland, B., Nicholson, P., Doonan, J. H., and Vain, P. (2012). T-DNA mutagenesis in *Brachypodium distachyon*. *J. Exp. Bot.* 63, 567–576. doi: 10.1093/jxb/err333

Ueda, T., Yamaguchi, M., Uchimiya, H., and Nakano, A. (2001). Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis thaliana*. *EMBO J.* 20, 4730–4741. doi: 10.1093/emboj/20.17.4730

Valko, M., Morris, H., and Cronin, M. T. (2005). Metals, toxicity and oxidative stress. *Curr. Med. Chem.* 12, 1161–1208. doi: 10.2174/0929867053764635

Vida, T. A., and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* 128, 779–792. doi: 10.1083/jcb.128.5.779

Vogel, J., and Hill, T. (2008). High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. *Plant Cell Rep.* 27, 471–478. doi: 10.1007/s00299-007-0472-y

Wu, X., Sinani, D., Kim, H., and Lee, J. (2009). Copper transport activity of yeast Ctr1 is down-regulated via its C terminus in response to excess copper. *J. Biol. Chem.* 284, 4112–4122. doi: 10.1074/jbc.M807909200

Xiao, Z., Loughlin, F., George, G., Howlett, G., and Wedd, A. (2004). C-terminal domain of the membrane copper transporter Ctr1 from *Saccharomyces cerevisiae* binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. *J. Am. Chem. Soc.* 126, 3081–3090. doi: 10.1021/ja0390350

Yordem, B. K., Conte, S. S., Ma, J. F., Yokosho, K., Vasques, K. A., Gopalsamy, S. N., et al. (2011). *Brachypodium distachyon* as a new model system for understanding iron homeostasis in grasses: phylogenetic and expression analysis of Yellow Stripe-Like (YSL) transporters. *Ann. Bot.* 108, 821–833. doi: 10.1093/aob/mcr200

Yuan, M., Chu, Z., Li, X., Xu, C., and Wang, S. (2010). The bacterial pathogen *Xanthomonas oryzae* overcomes rice defenses by regulating host copper redistribution. *Plant Cell* 22, 3164–3176. doi: 10.1105/tpc.110.078022

Yuan, M., Li, X., Xiao, J., and Wang, S. (2011). Molecular and functional analyses of COPT/Ctr-type copper transporter-like gene family in rice. *BMC Plant Biol.* 11:69. doi: 10.1186/1471-2229-11-69

Zhai, Z., Jung, H. I., and Vatamaniuk, O. K. (2009). Isolation of protoplasts from tissues of 14-day-old seedlings of *Arabidopsis thaliana*. *J. Vis. Exp.* 30:1149. doi: 10.3791/1149

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