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Influence of Soil Chemistry and Plant Physiology in the Phytoremediation of Cu, Mn, and Zn

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Different anthropogenic sources of metals can result from agricultural, industrial, military, mining and urban activities that contribute to environmental pollution. Plants can be grown for phytoremediation to remove or stabilize contaminants in water and soil. Copper (Cu), manganese (Mn) and zinc (Zn) are trace essential metals for plants, although their role in homeostasis in plants must be strictly regulated to avoid toxicity. In this review, we summarize the processes involved in the bioavailability, uptake, transport and storage of Cu, Mn and Zn in plants. The efficiency of phytoremediation depends on several factors including metal bioavailability and plant uptake, translocation and tolerance mechanisms. Soil parameters, such as clay fraction, organic matter content, oxidation state, pH, redox potential, aeration, and the presence of specific organisms, play fundamental roles in the uptake of trace essential metals. Key processes in the metal homeostasis network in plants have been identified. Membrane transporters involved in the acquisition, transport and storage of trace essential metals are reviewed. Recent advances in understanding the biochemical and molecular mechanisms of Cu, Mn and Zn hyperaccumulation are described. The use of plant-bacteria associations, plant-fungi associations and genetic engineering has opened a new range of opportunities to improve the efficiency of phytoremediation. The main directions for future research are proposed from the investigation of published results.

Keywords phytoremediation, copper, manganese, zinc, soil chemistry, genetic engineering, plant physiology

I. INTRODUCTION

In recent decades, the continuous growth of the world population and the quest for material goods has generated a massive expansion in industrial and agricultural production. Demand for food is quickly rising and will continue to rise with increases in global population (Godfray *et al.*, 2010). Since the 1960s, intensive irrigation and massive use of chemical fertilizers and pesticides has allowed an increase in food production obtained from the same amount of land (Vitousek *et al.*, 2009). However, extensive use of mineral fertilizers and pesticides has caused serious contamination of soil and water, decreasing the quality of water and land for human purposes (Melo *et al.*, 2012; Pinto *et al.*, 2010; Quinton *et al.*, 2010; Schwarzenbach *et al.*, 2010). Furthermore, several environmental pollution problems have been observed in cities and urban areas with concentrated population, large energy consumption, and high waste production, traffic emissions and industrial activity (Luo *et al.*, 2012). The continuous pollution of soil, air and water has had a direct adverse impact on ecosystems, cultural heritage, and human health (Phalan *et al.*, 2011).

Soil and water contamination by copper (Cu), manganese (Mn) and zinc (Zn) has been the subject of several studies in recent decades, and a large database has been already collected and presented in a number of reports. Several significant sources, such as fertilizers, sewage sludge, manure, agrochemicals and industrial by-product wastes, have contributed to the increased Cu, Mn and Zn levels observed in soil and water (Ahmed *et al.*, 2012; Arthur *et al.*, 2012; Buccolieri *et al.*, 2010; Hu *et al.*, 2009; Testiati *et al.*, 2013).

The complex behavior of metals in the environment is mainly caused by multiple processes occurring in the biosphere over both space and time (Csavina *et al.*, 2012). Physical, chemical and biological interactions that occur between plants and the surrounding environment of the soil are the most complex experienced by land plants. Recent years have seen great advances in the understanding of the complexity of some of these interactions, including the processes involved in nutrient and water uptake by roots under ideal conditions as well as when some nutrients are in short supply (Powlson *et al.*, 2011).

Phytoremediation relies on the use of plants and their associated rhizospheres to degrade, stabilize and/or remove soil contaminants, and it is a technology that has been of greatest interest in recent years. This environmentally friendly and low-cost technology can be used to decontaminate soils, water and sediments containing organic compounds and/or metals (Gerhardt *et al.*, 2009; Kramer, 2010). There is a great interest in applying phytoremediation to agriculture as well as to military and industrial fields that present contamination problems (Andreazza *et al.*, 2011; Mackie *et al.*, 2012; Panz and Miksch, 2012; Pignattelli *et al.*, 2012; Testiati *et al.*, 2013). Polluted waters that can be phytoremediated include sewage and municipal wastewater, agricultural runoff/drainage water, industrial waste water, landfill leachate and mine drainage (Agunbiade *et al.*, 2009; Anning *et al.*, 2013; Boojar and Tavakkoli, 2011; Vaseem and Banerjee, 2012).

Despite all its benefits, phytoremediation has some limitations regarding their specific technologies. For phytoextraction, plants should possess the ability to grow quickly, to produce large amounts of biomass, to be easily harvested and to accumulate a variety of metals in their harvestable parts (shoots). In phytostabilization, plants should have the capacity to retain contaminants in the roots and to produce large amounts of biomass (Andreazza *et al.*, 2011). As yet, no plant is known to fulfill all of these criteria. Limitations on plant growth in sites heavily contaminated with multiple compounds (both organic and inorganic) compromised the efficiency of phytoremediation (Chigbo *et al.*, 2013; Sirguey and Ouvrard, 2013). Thus, the use of transgenic plants could be an important tool to improve some of the characteristics necessary for phytotechnologies. Recent progress in plant physiology, biochemical and molecular fields provides a strong scientific basis for some strategies for achieving this goal (Palmer and Guerinot, 2009; Ramegowda *et al.*, 2012; Tan *et al.*, 2013; Turchi *et al.*, 2012).

The aim of this review is to compile information about plant processes involved in uptake, translocation, and sequestration of Cu, Mn and Zn to improve our understanding of the accumulation process and the distribution of these elements in the plant. The review will focus on the influence of the soil-plant system on phytoremediation. Recent improvements in this technology will be discussed in the field of plant physiology.

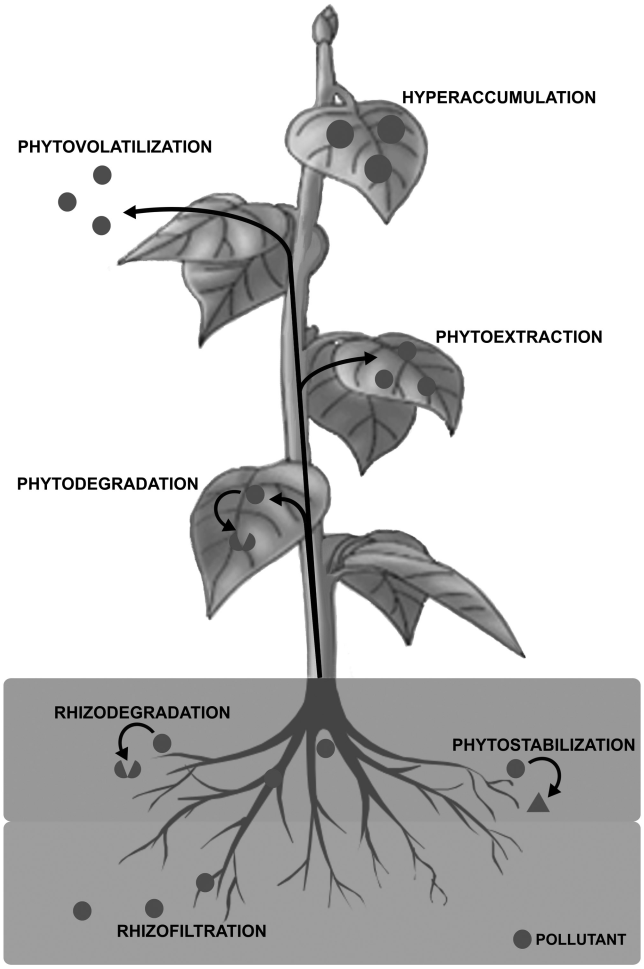

FIG. 1. Schematic presentation of phytoremediation processes.

II. PHYTOREMEDIATION TECHNOLOGIES

Plants can be used for phytoremediation in different ways (Figure 1) including removal of contaminants from water and aqueous waste streams in constructed wetlands or filtering pollutants through the root systems of hydroponically cultivated plants, a process called rhizofiltration (Chandra and Yadav, 2011; Valderrama *et al.*, 2012). Deep-rooted species, such as trees, can be used to provide a hydraulic barrier and plume containment through evapotranspiration, creating an upward water flow in the root zone and preventing the spread of contamination (Dominguez *et al.*, 2009). Phytostabilization, another phytotechnology, uses plants to stabilize pollutants in soil, preventing erosion, leaching or runoff, or by converting pollutants to less bioavailable forms. In this process, plants act as a ground cover, reducing animal contact with contaminants present in soil (Dasgupta-Schubert *et al.*, 2011; Pignattelli *et al.*, 2012; Van Nevel *et al.*, 2011).

Rhizodegradation is the ability of a plant to promote the enzymatic breakdown of organic pollutants by microbes in its rhizosphere (Slater *et al.*, 2011). Similarly, plants can degrade organic pollutants directly via their own enzymatic activities, a process called phytodegradation (Panz and Miksch, 2012). After uptake into plant tissue, certain inorganic and organic pollutants are capable of being volatilized; this process is called phytovolatilization (Dhillon *et al.*, 2010). Phytoextraction involves the use of plants to extract pollutants by the roots and transport them to aerial plant organs. Pollutants accumulated in stems and leaves are then harvested and removed from the site (Wu *et al.*, 2012b).

The phytotechnologies are not mutually exclusive, and they are often used in combinations such as degradation, accumulation, and volatilization. Because the phytoremediation process is natural, plants clean our environment constantly, without human interference (Gerhardt *et al.*, 2009; Kramer, 2010).

Desirable plant properties for phytoremediation are fast growth, high biomass, competitiveness, hardiness, and tolerance of pollution. Different phytoremediation technologies are suitable for different classes of pollutants; typically, different plant species are used for each process, as summarized in Table 1.

III. PLANT PHYSIOLOGY PROCESSES AFFECTING PHYTOREMEDIATION OF TRACE METALS

The efficiency of phytoremediation depends on several factors: trace metal bioavailability, trace metal uptake, translocation mechanisms, tolerance mechanisms (compartmentation), trace metal chelation and the movement of trace metals through ecosystems.

A. Bioavailability

As summarized in Figure 2, bioavailability of trace metals depends on environmental conditions such as oxidation state, moisture and temperature, soil properties such as soil organic matter (SOM) and soil pH, and biological activity promoted by microorganisms (Bravin *et al.*, 2012; Gadd, 2010; Yang *et al.*, 2012).

Cation exchange capacity (CEC) is influenced by the concentration of SOM and soil pH. This is an important parameter that controls the bioavailability of cations in soil. When soil pH becomes acid, the bioavailability of cations generally increases due to replacement of cations on soil CEC sites by H^+ ions. Clayey soils hold more water than sandy soils, and they have more binding sites for ions, especially cations (Vega *et al.*, 2010; Wu *et al.*, 2010). High temperatures accelerate physical, chemical, and biological processes. Precipitation stimulates general plant growth, and higher soil moisture increases the migration of water-soluble trace metals. The bioavailability of trace metals is also altered by biological activity. The microbial community present in the rhizosphere can produce chelators for delivering key plant nutrients and can enhance the availability/mobility of trace metals in the soil (Rajkumar *et al.*, 2012).

The rhizosphere includes the area surrounding the root (approximately 1 mm), and it plays an important role in phytoremediation. The physical, chemical, and biological interactions that take place between roots and the surrounding environment

TABLE 1
Phytoremediation technologies suitable for different classes of pollutants and favorable plant species for each process

Phytotechnologies	Type of pollutants	Favorable plant properties	Plant species	Reference
Rhizofiltration	inorganics (metals, nitrate, sulfate, ammonia, phosphate)	Good metal accumulators, grow fast, tolerant, attain a high biomass and can be harvested easily	*Typha angustifólia*; *Azolla filiculoides*; *Lemna minor*; *Pistia stratiotes*; *Azolla pinnata*	Chandra and Yadav, 2011 Valderrama *et al.*, 2012 Vaseem and Banerjee, 2012 Hua *et al.*, 2012
Rhizodegradation	hydrophobic organic compounds (PCBs, PAHs and others)	Large root surface area favors the degradation process, as it promotes microbial growth and the production of specific exudate compounds	*Salix alaxensis*; *Picea glauca*; *Glycine max*; *Oryza sativa*; *Medicago sativa*	Slater *et al.*, 2011 Panz and Miksch, 2012 Gerhardt *et al.*,2009
Phytoextraction	Trace metals (As, Co, Cu, Mn, Ni, Pb, Se, Zn and others)	High levels of plant uptake, translocation, and accumulation in harvestable tissues (hyperaccumulation occurs when concentration in above-ground tissues is between 0.1 – 1% of the plant dry weight)	*Arachis pintoi*; *Zea mays*; *Brassica alba*; *Oryza sativa*	Andreazza *et al.*, 2011 Murakami and Ae, 2009
			Hyperaccumulators: *Phytolacca americana* (Mn); *Alyssum bertolonii* (Ni, Co); *Noccaea caerulescens* (Cd, Zn, Ni, Pb); *Arabidopsis halleri* (Cd, Zn); *Sedum alfredii*, *Arabis paniculata* (Zn)	Kramer, 2010 Liu *et al.*, 2010b Tang *et al.*, 2009 Deinlein *et al.*, 2012
Phytodegradation	organics that are mobile in plants (herbicides, TPHs, TNT, BTEX and RDX)	Large, dense root systems and high levels of degrading enzymes	*Phalaris arundinacea*; *Lolium perenne*; *Abutilon avicennae*; *Phragmites australis*	Panz and Miksch, 2012 Gerhardt *et al.*,2009
Phytovolatilization	Volatile organic compounds (TCE and MTBE) and few inorganics (Se and Hg)	High transpiration rate facilitates the movement of these compounds through the plant	*Triticum aestivum*; *Brassica napus*; *Vigna sinensis*; *Saccharum officcinarum*; *Populus tremula* × *Populus alba*	Dhillon *et al.*, 2010 Gerhardt *et al.*,2009
Phytostabilization	organic and inorganic pollutants	High transpiration prevent leaching and runoff and deep-rooted grasses prevent the loss of top soil and sediments	*Quercus robur*; *Pinus sylvestris*; *Pseudotsuga menziesii*; *Silene paradoxa*; *Aldama dentata*	Nevel *et al.*, 2011 Dasgupta-Schubert *et al.*, 2011 Pignattelli *et al.*, 2012

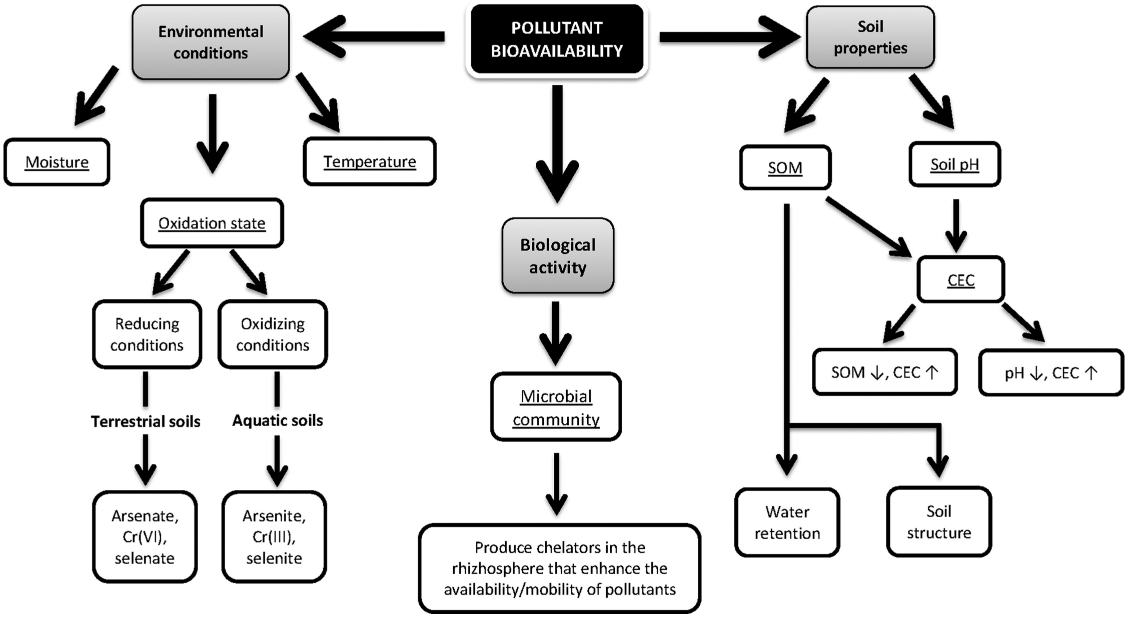

FIG. 2. Schematic diagram of the parameters that can influence pollutant bioavailability. SOM - soil organic matter; CEC - cation exchange capacity.

are largely controlled or directly influenced by roots and are often referred as rhizosphere processes. These include water uptake, exudation, nutrient mobilization, rhizosphere respiration, and rhizosphere-associated SOM decomposition (Cheng, 2009). The bioavailability of trace metals is highly dependent on the rhizosphere processes. Plants and their associated microorganisms can exude compounds that can enhance the uptake of trace metals or reduce their mobility in soil (Hinojosa *et al.*, 2010). For example, in aged polluted soils, the less or non-bioavailable trace metals tend to be more persistent than trace metals in newly contaminated soil, making phytoremediation more difficult to apply (Donner *et al.*, 2012).

Amendments may be added to soil that make metal cations more bioavailable for plant uptake (Wu *et al.*, 2012a). For instance, the effects of five amendments (EDDS, histidine, citric acid, rhamnolipid and sulfate) on the phytoextraction of Cu have been evaluated. The authors concluded that the combination of two or more amendments was effective for improving phytoremediation of metal contaminated sites by *Lolium perenne* (Gunawardana *et al.*, 2011). However, care should be taken when applying amendments to improve trace metal bioavailability because leaching to groundwater and thus groundwater pollution can occur (Perez-Esteban *et al.*, 2013).

Furthermore, rhizosphere processes may also be optimized to improve the phytoremediation efficiency. If a certain exudate compound that stimulates microbial degradation is found to enhance phytoremediation, plants and/or microorganisms can be selected or genetically engineered for use in remediation (Slater *et al.*, 2011; Techer *et al.*, 2011).

B. Plant Uptake

Trace metals enter the root either by crossing the plasma membrane of the root endodermal cells (symplastic transport) or by entering the root apoplast through the space between cells (apoplastic transport). They cannot pass through membranes without the aid of membrane transporter proteins. These transporter proteins occur naturally in several plant membranes (tonoplast, endoplasmic reticulum, mitochondria, chloroplasts) because trace metals are either nutrients (Cu, Mn, Zn) or are chemically similar to nutrients and are taken up involuntarily (e.g., Cd can be taken up by Zn transporters) (Leitenmaier and Kupper, 2011; Pilon *et al.*, 2009). Plants possess multiple transporters for most elements. For instance, rice (*Oryza sativa*) has at least seven Cu transporters (Yuan *et al.*, 2011). Each transporter has unique properties. When a low concentration of nutrients is present in the soil solution, their uptake usually requires a high-affinity transporter. By contrast, low-affinity transporters are more useful when high concentrations of nutrients are present, such as in agricultural soils after fertilization. Furthermore, the abundance of each transporter varies with tissue type and environmental conditions, making the uptake and movement of nutrients in plants complex processes (Cailliatte *et al.*, 2010; Vert *et al.*, 2009).

The accumulation of trace metals in tissues can cause toxicity by damaging the cell structure (e.g., stunting and chlorosis) and/or via replacement of other essential nutrients (Zhao *et al.*, 2012). Combinations of trace metals and organic pollutants may exert either alleviating or exacerbating effects on plant growth, depending on plant species, plant growth stage, the concentrations and properties of pollutants, and soil conditions such as pH and OM content (Chigbo *et al.*, 2013).

Depending on the type of phytoremediation, uptake of trace metals into the plant may be desirable, in the case of phytoextraction, or not, when phytostabilization is to be applied. In those situations, the selection of suitable plant species with the desired properties should be carefully considered (Murakami and Ae, 2009; Testiati *et al.*, 2013). Studies performed under similar conditions are a useful strategy to compare the uptake characteristics of different species for different trace metals (Chehregani *et al.*, 2009). Furthermore, phytoremediation efficiency can be enhanced through several types of changes such as by increasing oxygen levels in roots through aeration which may facilitate or difficult metal uptake (Zhao *et al.*, 2009) or by improving soil nutrient levels through fertilization in order to promote plant growth and thus trace metal uptake (Erenoglu *et al.*, 2011).

C. Root Chelation and Compartmentation

Plants and their associated microbial community can release compounds in the root/rhizosphere zone that play crucial roles in tolerance, sequestration, and transport of trace metals (Rajkumar *et al.*, 2012), as summarized in Figure 3.

Most plant-associated microorganism can produce siderophores in response to low Fe levels in the rhizosphere. These low-molecular-weight chelators play an important role in enhancing extracellular solubilization of Fe from minerals, making it available to the plant-microbial consortium (Schalk *et al.*, 2011). In addition to Fe, other trace metals, such as Cu, Mn and Zn, are also able to stimulate or inhibit siderophore production. Additionally, toxic metals may induce the production of some siderophores that have chelator capacity and can play a role in toxic metal tolerance (Braud *et al.*, 2010).

Organic acids released by plant-associated microbes also play an important role in the uptake of trace metals into roots as well as their roles in transport, sequestration, and tolerance of trace metals. In general, organic acids can bind trace metal ions in soil solution by complexation reactions, making them more available for plant uptake (Wang and Zhong, 2011). However, organic acids can also reduce the soil pH and solubilize some non-labile fraction of trace metals in soil, thus enhancing trace metal availability (Perez-Esteban *et al.*, 2013).

Biosurfactants are another group of important metabolites that have the potential to improve trace metal mobilization and phytoremediation. These amphiphilic molecules have the capacity to form complexes with trace metals at the soil interface and to desorb trace metals from soil matrix, further increasing their solubility and bioavailability in the soil solution (Venkatesh and Vedaraman, 2012).

Other processes based on plant-microbe associations can help promote trace metal mobilization or immobilization. The production of extracellular polymeric substances and glycoproteins (Mikutta *et al.*, 2012), redox reactions (Shi *et al.*, 2011) and biosorption mechanisms (Audet and Charest, 2013; Mrnka *et al.*, 2012) all have the capacity to alter the bioavailability and plant uptake of trace metals through metal mobilizing actions.

Inside the plant, trace metals can be chelated by glutathione (GSH) and phytochelatins (PCs) or metallothioneins (MTs). These chelators have a high affinity for metal cations due to the thiol (–SH) groups on their cysteine residues. After exposure to metal(s), PCs are immediately produced in cells and tissues, though their production is markedly influenced by the type of metal ion present. Phytochelatin synthesis was found to be catalyzed by PC synthase in the presence of metal ions such as Cu and Zn (Machado-Estrada *et al.*, 2012). Moreover, this process may work synergistically with secondary stress-defensive antioxidative systems to combat metal induced oxidative stress (Uraguchi *et al.*, 2009; Xu *et al.*, 2011). MTs are also able to bind a variety of trace metals (e.g., Cu and Zn) and to activate antioxidative enzyme defenses (Turchi *et al.*, 2012). Polyphenols and cell wall proteins and pectins are also involved in trace metal chelation and tolerance (Colzi *et al.*, 2012; Schmidt *et al.*, 2013).

After chelation, trace metals in roots may be stored in the vacuole and/or cell wall or exported to the shoot via the xylem. The vacuole is generally considered to be the main storage site for trace metals in plant cells and vacuolar compartmentalization of trace metals is also a part of the tolerance mechanism (Guo *et al.*, 2012).

D. Translocation

Translocation is of interest because some phytoremediation techniques demand the transfer of trace metals from roots to aerial tissues. Translocation from root to shoot first requires a membrane transport step from root symplast into xylem, during which solutes must pass the Casparian strip, a waxy coating that prevents solutes from entering the root xylem from the soil solution or root apoplast (Grebe, 2011; Xu *et al.*, 2011).

Although trace metal transporters are highly specialized in the movement of a certain metal, they can sometimes also translocate different metals because they use the same transport systems to be loaded into the xylem (Uraguchi *et al.*, 2009). Some trace metals are chelated by organic acids, which are involved in trace metal absorption by plant roots, translocation in the xylem, and storage in the vacuole of leaf cells (Larbi *et al.*, 2010; Wang and Zhong, 2011). Trace metals can also be bound by nicotianamine (NA) and mobilized by the yellow strike-like (YSL) family transporters (Chu *et al.*, 2010; Harris *et al.*, 2012). It should be emphasized that the translocation of trace metals is a complex process involving multiple networks between membrane transporters and metals. Specialized proteins can transport elements with similar characteristics (e.g., oxidative state), inducing competition that results in deficiency, toxicity and/or accumulation in the above-ground plant tissues (Barberon *et al.*, 2011; Zimmermann *et al.*, 2009).

Very few of these changes exclusively affect a single element, i.e., changes in a transporter that has high specificity for that element. Most of the above-mentioned changes will affect more

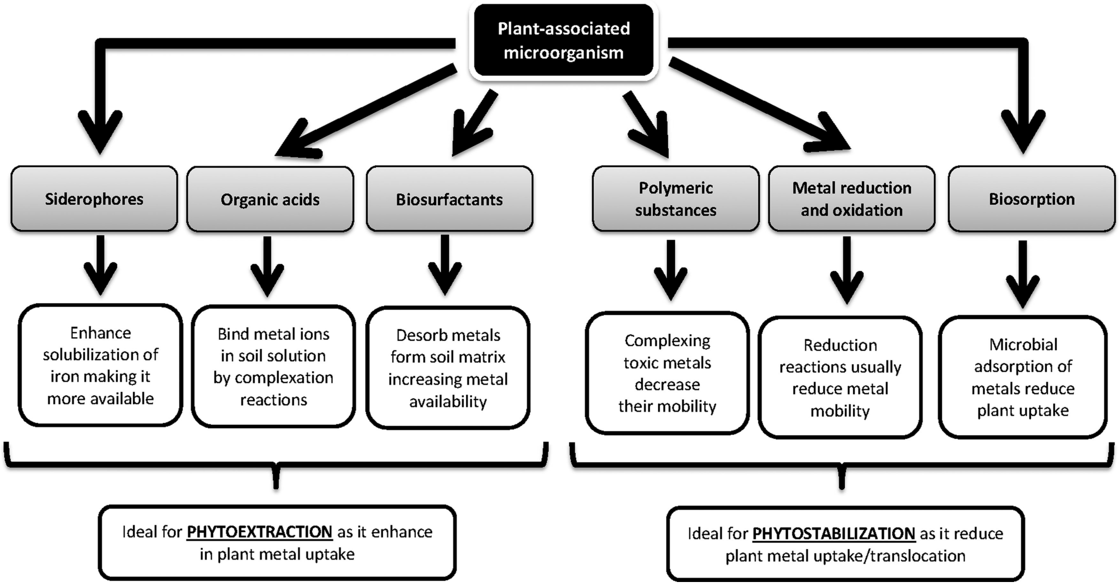

FIG. 3. Schematic diagram of the major plant associations and their roles in phytoremediation. The production of siderophores, organic acids and biosurfactants generally promotes trace metal uptake. The presence of polymeric substances, a reductive medium and biosorption processes reduce trace metal uptake.

than one element. Thus, experiments focused on single elements which do not take into account the total mineral nutrient and trace element content of the plant will not reveal the regulatory networks involved in the homoeostasis of the ionome (Salt *et al.*, 2008).

Transpiration rate is a key variable that determines the rate of chemical uptake for a given phytoremediation application. Bulk flow in the xylem from root to shoot is driven by transpiration from the shoot, which creates a negative pressure in the xylem that pulls up water and solutes. Plant transpiration depends on several aspects of plant species and the surrounding environment. For example, plant metabolic (e.g., C3/C4/CAM photosynthetic pathway) and anatomical differences (e.g., stomatal density) are important factors that affect transpiration rate (Alfonso and Bruggemann, 2012; Orsini *et al.*, 2012). Vegetation height and density, as well as environmental conditions, are also factors to consider (transpiration is normally maximal at high temperature, moderate wind, low relative air humidity, and high light) (Morandi *et al.*, 2012). Therefore, phytoremediation technologies that rely on translocation mechanisms are more efficient in climates with low relative humidity and high evapotranspiration rates.

E. Chelation and Compartmentation in Leaves

After transport inside the leaf symplast, the trace metal may be stored in certain tissues or cellular organelles. The general rule is that toxic metals are stored in places where they cause the least harm to essential cellular processes. At the tissue level, toxic metals are generally accumulated in the epidermis and trichomes (Sanchez-Pardo *et al.*, 2012); at the cellular level, they may be accumulated in the vacuole or chloroplasts (Boojar and Tavakkoli, 2011).

The distribution of trace metals in leaf tissues is generally asymmetric (Wu and Becker, 2012). Trace metal accumulation in leaf vacuoles makes sense because vacuoles do not contain a photosynthetic apparatus that would be sensitive to metal toxicity (Leitenmaier and Kupper, 2011). Trace metal storage occurs also in highly tolerant cells such as the leaf epidermis and the vein bundle sheath, as long as the metal import remains under control. Once the trace metal accumulation exceeds the tolerance threshold of the plant, the metals would be transported to mesophyll cells, which are more sensitive to toxic metals than other cell types, allowing photosynthesis to be threatened (Zhao *et al.*, 2012). At present, the physiological mechanisms involved in the sequestration of trace metals between different leaf tissues remain only partially understood. One possible mechanism is the differential expression of trace metal transporters in the plasma membrane and/or tonoplast between mesophyll and other tissues. In fact, Leitenmaier and Kupper (2011) show that metal storage in leaf epidermis cells is a rate-limiting step in metal hyperaccumulation, but it is not the only process that governs metal accumulation despite its role as an important driving force behind the complex phenomenon of metal hyperaccumulation. Several membrane transporters (e.g., HMA and VIT) have been proposed to play an important role in vacuolar sequestration (Morel *et al.*, 2009; Zhang *et al.*, 2012b).

Although the phytoremediation process mainly involves the uptake and translocation of trace metals from soil or water, air to leaf accumulation of trace metals is also an important entrance pathway (Saebo *et al.*, 2012). Air to leaf transfer can occur by both particulate and gaseous deposition, resulting in different distribution patterns across the leaf. Trace metals may be taken up directly via the stomata or be deposited on the leaf surface (Schreck *et al.*, 2012). Moreover, air to leaf accumulation of trace metals can cause various morphological, physiological and biochemical responses that may influence the phytoremediation process (Doganlar and Atmaca, 2011).

IV. PHYTOREMEDIATION OF TRACE ESSENTIAL METALS

Copper (Cu), manganese (Mn) and zinc (Zn) are trace essential metals for plants. Although they are essential, cellular concentrations of Cu, Mn and Zn must be strictly regulated to avoid deficiency or toxicity. Worldwide soil deficiency of Cu, Mn and Zn seems to be more common than potential excess because most soils have a small bioavailable fraction of these metals. However, very high metal concentrations in soils can be found in the literature where such excess was used to evaluate the plant status. Much knowledge has been obtained by comparing the physiology of plants grown under deficient versus toxic conditions. While such studies may provide an understanding of general plant responses to abiotic stress, this type of approach results in little useful information regarding metal homeostasis. In the next sections, the main findings achieved in the last years in the homeostasis of trace essential metals are summarized.

A. Copper

Copper is an trace essential element for both animals and plants and has an important role in some physiological processes (Stern, 2010). Nevertheless, it becomes toxic at high concentrations (Canning-Clode *et al.*, 2011). In soil, Cu concentrations range between 20 and 110 mg kg^{-1}. However, in soil solutions, Cu concentrations range between 30 and 241 μg L^{-1} (Buccolieri *et al.*, 2010; Kim and Owens, 2009; Mackie *et al.*, 2012). The continuous application of various Cu-based products such as pesticides, fertilizers and animal fodder has resulted in increased Cu concentrations in agricultural soils well above the European guideline limit of 100 mg kg^{-1} (Arthur *et al.*, 2012). Furthermore, Cu is a by-product in several industry and mining processes (Ahmed *et al.*, 2012; Testiati *et al.*, 2013).

Copper is present in several forms in soils, including free ions in the soil solution and complexes with other minerals or with organic components (Kasmaei and Fekri, 2012). Cu is a relatively immobile element in soil and shows relatively little variation in total content across soil profiles. Depending on soil pH, Cu may occur as Cu^+, Cu^{2+}, $CuSO_4$, $Cu(OH)_2$, $CuCO_3$, and other forms (Mackie *et al.*, 2012). Generally, increasing the pH decreases the solubility of Cu in soil (Wang *et al.*, 2013). Most common forms of Cu in soil solutions are soluble organic chelates of this metal (Kasmaei and Fekri, 2012; van Schaik *et al.*, 2010). The two major parameters that influence the variability of total Cu concentration as well as the available Cu concentration in soil are the CEC and SOM (Bravin *et al.*, 2012; Vega *et al.*, 2010; Wu *et al.*, 2010). Cu mobility is especially reduced in the presence of large mineral colloids in the clay fraction, as the presence of Fe and Mn oxides enhances Cu fixation to the soil components (Komarek *et al.*, 2008). It is widely accepted that Cu in soil competes with other elements for sorption sites. Therefore, the mineral composition (particularly the content of other metal cations) will influence the availability of Cu in soil (Merdy *et al.*, 2009). Cu is not readily leached from the soil horizons due to its high affinity for OM, and it tends to accumulate in surface soils (van Schaik *et al.*, 2010).

1. Copper uptake by plants

Figure 4 addresses the main processes that control Cu distribution within the plant. Because the most bioavailable form of Cu in soils is Cu^{2+}, root uptake is most often facilitated by reduction. The ferric reductase oxidases FRO1, FRO2, FRO3, FRO4 and FRO5, expressed in *Arabidopsis thaliana*, seem to be involved in this process (Bernal *et al.*, 2012; Jeong and Connolly, 2009). Cu is likely to enter the cytosol of root cells through a cell surface COPT/Ctr-family transporter. Six members of the Ctr family (COPT1–6), which mediate the influx of Cu, have been identified in Arabidopsis. AtCOPT1, the first COPT family member identified and characterized, is expressed in the plasma membrane of roots and has an important role in the acquisition of Cu monovalent ions. AtCOPT2 and AtCOPT3 are also present in the plasma membrane of root tissues and seem to play a role in Cu acquisition from the rhizosphere (Andres-Colas *et al.*, 2010). Furthermore, it is hypothesized that members of the Zinc-Regulated Transporter and Iron-Regulated Transporter (ZRT-IRT)-like proteins (ZIP) family can mediate the uptake and transport of Cu in both *Arabidopsis thaliana* and *Medicago truncatula* (del Pozo *et al.*, 2010; Stephens *et al.*, 2011). Members of the Yellow Stripe family (ZmYS1) are also involved in the transport of Cu-phytosiderophore (PS) complexes (Murata *et al.*, 2008).

The export of Cu from the root symplast to the xylem requires Cu-transporting P-type ATPases. The P-type ATPases, known as heavy metal P-type ATPases (HMAs) in plants, include at least eight (HMA1-HMA8) identified members in *Arabidopsis thaliana*. AtHMA1 to AtHMA4 belong to the group implicated in divalent cation transport; AtHMA5 to AtHMA8 act on monovalent Cu ion transport (Zimmermann *et al.*, 2009; Zorrig *et al.*, 2011). Efflux of Cu into the vascular tissues is thought to occur through an HMA family transporter. AtHMA5, mostly expressed in roots, flowers and pollen, is likely responsible for root Cu detoxification. This phenotype function is the opposite of COPT, corroborating the idea that AtCOPT1 and AtHMA5 transport Cu in opposite directions (del Pozo *et al.*, 2010; Kobayashi *et al.*, 2008). Cu translocation may involve chelators such as the nicotianamine (NA) and several amino acids (Harris *et al.*, 2012; Irtelli *et al.*, 2009). Moreover, Cu in the xylem sap of rice seems to be bound to deoxymugineic acid (DMA), while in the phloem sap, Cu mainly complexes with NA and histidine (Ando *et al.*, 2013). Regarding transporters, the recently identified HvYSL2, which is localized in the root endodermis, also seems to be involved in the transport of Cu

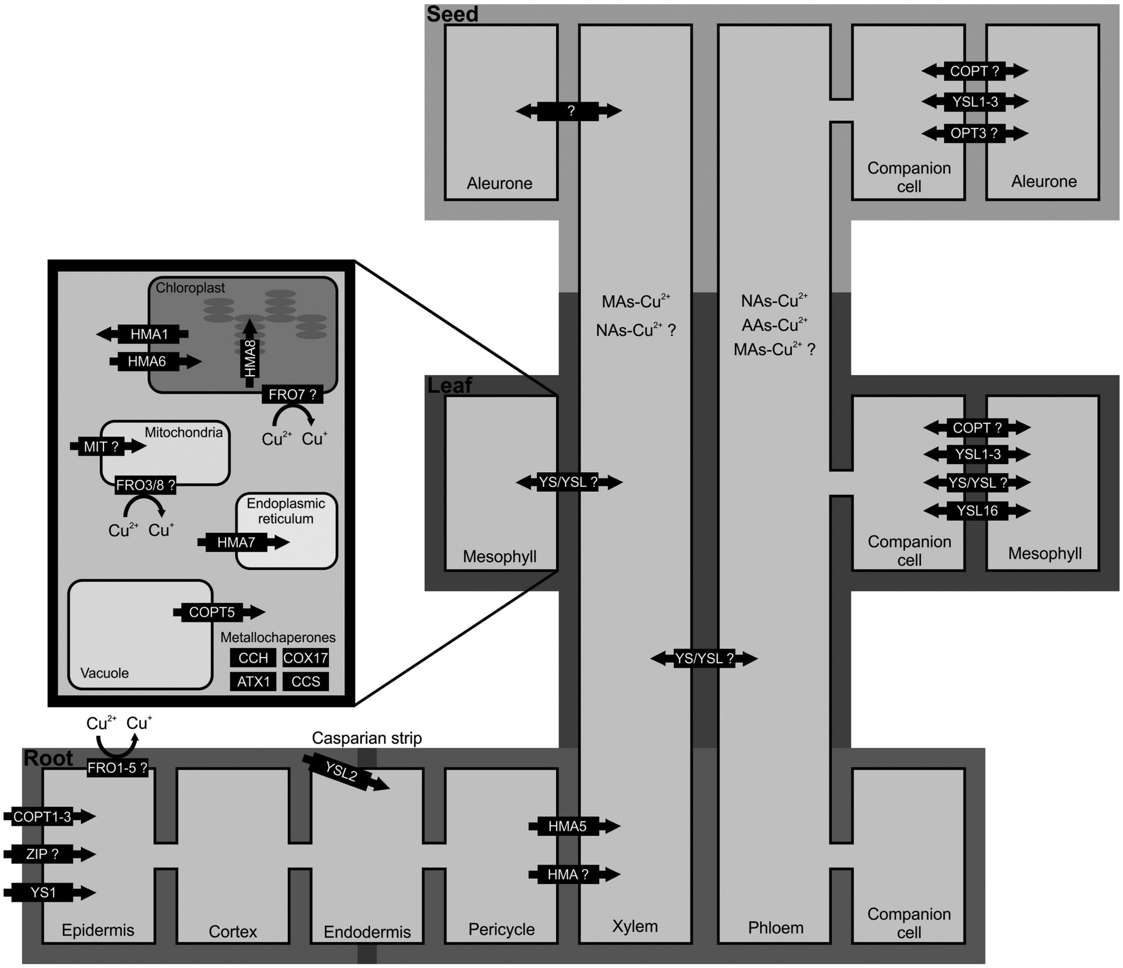

FIG. 4. Copper transport in a model plant. Cu is taken up into the symplast by transporters in the epidermis in the free form (COPT1–3 and ZIP) and/or complexed with phytosiderophores (YS1). Possible reduction of Cu^{2+} to Cu^{+} could be performed by FRO1–5. To enter the xylem, Cu must pass the Casparian strip. YSL transporters (e.g., YSL2) appear to have a role in the mobilization of Cu from the cortex to the pericycle. Transport into the xylem is mediated by members of the HMA family. In the xylem, Cu is thought to be moved in the form of complexes (with MAs and NAs) to the shoot, where they are unloaded, most likely by members of the YSL family. The xylem transport of Cu to seed is not well characterized. The YSLs may also translocate metals to the phloem, where they can then be delivered to the leaf and seed tissues by various transporters (YSL, COPT, OPT). After transport inside the leaf symplast, Cu may be stored in certain tissues or cellular organelles. If Cu is present in the divalent form, reduction must be performed before it can enter the cell organelles. FRO7 (expressed in the chloroplasts) and FRO3/8 (expressed in the mitochondria) are hypothesized to have a role in Cu reduction. Members of the HMA family (HMA1 and HMA6) mediate the influx and efflux of Cu in the chloroplast. Moreover, HMA8 is expressed in the thylakoid membrane and supplies Cu to plastocyanin. In the mitochondria, Cu influx is thought to occur via MIT. HMA7 is responsible for supplying Cu at the endoplasmic reticulum. COPT5 is important for Cu export from the vacuole. Several metallochaperones (ATX1, CCH, COX17, CCS) ensure proper metal delivery to target proteins and maintain Cu homeostasis. (Figure layout based on Palmer and Guerinot, 2009).

(and other metals) from the cortex to the pericycle (Araki *et al.*, 2011). OsYSL16 is a Cu-NA transporter required to deliver Cu to developing young tissues and seeds through phloem transport (Lee *et al.*, 2012; Zheng *et al.*, 2012). In Arabidopsis root, the plasma membrane AtYSL1 and AtYSL3 were proven to be involved in the root-to-shoot and shoot-to-seed transport of Cu as well as other metals (Chu *et al.*, 2010; Waters and Grusak, 2008). The COPT-family transporters are also responsible for Cu uptake in leaves and other shoot organs. In addition to their expression in root, AtCOPT1 and OsCOPT1 also showed high expression in the plasma membrane of leaves supporting the idea of a role in the Cu transport in shoot (Andres-Colas *et al.*, 2010; Yuan *et al.*, 2011). OsCOPT2 and OsCOPT3 had relatively high expression levels in leaf and panicle (Yuan *et al.*, 2011). In Arabidopsis, COPT6 is expressed in different cell types in different plant compartments, but the bulk of its expression is located in the vasculature (Jung *et al.*, 2012). Similarly, OsCOPT6 is not expressed in root but is highly expressed in leaf, stem and sheath. COPT7 was only found in rice and had relatively high expression levels in leaves; similar responses to OsCOPT5 were observed in both Cu deficiency and overdose environments (Yuan *et al.*, 2011). Furthermore, the oligopeptide transporter AtOPT3 and its orthologue TcOPT3 seem to participate in the mobilization of Cu (Hu *et al.*, 2012; Stacey *et al.*, 2008).

Again, if Cu is present in its divalent form, reduction must be performed before it can enter the cell organelles. AtFRO7 (expressed in the chloroplasts) and AtFRO3/8 (expressed in the mitochondria) play a central role in Fe reduction, and it is hypothesized that they also participate in Cu reduction (Jeong and Connolly, 2009). AtHMA6 (or PAA1), localized in the inner chloroplast envelope, is responsible for the delivery of Cu to chloroplasts (Catty *et al.*, 2011). AtHMA8 (PAA2), closely related to AtHMA6 (PAA1), is expressed in the thylakoid membrane and supplies Cu to plastocyanin (Tapken *et al.*, 2012). AtHMA1 and HvHMA1, present in the chloroplast envelope, are broad-specificity exporters of metals from chloroplasts and may play a specialized role in Cu mobilization (Mikkelsen *et al.*, 2012). AtHMA7 (RAN1), the first functionally characterized heavy metal ATPase, is responsible for the biogenesis of ethylene receptors by supplying Cu at the endoplasmic reticulum and also for Cu homeostasis in seedling development (Binder *et al.*, 2010). The rice mitochondrial Fe transporter (MIT) also appears to regulate the influx of Cu, although more studies are needed to confirm this (Bashir *et al.*, 2011). The tonoplast-localized AtCOPT5 is important for Cu export from the vacuole and is involved in the remobilization of Cu ions (Garcia-Molina *et al.*, 2011; Klaumann *et al.*, 2011). Until now, none of the eight identified FRO present in Arabidopsis have been expected to play a role in the reduction process of Cu on the vacuolar membrane, raising the possibility that other reductase enzymes may function as ferric reductases in plants (Jeong and Connolly, 2009). Finally, several metallochaperones expressed in Arabidopsis (e.g., ATX1, CCH, COX17, CCS) ensure proper metal delivery to target proteins and also maintain Cu homeostasis, preventing metal toxicity (Puig and Penarrubia, 2009).

Normally, Cu content ranges from 2 to 50 μg g^{-1} DW, depending on the plant species. However, 5 - 20 μg g^{-1} DW seems to be optimal, as toxicity symptoms appear above and deficiency symptoms below this critical range (Hansch and Mendel, 2009; White and Brown, 2010). Cu deficiency/toxicity affects plant physiological processes and ultimately plant production (Ravet *et al.*, 2011; Sanchez-Pardo *et al.*, 2012).

2. *Phytoremediation of Cu-contaminated sites*

Phytoremediation of Cu-contaminated sites can be observed in both soil and water. Regarding water remediation, Cu accumulation and translocation was observed in the well-known macrophyte *Hydrilla verticillata* at higher extent. Plant shoots accumulate Cu to a maximum of 30830 μg g^{-1} DW after exposure to 4 mg L^{-1} Cu for 4 days (Xue *et al.*, 2010). The duckweed *Lemna minor*, the macrophyte *Azolla pinnata* and the water crowfoot *Ranunculus tricophyllus* also show accumulation of Cu, suggesting that all three species can be used for remediation of this metal in polluted waters (Vaseem and Banerjee, 2012). Furthermore, *Eleocharis acicularis* can accumulate a maximum of 20200 μg g^{-1} of Cu in its shoots, suggesting great potential for use in the phytoremediation of water environments (Sakakibara *et al.*, 2011). The amphibious water plant *Crassula helmsii* can also hyperaccumulate Cu (Kupper *et al.*, 2009). Based on the Cu accumulation of *Azolla filiculoides* (6013 μg g^{-1}), this species can be regarded as a potential phytoremediation organism with high potential for cleaning water polluted with Cu (Valderrama *et al.*, 2012). In another study, Liu *et al.* (2010a) compiled information about 19 wetland plant species and concluded that the selection of appropriate plant species in constructed wetland is crucial for the improvement of metal removal efficiency.

The phytoremediation of Cu-contaminated sites has been accomplished by numerous plant species in metal-polluted soils. An area contaminated with metals due to mining activities was phytoremediated by *Zygophyllum fabago*, which mostly accumulated Cu in its aerial part, particularly in leaf vacuoles (Boojar and Tavakkoli, 2011). In addition to mining sites, other Cu-contaminated areas such as vineyard soils have also been phytoremediated. The potential of the perennial peanut (*Arachis pintoi*) was evaluated in those soils, and the authors concluded that this species has high potential for Cu removal and can be an important candidate for use in the phytoextraction and phytostabilization of vineyard soils contaminated with Cu (Andreazza *et al.*, 2011). Phytoremediation of Cu-contaminated sites was also performed by *Sedum plumbizincicola* in agricultural fields (Wu *et al.*, 2012b). Furthermore, maize (*Zea mays*), white mustard (*Brassica alba*) and rice (*Oryza sativa*) also show great potential for Cu phytoextraction (Brunetti *et al.*, 2012; Murakami and Ae, 2009). Other plants that can be used in the phytoremediation Cu-contaminated sites are *Euphorbia prostrate, Dyssodia setifolia, Parthenium incanum*, and *Zinnia acerosa* (Machado-Estrada *et al.*, 2012). Aside from phytoextraction, the stabilization of Cu by plant species is also a reliable technology for improving soil quality in heavily contaminated sites (Kumpiene *et al.*, 2009; Testiati *et al.*, 2013). The high Cu storage in the roots of *Aldama dentata* makes it attractive as a possible Cu phytostabilizer (Dasgupta-Schubert *et al.*, 2011). The salt-marsh shrub *Halimione portulacoides* can tolerate external Cu levels of up to 1000 mg L^{-1} without suffering adverse physiological effects (Cambrolle *et al.*, 2012). Moreover, the cuprophyte *Haumaniastrum katangense* was proved to be highly tolerant to Cu (Chipeng *et al.*, 2010).

B. Manganese

Typical soil concentrations of Mn vary from 450 to 550 mg kg^{-1}. In soil solution, Mn concentrations are normally between 50 and 2000 μg L^{-1} (Mundus *et al.*, 2012; Rajapaksha *et al.*, 2012). Mn commonly occurs in more than one oxidation state (e.g., Mn^{2+}, Mn^{3+}, MnO_4^- and others). Because Mn^{3+} is unstable in solution and Mn^{4+} compounds are slightly soluble, Mn^{2+} is the only important soluble form of Mn in soils (Das *et al.*, 2011; Mundus *et al.*, 2012). The soil distribution of Mn is not uniform and is known to be concentrated in certain spots, which are usually enriched with other trace elements (Heredia and Cirelli, 2009). The dynamics and availability of soil Mn are determined by many factors such as pH, Eh, the nature and concentration of cations and anions, clay mineralogical composition, OM content, and microorganisms. Mn availability is higher in acid soils due to the higher solubility of Mn compounds under low-pH conditions (Husson, 2013). Mn oxides show a great affinity for adsorption of both cationic and anionic forms of elements. Thus, Mn oxides have a major impact in the mobilization of trace metals in soils (Manh *et al.*, 2011; Rajapaksha *et al.*, 2012). Mn-oxidizing microbes increase Mn oxidation rates in soils by up to five orders of magnitude, affecting the availability of Mn to plants (Gadd, 2010).

The main sources of Mn for soils are organic manures, chemical fertilizers, and microbial biomass. The principal Mn depletion mechanisms are uptake by crop plants, loss through soil erosion, leaching, and adsorption on organic compounds or microbial biomass (Cheraghi *et al.*, 2012; Geissen *et al.*, 2010). The major anthropogenic sources of Mn are municipal wastewaters, sewage sludge, and metal smelting processes (Hu *et al.*, 2009; Ning *et al.*, 2010). Mn is widely used in metallurgy as well as in the electrical industry. It also has applications in the production of pigments, ceramics, and glass. Further, methylcyclopentadienyl manganese tricarbonyl (MMT) is used as an octane-enhancing agent (Das *et al.*, 2011; Joly *et al.*, 2011). Certain areas are exposed to higher Mn inputs, leading to environmental as well as human consumption problems (Geissen *et al.*, 2010; McArthur *et al.*, 2012).

1. Manganese uptake by plants

The oxidized forms Mn^{3+} and Mn^{4+} are not bioavailable to plants and cannot be accumulated. It is the reduced form of this element, Mn^{2+}, that is absorbed by root cells (Mundus *et al.*, 2012). The mechanisms of Mn homeostasis, including uptake, distribution and storage, remain poorly understood. However, some transporters have been identified as participating in the transport of Mn; they are described in Figure 5. The plasma membrane IRT1, expressed in both Arabidopsis and barley, can transport Mn as well as Zn (Barberon *et al.*, 2011; Pedas *et al.*, 2008). Furthermore, overexpressing of AtIRT2 leads to overaccumulation of Mn in transgenic plants, but the role of this transporter in Mn accumulation remains to be clarified (Vert *et al.*, 2009). The plasma membrane-localized NRAMP1 and NRAMP5 were shown to be high-affinity Mn transporters in Arabidopsis and rice, respectively. Both AtNRAMP1 and OsNRAMP5 have broad selectivity, and their expression is restricted to the root (Cailliatte *et al.*, 2010; Ishimaru *et al.*, 2012). Furthermore, Mn uptake can also be performed by the ZmYS1 transporter, which was confirmed to be capable of acquiring Mn-PS complexes from the rhizosphere (Murata *et al.*, 2008).

The translocation of Mn involves chelators such as NA, amino acids and carboxylic acids (Harris *et al.*, 2012; Kato *et al.*, 2010). Despite the preference for the transport of Fe-PS complexes, the ZmYS1 transporter is also able to translocate Mn-chelated species (Murata *et al.*, 2008). OsYSL6 is a Mn-NA transporter that is required for the detoxification of excess Mn in rice (Sasaki *et al.*, 2011). Likewise, OsYSL2 is involved in Mn translocation in the phloem. In the knockout *ysl2* line, seeds and shoots contain lower Mn content compared with the wild-type (Ishimaru *et al.*, 2010). Moreover, the same transporter (YSL2) shows the ability to transport Mn-PS complexes in barley roots (Araki *et al.*, 2011). In Arabidopsis, AtZIP2 seems to participate in Mn transport into the root vasculature for translocation to the shoot (Milner *et al.*, 2013). The oligopeptide transporter AtOPT3 seems to play an important role in the long-distance transport of Mn (Stacey *et al.*, 2008).

After uptake and translocation, Mn must be distributed into various cell compartments. The P-type ATPases AtECA1 (localized at the endoplasmic reticulum) and AtECA3 (expressed in the Golgi complex) participate in the influx of Mn (Li *et al.*, 2008; Mills *et al.*, 2008). The cation/H exchanger (CAX) transporters AtCAX2 and AtCAX4, which were originally identified as Ca transporters, also have the ability to transport Mn into the vacuole (Connorton *et al.*, 2012; Korenkov *et al.*, 2007). Likewise, vacuolar Mn/H antiporter activity in the Arabidopsis *cax2* knockout mutant is significantly reduced compared with wild-type, although it is not completely absent, suggesting the presence of additional vacuolar transporters (e.g., AtCAX5) that contribute to Mn transport (Edmond *et al.*, 2009). AtMTP11, which belongs to the cation diffusion facilitator (CDF) family, is implicated in the pre-vacuolar compartmentation of Mn as well as in the Mn homeostasis mechanisms (Delhaize *et al.*, 2007). Other MTPs also contribute to Mn transport (Gustin *et al.*, 2011). Furthermore, it seems that the tonoplast-localized transporters OsVIT1 and OsVIT2 participate in Mn influx to the vacuole (Zhang *et al.*, 2012b). Regarding vacuolar export, AtNRAMP3 and AtNRAMP4 are expressed in the tonoplast and can transport Mn and other metals (Lanquar *et al.*, 2010). AtZIP1 probably plays a role in Mn vacuolar efflux, based on the increased sensitivity to low Mn and increased accumulation of Mn in roots in the *zip1* knockout line (Milner *et al.*, 2013). The rice Fe transporter MIT appears to play a role in Mn transport into mitochondria (Bashir *et al.*, 2011). The mechanisms of Mn transport in the chloroplast remain unknown, despite the fact that Mn has a critical role in photosynthesis and the chloroplast is the one of the major sinks for Mn (Millaleo *et al.*, 2013; Yao *et al.*, 2012).

Mn content is remarkably varied across plant species, growth stage and different organs and ecosystems. Normally, Mn content in plants varies from 10 to 100 $\mu g\ g^{-1}$ (Hansch and Mendel, 2009; White and Brown, 2010). Below 10 and above 200 $\mu g\ g^{-1}$, Mn deficiency/toxicity occurs and plant physiological processes are compromised (Najeeb *et al.*, 2009; Zhao *et al.*, 2012).

2. Phytoremediation of Mn-contaminated sites

Phytoextraction of Mn-contaminated sites can be observed in both soil and water. In water, Mn remediation has been performed by *T. latifolia*, *Scirpus americanus* (Santos-Diaz and

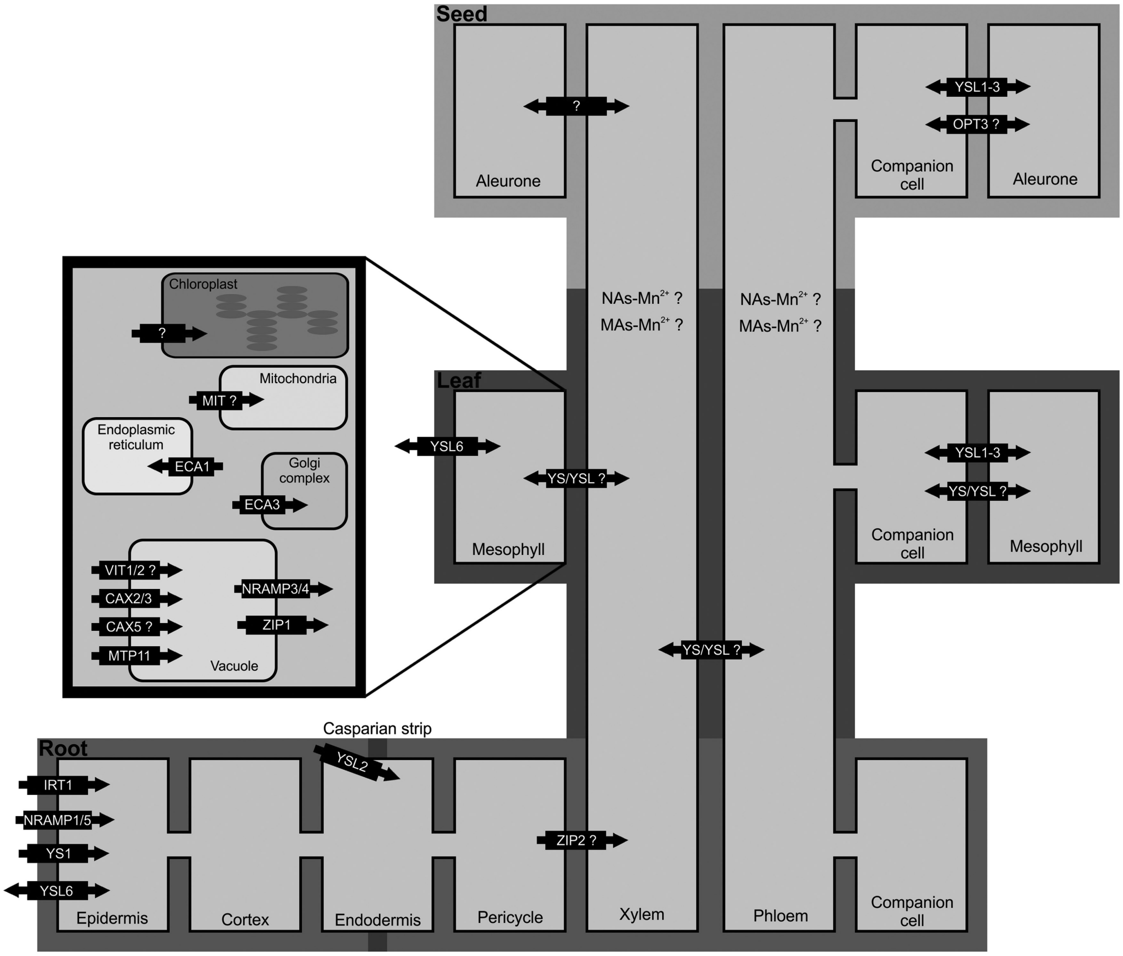

FIG. 5. Manganese transport in a model plant. Mn is taken up into the symplast by membrane transporters in the epidermis in the free form (IRT1 and NRAMP1) and/or complexed with phytosiderophores (YS1). To enter the xylem, Mn must pass the Casparian strip. YSL transporters (e.g., YSL2) appear to have a role in the mobilization of Mn from the cortex to the pericycle. Transport of free Mn into the xylem is thought to occur via members of the ZIP family. In the xylem, Mn is thought to move in the form of Mn-PS complexes to the shoot, where they are unloaded, most likely by members of the YSL family. Xylem transport of Mn is not well characterized. The YSLs may also translocate metals to the phloem, where they can then be delivered to the leaf and seed tissues by various transporters (YS, YSL, OPT). After transport inside the leaf symplast, Mn may be stored in certain tissues or cellular organelles. Manganese transport into mitochondria is hypothesized to occur via MIT. Mn is supplied to the Golgi complex by ECA3 and to the endoplasmic reticulum by ECA1. The transporters CAX2/3 and MTP11 mediate the influx of Mn across the vacuolar membrane into the vacuole. Furthermore, other CAXs (e.g., CAX5) are thought to have a similar role in the influx of Fe into the vacuole, as is VIT1/2. Mn transport into the chloroplast is unknown. (Figure layout based on Palmer and Guerinot, 2009).

Barron-Cruz, 2011), *P. cummunis, T. angustifolia, C. esculentus* (Chandra and Yadav, 2011), *A. caroliniana* (Pandey, 2012), *Alternanthera philoxeroides*, *Eichhornia crassipes* and *P. stratiotes* (Agunbiade *et al.*, 2009; Hua *et al.*, 2012). Of those, the water lettuce (*P. stratiotes*) accumulates high levels of Mn in both roots and shoots (28290 and 26240 μg g^{-1}, respectively) and has been found to meet the characteristics of a good phytoremediator (Lu *et al.*, 2011). Pokeweed (*Phytolacca americana*) was also able to accumulate high levels of Mn (approximately 20000 μg g^{-1} DW) (Dou *et al.*, 2009). Furthermore, two macrophytes, *A. pinnata* and *L. minor*, have shown great potential to remediate Mn (Vaseem and Banerjee, 2012).

For Mn phytoremediation in soil, eight plants were evaluated according to their capacity to tolerate or accumulate Mn. An exclusion strategy was performed with *Equisetum hyemale* and *Telypteris kunthii*, while *Cnidoscolus multilobus, Platanus mexicana, Solanum diversifolium, Asclepius curassavica* and *Pluchea sympitifolia* were employed for an accumulation strategy. Accumulation of Mn in plant leaves was in the range of 182 – 1507 μg g^{-1} DW (Juarez-Santillan *et al.*, 2010). In another study, the same Mn tolerance and accumulation mechanism was tested in six species. Of those, the authors found that *P. perfoliatum*, *P. hydropiper*, and *P. americana* were Mn hyperaccumulators and that *P. perforliatum* had superior Mn accumulation and tolerance (shoots of *P. perfoliatum* reached 18342 μg g^{-1}) over the other five species (Liu *et al.*, 2010b). Five woody plants species (*T. arjuna, P. juliflora, P. alba, E. tereticornis* and *D. strictus*) were evaluated for their capacity to remediate metals from tannery sludge dumps. All plants show the capacity to accumulate Mn in their tissues, but *E. tereticornis* was considered the most effective plant in the remediation of Mn (Shukla *et al.*, 2011). For phytostabilization purposes, the use of *S. paradoxa* may be suitable due to its low root-to-shoot translocation of Mn (Pignattelli *et al.*, 2012).

C. Zinc

In worldwide soils, total Zn content ranges between 60 and 100 mg kg^{-1}; and in soil solution range between 20 and 570 μg L^{-1} (Behera *et al.*, 2011). In soil solution, Zn occurs as both free and complexed ions such as Zn^{2+}, $ZnNO_3^+$, $ZnOH^+$, $ZnHCO_3^+$, $ZnSO_4$ and $Zn(HPO_4)$, which are the most common and mobile Zn species in soil. Zn-organic species may also occur in soil solution (Stephan *et al.*, 2008). The soil–plant system is highly dynamic, and the availability of elements such as Zn is altered due to physical, chemical, and biological changes. The main factors that control the mobility of Zn in soils are very similar to those mentioned for Cu and Mn, and they include pH, SOM, Eh, CEC, the type and concentration of ionic species, carbonate content, particle-size distribution and presence of oxide and hydroxide species. By lowering the soil pH, the adsorption of Zn is reduced, resulting in the mobilization and leaching of Zn. By increasing the Eh in soils, Zn is shifted from unstable to more stable fractions, thus decreasing its mobility (Yang *et al.*, 2012). Clay fraction and SOM are capable of immobilizing Zn, especially at neutral and alkaline pH values (Luo *et al.*, 2011; Zhong *et al.*, 2011). Concentrations of Zn in the soil particle fractions tended to increase with decreasing particle size (Ghiri *et al.*, 2012). The presence of other ionic species in the soil can influence the mobility of Zn in soil (Jalali and Zinli, 2012). Moreover, oxides and hydroxides of Al, Fe, and Mn seem to be important for binding Zn in soils (Zhao *et al.*, 2011).

Zinc is widely used in many industry activities. It is a major component of alloys, batteries, automobile equipment, pipes, and household devices, and it is used as catalyst in the production of rubber, pigments, plastic, lubricants, and pesticides (Wilkinson *et al.*, 2011). Despite the usual low concentrations of Zn in soil, the content of this element can rise to several thousands of mg kg^{-1} due to anthropogenic activities such as agricultural practices and industry and mining activities (Ahmed *et al.*, 2012; Buccolieri *et al.*, 2010). Water pollution by Zn is more notable near industry zones due to waste discharges. Leaching of Zn can occur due to its mobility in soil, increasing water contamination (Gonzalez-Fernandez *et al.*, 2011; Yi *et al.*, 2011).

1. Zinc uptake by plants

The homeostasis of Zn in plants is a complex process. The major mechanisms involved in this process are described in Figure 6. Zn is readily available to plants in its soluble forms, including water-soluble Zn, exchangeable Zn and Zn adsorbed to colloids/OM surface. However, the soluble fraction accounts for less than 10% of total soil Zn content (Kim *et al.*, 2010). Therefore, solubilization of tightly bound Zn is necessary, and it seems to occur by the acidification of the rhizosphere and the secretion of chelating species (Li *et al.*, 2011; Widodo *et al.*, 2010). Then, Zn is taken up across the plasma membrane of root cells as a free ion and/or complexed with phytosiderophores (Ptashnyk *et al.*, 2011; Suzuki *et al.*, 2008). The ZIP transporter family includes the best candidates for facilitating Zn influx into the plant cytoplasm. In addition to its role in Fe acquisition, AtIRT1 is also responsible for the uptake of Zn from the soil solution (Fukao *et al.*, 2011). The broad selectivity of AtIRT1 allows this transporter to mediate the uptake of several divalent metal cations, including Zn (Barberon *et al.*, 2011; Shanmugam *et al.*, 2011). This was also demonstrated in rice plants overexpressing OsIRT1, which accumulate elevated levels of Zn in the shoots, roots and mature seeds (Lee and An, 2009). Like their close homolog IRT1, IRT2 and IRT3 are also able to transport Zn into roots. Arabidopsis overexpressing IRT2 and IRT3 accumulated more Zn than did wild-type plants (Lin *et al.*, 2009; Vert *et al.*, 2009). Other ZIP transporters are involved in the uptake of Zn from soil. For example, OsZIP1 and OsZIP3 are likely to play a role in Zn uptake from soil (Bashir *et al.*, 2012). In Arabidopsis, ZIP4 expression is induced upon Zn deficiency, showing its function as a Zn transporter (Assunção *et al.*, 2010). The high affinity of the divalent metal transporters MtZIP5 and MtZIP6 suggest a role in the uptake of Zn from the rhizosphere (Stephens *et al.*, 2011). This trend was confirmed in a similar study, in which overexpression of OsZIP5 improved Zn uptake from the soil (Lee *et al.*, 2010a). ZIP8, localized in the plasma membrane of rice, seems to be a Zn transporter that functions in Zn uptake (Lee *et al.*, 2010b). Furthermore, AtNRAMP1 is also able to transport Zn from the soil (Cailliatte *et al.*, 2010). Zn is also transported from the rhizosphere in the form of Zn–PS complexes by ZmYS1, although its orthologue in barley, HvYS1, is not able to perform this transport (Murata *et al.*, 2008).

Zinc is exported from root by two members of the P-type ATPase family, HMA2 (expressed in Arabidopsis, rice and barley) and HMA4 (Mills *et al.*, 2012; Takahashi *et al.*, 2012; Wong and Cobbett, 2009). However, other transporters can also perform the export of Zn to vascular tissues. In Arabidopsis, *pcr2* loss-of-function mutants accumulate Zn in roots, suggesting a role of in root-to-shoot translocation of Zn, independent of HMA2 and HMA4 (Song *et al.*, 2010). Furthermore, it was proved that AtFRD3 is involved in loading Zn into xylem (Pineau *et al.*, 2012). Zn can also be exported from the roots in the form of Zn complexes. Before export from the roots, Zn must be mobilized to pericycle cells, apparently by HvYSL2 (Araki *et al.*, 2011). After xylem loading, Zn can be transported to aboveground tissues by the YS and YSL transporters. There is some evidence that AtYSL1, AtYSL2 and AtYSL3 can be involved in the transport of Zn (Chu *et al.*, 2010). Phytosiderophores such as NA and DMA are important in the distribution of Zn within the plant (Harris *et al.*, 2012; Nishiyama *et al.*, 2012; Suzuki *et al.*, 2008). Although until now no Zn-PS transporters have been identified, the presence of these complexes in the xylem and phloem sap suggests that members of the YSL family may

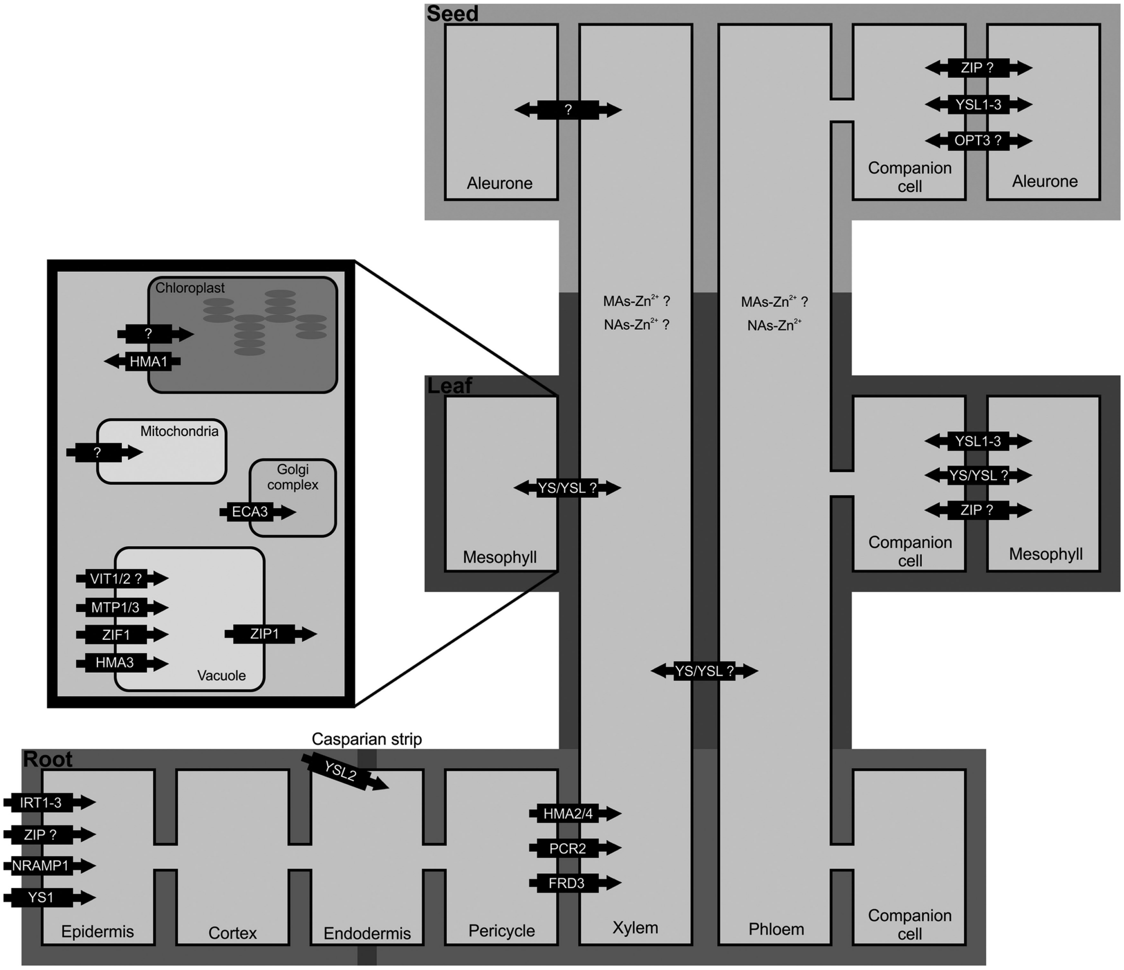

FIG. 6. Zinc transport in a model plant. Zn is taken up into the symplast by membrane transporters in the epidermis as a free cation (IRT1–3, NRAMP1 and ZIP) and/or complexed with phytosiderophores (YS1). To enter the xylem, Zn must pass the Casparian strip. YSL transporters (e.g., YSL2) appear to have a role in the mobilization of Zn from the cortex to the pericycle. Transport of free Zn into the xylem is thought to occur via the HMA2/4, PCR2 and FRD3. In the xylem, Zn is thought to move in the form of Zn-PS complexes to the shoot, where they are unloaded, most likely by members of the YSL family. Xylem transport of Zn is not well characterized. The YSLs may also translocate metals to the phloem, where they can then be delivered to the leaf and seed tissues by various transporters (ZIP, YS, YSL, OPT). After transport inside the leaf symplast, Zn may be stored in certain tissues or cellular organelles. Zn transport into mitochondria has not been characterized. The supply of Zn to the Golgi complex is performed by ECA3. The transporters HMA3, ZIF1 and MTP1/3 mediate the influx of Zn across the tonoplast into the vacuole. Furthermore, both VIT1 and VIT2 are thought to have a similar role in the influx of Fe into the vacuole. The efflux of Zn from the vacuole appears to be performed by ZIP1. Zn influx to the chloroplast is unknown, but its efflux is mediated by HMA1. (Figure layout based on Palmer and Guerinot, 2009).

be involved in the transport of Zn complexes (Deinlein *et al.*, 2012; Rellan-Alvarez *et al.*, 2008). Moreover, members of the ZIP family are expressed in the vascular bundle, suggesting that they contribute to the distribution of Zn along the plant (Bashir *et al.*, 2012). AtOPT3 and its orthologue TcOPT3 seem to participate in the long-distance transport of Zn (Hu *et al.*, 2012; Stacey *et al.*, 2008).

The delivery of Zn to plant organelles is also mediated by specific transporters. The vacuolar membrane transporter AtMTP1 and its orthologue HvMTP1 have an essential role in detoxification of excessive Zn. When grown in excessive Zn, the mutant line of Arabidopsis that lacks MTP1 was not able to accumulate Zn in vacuoles, unlike wild-type roots (Kawachi *et al.*, 2009; Podar *et al.*, 2012). AtMTP3 is also localized in the tonoplast, where it contributes to basic cellular Zn tolerance and controls Zn partitioning (Arrivault *et al.*, 2006). Similarly, AtHMA3 seems to play a role in the detoxification of Zn by participating in its vacuolar sequestration (Morel *et al.*, 2009). It has been speculated that ZIF1 is involved in a mechanism of Zn sequestration. Specifically, AtZIF1 has been implicated in the transport of Zn complexes (mainly with NA) into the vacuole, as overexpression of ZIF1 leads to strongly enhanced vacuolar

Zn accumulation (Haydon *et al.*, 2012). The tonoplast-localized OsVIT1 and OsVIT2 seem to transport Zn into the vacuoles of plant cells (Zhang *et al.*, 2012b). AtZIP1 may contribute to remobilizing Zn from the vacuole to the cytoplasm (Milner *et al.*, 2013). In the chloroplasts of both Arabidopsis and barley, HMA1 contributes to Zn detoxification by exporting Zn from the plastids to the cytoplasm (Kim *et al.*, 2009; Mikkelsen *et al.*, 2012). It has been proposed that the Golgi-localized transporter AtECA3 may have a role in the mobilization of Zn from the cytoplasm to the Golgi complex (Barabasz *et al.*, 2011; Mills *et al.*, 2008).

In most crops, the optimal Zn content ranges between 15 and 50 μg g^{-1} DW (Hansch and Mendel, 2009; White and Brown, 2010). Below 15 μg g^{-1}, plants exhibit Zn deficiency symptoms. Zn toxicity is usually observed when plant Zn content is above 100 μg g^{-1} (Hajiboland and Amirazad, 2010; Zhao *et al.*, 2012).

2. *Phytoremediation of Zn-contaminated sites*

The phytoremediation of Zn-contaminated sites can be performed in soil and water. In water, phytoremediation of Zn is well performed by *E. crassipes*, which achieves a 95% removal of this metal (Mishra and Tripathi, 2009). Similar percentages of Zn removal were also observed when *A. pinnata* and *L. minor* were used (Vaseem and Banerjee, 2012). Other plant species such as *T. latifolia* (Sasmaz *et al.*, 2008), *C. ligulata, J. imbricatus* (Miguel *et al.*, 2013), *E. acicularis* (Ha *et al.*, 2009), *P. cummunis, T. angustifolia*, *C. esculentus* (Chandra and Yadav, 2011) and *P. stratiotes* (Lu *et al.*, 2011) have shown potential to remediate Zn-contaminated waters.

In soil, phytoremediation of Zn can be conducted by several plant species. Some examples are *Pennisetum americanum* × *Pennisetum purpureum, Paspalum atratum* (Zhang *et al.*, 2010), *Z. fabago* (Boojar and Tavakkoli, 2011), *Betula pendula, Robinia pseudoacacia, Populus tremula* (Van Nevel *et al.*, 2011), *Noea mucronta* (Chehregani *et al.*, 2009), *Glycine max* (Murakami and Ae, 2009) and *Salix smithiana* (Puschenreiter *et al.*, 2013). Hyperaccumulation of Zn can also be observed. A wild population of *Arabis paniculata* was shown to be hypertolerant of extremely high concentrations of Zn and could accumulate an average of 20800 μg g^{-1} of this element in its shoots (Tang *et al.*, 2009). Furthermore, plants such as *Arabidopsis halleri, Sedum alfredii* and *Noccaea caerulescens* are widely known Zn hyperaccumulators (Deinlein *et al.*, 2012; Kramer, 2010). Phytostabilization of Zn is also a viable technology for application in contaminated soils, and several plants have been well established to perform this task. For example, the perennial shrub *Sesbania virgata* is an excellent species for use in Zn contaminated soils (Branzini *et al.*, 2012). Other plant species that are good candidates for phytostabilization are *Quercus robur, Quercus petraea, Pinus sylvestris, Pseudotsuga menziesii* (Van Nevel *et al.*, 2011) and *S. paradoxa* (Pignattelli *et al.*, 2012).

V. STRATEGIES FOR IMPROVING THE PHYTOREMEDIATION OF TRACE ESSENTIAL METALS

Aging of soil promotes the immobilization of Cu, Mn and Zn by shifting these elements from labile to non-labile fractions. The reduction of the available fraction of these trace metals is one of the major constraints of phytoremediation efficiency (Donner *et al.*, 2012). Moreover, the application of phytoremediation in heavily contaminated soils may become very difficult because plants cannot yield sufficient biomass and/or withstand the metal stress in such severe conditions (Sirguey and Ouvrard, 2013). To surpass such limitations, several strategies have been proposed, including the application of amendments, the use of bacteria and fungi associated with plants and the application of genetic engineering to improve plant processes.

To improve the phytoremediation of Cu, Mn and Zn, the application of amendments (organics and inorganics) has been widely studied and has proven to be a useful way to manage some of the problems related to trace metal bioavailability and plant growth (Gunawardana *et al.*, 2011; Najeeb *et al.*, 2009; Perez-Esteban *et al.*, 2013). Amendments can be used to enhance the bioavailability of trace metals, which will improve phytoextraction, or to reduce the labile fraction of trace metals, to assist in phytostabilization (Padmavathiamma and Li, 2010; Wu *et al.*, 2012a).

Another way to improve the phytoremediation of Cu, Mn and Zn relies on the use of endophytic bacteria. Endophytes are microbes that live inside plant tissues without causing harm to the host and can facilitate plant growth as well as increase resistance to pathogens, drought and herbivores (Rajkumar *et al.*, 2012). Plant growth-promoting bacteria can be exploited for promoting plant biomass production and metal phytoremediation in contaminated soils (Andreazza *et al.*, 2010; Kumari and Singh, 2011). In addition to bacteria, the arbuscular mycorrhizal fungi (AMF) are also involved in phytoremediation of Cu, Mn and Zn. AMF are ubiquitous terrestrial symbionts involving functioning parts of plants living in mutualistic association with the fungi (Meier *et al.*, 2012). Symbiotic mycorrhizal associations can increase the efficiency of phytoremediation due to improvements in the uptake and delivery of nutrients to the plant and in tolerance to metal toxicity (Bissonnette *et al.*, 2010; Cornejo *et al.*, 2013; Hernandez-Ortega *et al.*, 2012). For example, the nonpathogenic *Fusarium fungus* was able to increase *S. alfredii* root system function, metal availability and accumulation, plant biomass, and thus Zn phytoextraction efficiency (Zhang *et al.*, 2012a).

Phytoremediation of Cu, Mn and Zn can also be improved by controlling the biochemical processes that occur inside and outside the plant at the cellular and molecular level. The expression of specific membrane transporters in transgenic plants that are responsible for metal uptake, translocation and storage is currently being used to improve phytoremediation processes. The expression of OsZIP1 in finger millet and tobacco (*Nicotiana tabacum*) results in significantly higher accumulation of Mn and

Zn (Ramegowda *et al.*, 2012). Overexpression of *Ta*HMA2 in rice increased the elongation and decreased the seed-setting rate and root-to-shoot Zn translocation (Tan *et al.*, 2013). Heterologous expression of AhHMA4 in tomato facilitates root-to-shoot Zn translocation and induces Zn uptake (Barabasz *et al.*, 2012). Similarly, expression of AtHMA4 in tobacco could be a candidate transporter for engineering modifications of Zn translocation (Siemianowski *et al.*, 2011). The Golgi-localized AtECA3 was introduced into tobacco, resulting in better growth of the plants and enhanced tolerance of high Mn and Zn concentrations (Barabasz *et al.*, 2011). Further, the expression of AtCAX2 or AtCAX4 in *Nicotiana tabacum* results in enhanced tolerance of Mn (Korenkov *et al.*, 2007). The chelation capacity of PCs and MTs to detoxify metals in plant can also be improved through plant engineering (Machado-Estrada *et al.*, 2012; Vurro *et al.*, 2011). The overexpression of phytochelatin synthase 1 (PtPCS1) in poplar results in a two-fold increase of Zn accumulation in leaf tissues (Adams *et al.*, 2011). Transgenic poplar lines expressing MT type 2 gene (PsMTA1) from *Pisum sativum* show increases in the ability to translocate and accumulate Cu during metal stress without a significant increase in reactive oxygen species (Turchi *et al.*, 2012). Transgenic tobacco expressing EhMT1 (metallothionein type 1 protein from *Elsholtzia haichowensis*) shows high tolerance to and accumulation of Cu and a more efficient antioxidant system, suggesting that MT1 should be considered as a potential candidate for enhancement of Cu tolerance in plants (Sekhar *et al.*, 2011). In the same way, the metallothionein type 1 protein from *Cajanus cajan* (CcMT1) was expressed in *Arabidopsis thaliana*, resulting in higher accumulations of Cu and providing a marked tolerance of metal stresses (Sekhar *et al.*, 2011). Another metallothionein (pCeMT) was isolated from *Colocasia esculenta* and overexpressed in tobacco, which exhibited enhanced Cu tolerance and accumulation and better growth compared with control (Kim *et al.*, 2013). Moreover, the biosynthesis of other metal chelators such as NA and DMA can be improved to enhance metal accumulation in different plant parts. The overexpression of the barley NA synthase HvNAS1 in rice showed increases in endogenous phytosiderophore content in shoots, roots, and seeds, resulting in higher Zn levels in rice seeds (Masuda *et al.*, 2009). Similarly, overexpression of OsNAS2 in rice results in a 16-fold increase in the total content of Zn complexes with both NA and DMA. Moreover, high levels of NA inside the plant led to greater exudation of PS from the roots, thereby stimulating Zn uptake, translocation and seed-loading (Lee *et al.*, 2011). Finally, the expression of genes from non-plant organisms can also improve the phytoremediation efficiency. Transgenic Arabidopsis overexpressing the yeast transcription factor ACE1 increased the activity levels of superoxide dismutase and peroxidase, benefiting the cell in response to toxic levels of Cu (Xu *et al.*, 2009). Transgenic poplar plants expressing the heavy metal resistance gene ScYCF1 (yeast cadmium factor 1) showed enhanced growth, reduced toxicity symptoms, and increased Zn content (Shim *et al.*, 2013).

VI. FINAL REMARKS

In recent years, phytoremediation has arisen as a practical, low cost and environmentally friendly technology. It is considered the best approach for removing metal pollution. The major drawbacks of metal phytoextraction processes are the bioavailability of the target metal(s) and the ability of plants to accumulate metals within their aboveground biomass at levels well above their nutrient requirements. Recent research in the field of phytoextraction has been focused on finding the ideal metal-accumulating plants and metal uptake and translocation mechanisms. Currently, several plant species are known to hyperaccumulate Cu, Mn and Zn. Although metal hyperaccumulation in plants is poorly understood, outstanding progress has been made in the last few years to understand the biochemical and molecular mechanisms of that process.

During the last few years, several reports of attempts to increase metal bioavailability through the addition of various amendments have been published. Although this approach often works on a laboratory scale, it is much less effective in the field. The use of plant-bacteria associations to improve metal phytoremediation can significantly help the growth of plants in the presence of high (and often phytotoxic) levels of metals, but the bacteria typically do little or nothing to increase metal phytoavailability. However, bacterially assisted phytoremediation is a recent technology, and better understanding of how different types of bacteria contribute to phytoremediation is needed. Regarding plant-fungi associations, there is plenty of evidence in the literature that demonstrates the important role of these associations in promoting plant status in metal-polluted soils. Although there have been recent advances in understanding the physiological mechanisms behind these associations, more investigation is needed to ascertain their functional compatibility and to prove their efficiency in promoting phytoremediation.

Key processes in the metal homeostasis network whose alteration drastically modifies metal tolerance and distribution in plant tissues have been identified. Several genes (e.g., COPT, ZIP, NAS, YSL, HMA) have been functionally cloned, and their importance in metal accumulation has been established. More broadly, genetic engineering has opened a new range of opportunities for understanding metal hyperaccumulation and hypertolerance. Though much remains to be learned about Mn and Zn uptake, transport and homeostasis, a full understanding of Cu homeostasis is near. In addition, an integrative approach using diverse plant species will lead to a better understanding of metal uptake, transport and distribution. Although there is limited evidence for coordination of the regulatory networks that control Cu, Mn and Zn homeostasis in plants, there is some crosstalk. Because different metals use the same transporters, there is clear evidence for interaction. For example, the IRT1 transporter performs both Mn and Zn uptake; therefore, these metals affect each other's uptake. Moreover, low Fe leads to IRT upregulation, which in turn allows more Zn uptake. Zn toxicity may therefore be a secondary effect of Fe deficiency. Other membrane transporters have the same capacity to transport different metals, so a complex coordination process is thought to occur.

Many of the challenges in plant metal homeostasis, from uptake to storage, have been overcome. There is significant evidence that the regulation of metal ion homeostasis is mediated by membrane transporters. A multigene strategy must be carried out by the use of modern molecular techniques to further understand the complex regulatory processes in plants in order to improve metal accumulation in high biomass non-hyperaccumulator species useful for phytoremediation. More work is necessary to transfer genes into high-biomass or crop species for phytoremediation. There is a need to be cautiously optimistic about the application of phytoremediation, taking into account the limits of this strategy. Bringing this technology into common practice is also a challenge. Laboratory conditions using optimal hydroponic growth conditions and exposure to single metals are excellent for understanding physiological processes and the functions of new genes, but they do not represent conditions in the field. Therefore, it is worthwhile to perform phytoremediation tests of transgenic and non-transgenic plants in actual metal-contaminated sites.

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