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REVIEW

Low-molecular weight organic acids and peptides involved in the long-distance transport of trace metals

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Abstract Higher plants evolved mechanisms of uptake, distribution and accumulation of trace metals essential for the proper functioning of the organism (e.g., copper, zinc). Non-essential metals (e.g., cadmium, arsenic, lead) can also enter plant cells using the routes dedicated to the essential ones, because of the shared similar chemical and physical properties. Generally, trace elements are very reactive, able to generate reactive oxygen species and to interact or bind various organic ligands composed of C, H, O, N, P or S. Thus, after entering to the cells, metals are transported and sequestered mainly in a complex form, bound with amino acids, organic acids, peptides or specific metal-binding ligands. Considering diverse properties (e.g., pH value, abundancy of ions, redox state) characterizing cells, tissues and phloem or xylem sap, plants use various ligands to form stable complexes in different conditions. This literature review aims to provide a comprehensive overview on the role of low-molecular weight acids and peptides in trace metals translocation.

Keywords Phytoextraction · Trace metals · Long-distance transport · Source-to-sink transport

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Introduction

The use of plants to extract trace metals (TMs) from polluted soils has been extensively studied since early 90s of the twentieth century. In 1994, the terms 'phytoremediation' and 'phytoextraction' were coined (Raskin et al. 1994; Raskin 1996), but even before then the idea of using plants for a clean-up of the environment was present as a notion of green remediation (Baker and Brooks 1989; Baker et al. 1994; Fellet and Marchiol 2011). Phytoextraction has three prime areas of interest: metal uptake, plant's response to metals and mechanisms of metal translocation to the above-ground tissues. At first, researchers focused on root uptake, because the flow of trace metals in plants begins underground (Baker 1981; Baker and Walker 1990; Verbruggen et al. 2009a). Then, their focus shifted to the influence of trace metals on defense systems (antioxidative and detoxicative), metal chelation, sequestration, and accumulation (Steffens 1990; Chaney et al. 1997; McGrath and Zhao 2003, Pal and Rai 2010; Yadav 2010; Anjum et al. 2012). Now, authors study the translocation of trace metals to above-ground parts of plants to improve efficiency and cost-effectiveness of phytoextraction, or enrich seeds with essential micronutrients (Palmgren et al. 2008; Cakmak et al. 2010; Krämer 2010; Rascio and Navari-Izzo 2011; Waters and Sankaran 2011).

Trace metals can be subdivided into two groups based on the organism's demand: essential (Cu, Zn, Ni, Fe, Mn, Co) and non-essential (Cd, Hg, As, Pb) elements (Nagajyoti et al. 2010; Vamerali et al. 2010). Both essential and non-essential metals threaten cell's homeostasis as their chemical structure—an incomplete atomic sub-shell—grants them high reactivity towards organic molecules and ability to generate reactive oxygen species (Diwan et al.

2012; Lin and Aarts 2012; Pandey and Singh 2012). Due to their reactivity, trace metals are transported for longer distances and stored in cells mainly in a complex form, bound with amino acids, organic acids, peptides or by specific metal-binding ligands (Nagajyoti et al. 2010).

This review aims to discuss the recent experimental and computational data on the translocation of trace metals. Special attention is given to the long-distance transport in vascular bundles (xylem and phloem) and the role of low-molecular organic acids and peptides in this process.

Biochemical properties of metal–ligand complex formation in plants

Metals in plants function in a number of different ways (Roat-Malone 2007):

1. macronutrients, e.g., Mg^{2+} is structural element of chlorophyll, Ca^{2+} is involved in signal transduction pathways as a intracellular messenger molecule, they also maintain charge and osmotic balance;
2. transition metal ions existing in single oxidation states are building elements, e.g., Zn^{2+} in superoxide dismutase and zinc fingers;
3. trace metals with multiple oxidation states are electron carriers (Fe ions in cytochromes, Fe–S clusters of the nitrogenase, Cu^{2+} in cytochrome c oxidase) and facilitate enzymatic catalysis (Cu^{2+} in superoxide dismutase, Fe cations in cytochrome c oxidase or Fe and Mo^{2+} cations in nitrogenase).

Due to the high reactivity, metal ions in biological systems are transported and sequestered in a complex form as simple hydrated cations or structures formed by interactions with various organic ligands. The most important principles of metal–ligand binding are possible coordination geometries of the cationic center and ligand preference (Table 1). Coordination by ligands is closely related to the hard–soft acid–base theory (HSAB theory), which determines interaction preferences—hard metal cations (e.g., Mg^{2+}, Ca^{2+}, Co^{3+}, Fe^{3+}) form most stable compounds with hard ligands (e.g., carboxylates, sulfates, alcohols, amines, phosphates) in opposition to soft metal cations (e.g., Cu^{+}, Hg^{2+}, Cd^{2+}), which strongly bind to the soft ligands (e.g., thiols, phenyl groups) (Hay 1984). Apart from the HSAB theory, the stability of metal–ligand complex is influenced by the properties of surrounding solution (cell plasma, phloem sap, xylem sap, etc.), which is a thermodynamic factor, as well as the rate of the ligand exchange in enzymatic reactions, which is a kinetic factor.

Thermodynamic stability depends on concentrations of the metals and ligands at the local complexation site. The type of complex formed is a result of many competing equilibria constants e.g., solubility of products, complexation and acid–base balance (Roat-Malone 2007). The kinetic factor of the complex formation is a separate issue. There is no proved connection between its thermodynamic stability and kinetic lability. Metal complexes can be divided on kinetically passive and labile. In passive complexes, ligand substitution occurs slowly, opposite to labile complexes where ligands are substituted rapidly (Hay 1984).

Biochemical properties of metal (Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Fe^{3+}) complex formation in plants were studied by Curie and coworkers (Curie et al. 2009), with the example of nicotianamine, deoxymugineic acid and citrate as ligands. As a conclusion, the pH value of the surrounding medium was pointed as a dominant factor of ligand exchange. Moreover, it was suggested that in case of trace metals with multiple oxidation states that act as electron carriers (Fe^{2+}/Fe^{3+} and Cu^{+}/Cu^{2+}), the ligand switch is determined by the adjacent redox reaction.

From root to shoot

Plant's demand for essential metals must be fulfilled by acquisition of ions present in soil. Close similarities in chemical and physical nature of essential and non-essential metals challenge plants to uptake unwanted trace elements. To maintain balance in metal homeostasis and to keep the toxicity within physiological limits, plants employ a range of different chelates to transport, sequestrate and prevent cytoplasmic precipitation of metals (Pal and Rai 2010; Hassan and Aarts 2011; Sinclair and Krämer 2012). Studies indicate that trace metals can be bound in cells by: low-molecular weight organic acids (LMWOA; citrate, malate, malonate, oxalate), aminoacids (histidine, cysteine, asparagine, glutamate), short peptides (glutathione, phytochelatins, nicotianamine, phytosiderophores), and proteins (eg., metallothioneins) (Haydon and Cobbett 2007; Harris et al. 2012). Plants use LMWOA and short peptides for metal-trafficking, and peptides for sequestration (Freisinger 2008; Haydon et al. 2012).

Mechanisms of trace metals uptake to root have been extensively studied (reviewed by: Palmer and Guerinot 2009; Morrissey and Guerinot 2009; Hassan and Aarts 2011; Sinclair and Krämer 2012; Milner et al. 2013). Once metals cross the root plasma membrane of epidermis (or endodermis, if they were transported apoplastically till Casparian strip), they are either transported into vacuoles or undergo symplastic transport towards the vascular cylinder (Mendoza-Cozatl et al. 2011).

Xylem loading involves export of metals from root cells symplast through their membranes and into the vascular

Table 1 Chemical properties and preferred ligands of metals

Element	Coordination number and geometry	Preferred ligands and examples	Function and examples
Macro- and microelements			
Magnesium (Mg^{2+})	6, octahedral	O-Carboxylate and phosphate *Phloem sap—hydrated ion, citrate*	Structure in hydrolases, chlorophyll, and isomerases, phosphate transfer, triggers reactions
Calcium (Ca^{2+})	6–8, flexible	O-Carboxylate, carbonyl and phosphate *Phloem sap—hydrated ion, citrate*	Structure, charge carrier, phosphate transfer, signal transduction, triggers reactions
Manganese (Mn^{2+}, Mn^{3+})	Mn(II)—6, octahedral Mn(III)—4 tetragonal	O-Carboxylate, phosphate, Mn(II)—N-imidazole, Mn(III)—hydroxide *Xylem sap—citrate and malate* *Phloem sap—free ion, NA, Glu*	Structure in oxidases, photosynthesis
Iron (Fe^{2+})	4, tetrahedral 6, octahedral	S-Thiolate (4) O-Carboxylate, alkoxide, oxide and phenolate (6) *Xylem sap—NA, citrate* *Phloem sap—NA*	4—Electron transfer, nitrogen fixation in nitrogenases 6—Electron transfer in oxidases
Iron (Fe^{3+})	4, tetrahedral 6, octahedral	S-Thiolate (4) O-Carboxylate, alkoxide, oxide and phenolate (6) *Xylem sap—DMA, citrate* *Phloem sap—Glu, NA, citrate*	4—Electron transfer, nitrogen fixation in nitrogenases 6—Electron transfer in oxidases
Nickel (Ni^{2+})	4, square planar	S-Thiolate, thioether, N-imidazole and polypyrrole *Xylem sap—hydrated ion, citrate* *Phloem sap—NA, His*	Hydrogenases, hydrolases
Copper (Cu^{2+})	4, square planar 5, square planar 6, tetragonal	O-Carboxylate and N-imidazole *Xylem sap—free ion, NA, DMA and amino acids* *Phloem sap—NA, His, Asn, Gln, Ser*	Type II copper oxidases, hydroxylases Type III copper hydroxylases
Zinc (Zn^{2+})	4, tetrahedral 5, square pyramid	O-Carboxylate (4, 5), phosphate (4, 5), N-imidazole (4, 5) and S-thiolate (4) *Xylem sap—citrate and malate, hydrated ion* *Phloem sap—NA, Cys*	4—Structure in zinc fingers, gene regulation, anhydrases, dehydrogenases 5—Structure in hydrolases, peptidases
Non-essential metals			
Arsenic (As^{3+}, As^{5+})	As(III)—3, trigonal pyramidal As(V)—6, octahedral or distorted octahedral	*Phloem sap—As(III)—GSH and PCs*	Toxic
Cadmium (Cd^{2+})	6, octahedral	Sulfhydryl groups *Xylem sap—citrate* *Phloem sap—GSH and PCs*	Toxic
Lead (Pb^{2+})	4, tetrahedral	*Xylem sap—citrate* *Phloem sap—GSH and PCs*	Toxic

NA nicotianamine, *Glu* glutamate, *DMA* 2′-deoxymugineic acid, *His* histidine, *Asn* asparagine, *Gln* glutamine, *Ser* serine, *Cys* cysteine, *GSH* glutathione, *PCs* phytochelatins

bundles. HMA2 and HMA4 (heavy metal ATPase) belonging to the family of P_{1B}-type ATPase are probably the most important transporters involved in this process. They are localized to the plasma membrane (PM) and mediate xylem loading for divalent cations (Hussain et al. 2004; Hanikenne et al. 2008; Wong and Cobbett 2009; Siemianowski et al. 2011). Transpiration imposes fluid movement from root to shoot and enables fast translocation of water, nutrients and micro- and macroelements above the ground (Fig. 1).

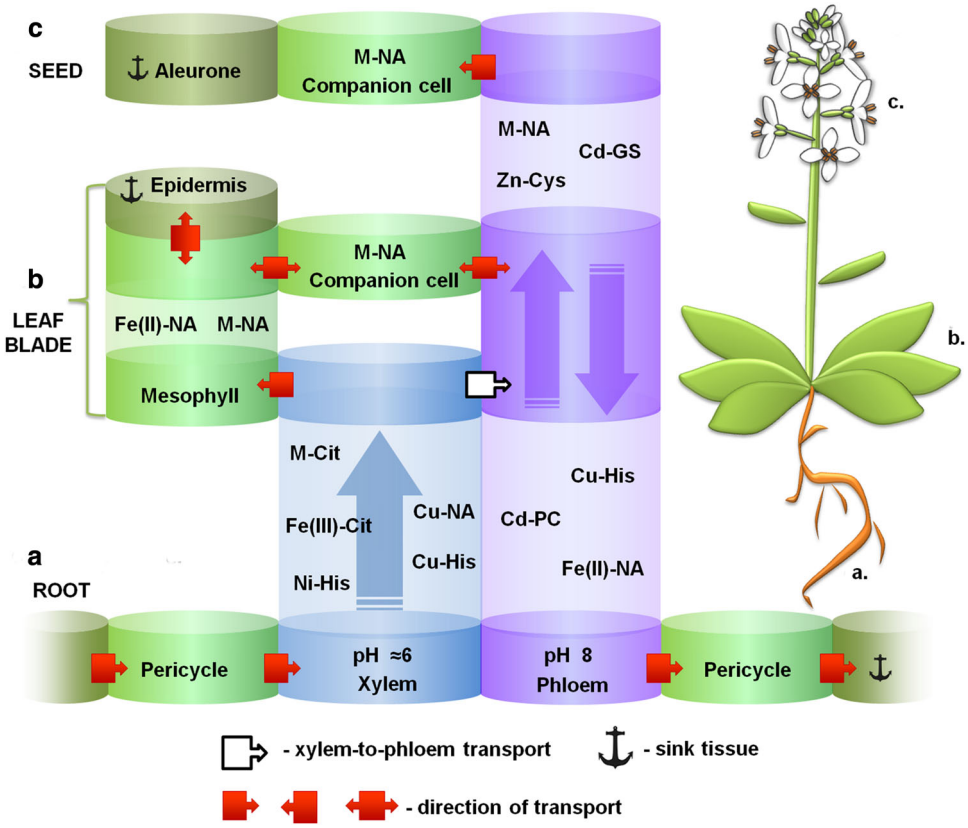

Fig. 1 Mechanisms of long-distance and source-to-sink transport of trace metals (TMs) in plants. *Anchor* symbol indicates sink tissues, where TMs can be sequestered. **a** Once loaded to the xylem, in a slightly acidic pH TMs (Fe, Zn, Cu, Mn, Pb, Cd) are mainly bound to citric acid. Histidine plays a role as a ligand for Ni and Cu (supplied in excess), while nicotianamine (NA) complexes Cu under copper starvation. **b** After xylem unloading in leafs, TMs are transported bound to NA and distributed symplastically in leaf blade or stored in vacuoles, preferably of epidermis. Re-mobilization of TMs from sink vacuoles occurs after new leaf formation or shift to the reproductive growth. Metals are transported in symplast towards companion cells which are connected with the phloem and involved in phloem loading. Nicotianamine is a main ligand of TMs in the phloem (pH 8). Other ligands, including amino acids, glutathione and phytochelatins are also involved in metals chelation (Fig. 4). Transport in phloem can occur both *upwards* (**c**) and *downwards* (**a**) to sink tissues—seeds, new leafs and root cells. Transfer of TMs directly from the xylem to the phloem had been already identified in rice and occurs in nodes. Iron oxidation state changes in different tissues: ferric form Fe(III) is dominant in xylem and after sequestration in vacuoles of the sink tissues, while ferrous Fe(II) is prevalent in phloem and during short-distance transport in symplast of root, mesophyll and companion cells. Graminaceous plants can substitute nicotianamine with phytosiderophores (2′-deoxymugineic acid) for transport of TMs

Xylem sap has a slightly acidic pH of 5–6.5. It contains a mixture of carboxylic acids (2–9 mM) and amino acids (1–3 mM) (White et al. 1981; Lopez-Millan et al. 2000, 2001). This information came from the studies on the xylem sap from *Beta vulgaris, Pyrus communis L, Glycine max, Lycopersicon esculentum.* This environment favors complexation of metals with the carboxylic acids rather than amino acids. Characterizations of xylem and even computational analyses of metal speciation have been ongoing for several decades, because xylem sap collection is relatively easy (White et al. 1981; Mullins et al. 1986). Both approaches—experimental and in silico simulations—for many years suggested a great role of LMWOA as ligands of various metals in xylem sap.

Translocation to shoots ends with xylem unloading. Trace metals present in xylem sap are taken up by surrounding cells and transported symplastically in leaf tissue, or they continue to be distributed apoplastically in the leaf blade. Symplastic transport is most likely ensured by the chelation with nicotianamine (NA) coupled with action of trafficking transporters from the YSL family (reviewed by: Curie et al. 2009; Conte and Walker Elsbeth 2011; Hassan and Aarts 2011). If this proposed model for xylem unloading is correct, some questions remain unanswered. Before surrounding cells can uptake trace metals complexed with nicotianamine, TMs have to change their ligands from citrate to NA. Relatively, acidic pH of the apoplast favors complexation of metals with carboxylic acids, therefore rendering this ligand-shift unlikely, especially for iron (Rellan-Alvarez et al. 2009). This suggest two possibilities: (1) additional biochemical transformation at site of xylem unloading, and/or (2) involvement of other transporters.

Arabidopsis thaliana genome encodes four genes of niocotianamine synthase (NAS) with varying tissue expression patterns (in roots, leaves, petals, sepals) and differently regulated. Single loss-of-function mutations in *NAS* genes in *Arabidopsis* did not change significantly NA contents in mutants compared to the wild type, because NA can be transported throughout the whole plant. Triple mutants (*nas4x*-1) had reduced NA levels to 30–40 % of wild type, while quadruple *Arabidopsis* mutants *nas4x-2* with knock-out in all four functional genes showed no production of nicotianamine (Schuler and Bauer 2011).

Leaf cell vacuoles are used for sequestration of excess essential and non-essential metals, whether for detoxification or temporary storage. Epidermis appears to be preferred for TMs sequestration, of all cells present in leaf blade, because most epidermic cells lack chloroplasts and are photosynthetically inactive (Hassan and Aarts 2011). TMs compartmentation in epidermis occurs also in idioblasts (Katayama et al. 2013). If metals are not fully sequestered in leaf vacuoles or if they are being re-mobilized during reproductive growth, they can reach phloem parenchyma and companion cells through plasmodesmata. Complexes with TMs formed within companion cells or transported to them are likely to enter phloem and begin transport towards sink tissues (e.g., seeds, roots, young leaves) (Chen et al. 2006; Li et al. 2006). This route is coincident with transport of photoassimilates from source-to-sink.

From shoot to root

Phloem sap serves as medium nourishing different parts of the plant. It is rich in sugars, amino acids and metal ions re-mobilized from leaves. Depending on the type of plant, collection of phloem sap can be very problematic. In case of most plants, phloem clogs very easily, due to its high viscosity. Clogging can be prevented by insertion of freshly cut end of the plant stem in a solution containing EDTA. However, dilution of unknown volume of phloem sap in a collection solution hinders calculations of the absolute concentrations of the sap components (King and Zeevaart 1974). Another method of sap collection involves use of aphids. Aphids insert their stylets into the plant and extract phloem from the incision. After aphid starts to feed, stylets are severed by a high-power laser and sap continues to flow to a capillary. The volume collected from each stylet is typically 10–150 µL (Harris et al. 2012).

Harris et al. (2012) proposed recently a sophisticated computer model for the speciation of trace metals (Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}) present under normal conditions in a *generic* phloem sap. Generic phloem sap composition has a pH of 8 and contains, among other parameters: 150 mM of a mixture of 20 amino acids, 200 µM of nicotianamine, 6.9 mM of phosphate, 15 mM of dicarboxylic acids (Harris et al. 2012). This model was developed with data gathered from available analytical studies of the chemical composition of phloem from various plants; and will be used to present metal speciation in phloem sap in subsequent parts of this review.

Iron trafficking

Iron is an essential element and plants developed two strategies to acquire it. Non-graminaceous plants (called Strategy I plants) lower rhizospheric pH by extruding organic acids and protons reduce Fe to Fe^{2+} by root ferric chelate reductase and then use ferrous transporter IRT1 for metal uptake. Graminaceous plants (Strategy II plants) enzymatically synthesize phytosiderophores (PS) (e.g., 2′-deoxymugineic acid) from S-adenosyl methioninie via the intermediate nicotianamine (NA), and secrete them into rhizosphere (Fig. 2). Phytosiderophores increase Fe solubility and form complexes with metals (e.g., Fe(III)-PS, Zn(II)-PS) that are then taken up into the root by YSL (yellow stripe like) family of transporters. Non-graminaceous plants do not synthesize PS, though they have the ability to produce nicotianamine. Both strategy type plants use nicotianamine in intracellular metal chelation and in long-distance transport in phloem and xylem (Curie et al. 2009). In addition, phytosiderophore 2′-deoxymugineic

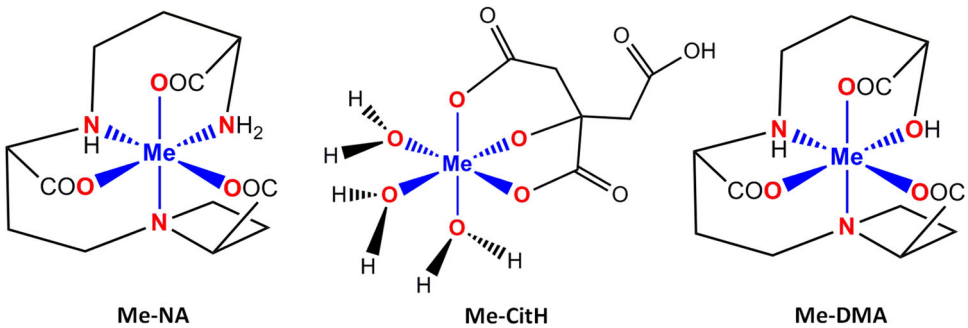

Fig. 2 Simplified structure of a metal complex with nicotianamine (NA), citric acid (CitH) and phytosiderophore 2′-deoxymugineic acid (DMA) in neutral pH. Me metal, *blue label* metal coordination site, *red label* ligand atoms directly interacting with metal. Generated with ChemBioDraw 12.0 (color figure online)

acid may play a similar role as a ligand for metals in xylem sap of graminaceae (Ando et al. 2012).

Fe translocation in xylem strongly depends on the action of FRD3 transporter (member of the MATE family) that loads citrate to xylem. FRD3 is expressed only in pericycle and cells surrounding the vascular tissue (Green and Rogers 2004). Citrate abundance in xylem enables Fe solubilisation and translocation to shoot (Roschzttardtz et al. 2011; Kobayashi and Nishizawa 2012). Studies on *Arabidopsis* plants showing natural variations in *FRD3* gene have shown that FRD3 is probably important as a control point mediating cross-talk between Zn and Fe. High concentrations of Zn in the medium decreased Fe content in shoots of studied plants, and vice versa (Pineau et al. 2012). A candidate transporter for xylem loading of Fe has been identified by Morrissey et al. (2009). FPN1 (ferroportin1, also called IREG1: iron-regulated protein1) expression patterns: in the stele of the root, at the root–shoot junctions and in the leaf veins, indicate a role in efflux into the apoplast and/or xylem. Plants with *fpn1* mutations show leaf chlorosis related to Fe deficiency and translocate decreased Co levels to shoots (Morrissey et al. 2009). A part of iron loaded into xylem is transported by YSL family of proteins. YSL proteins are known to transport both metal-2′-deoxymugineic acid (M-DMA) and metal-nicotianamine (M-NA) complexes (Fig. 3). In rice, a graminaceous plant model, Fe is effluxed into xylem by OsYSL16, a transporter of the Fe(III)-DMA complex (Kakei et al. 2012). Supposedly, in non-graminaceous plants, another YSL protein loads Fe into xylem in form of Fe(II)-NA. Structural differences accounting for 15 % between OsYSL16 and OsYSL2 transporters enable specificity shift (accordingly): from towards Fe(III)-DMA to Fe(II)-NA complexes (Kakei et al. 2012).

In 2009, Rellan-Alvarez at el. identified in xylem sap of Fe-deficient tomato (*Solanum lycopersicum*) complexes of Fe(III)-Citrate (Fe_3Cit_3 and Fe_2Cit_2). This experiment proved what has been hypothesized since early works of Brown (1996) and Tiffin 1996a, b, 1997). Fe_3Cit_3 complex formation is favored in xylem (with prevalence higher than 75 %) when the ratio of Fe to Cit is >1:10. This ratio is observed under normal conditions in a wide range of species regardless of their Fe nutrition status. Fe_2Cit_2 is favored when the threshold ratio is <1:75, a situation observed in some Fe-deficient plant species (Rellan-Alvarez et al. 2009). This analysis confirmed also that iron is transported in xylem sap as a ferric ion (Palmer and Guerinot 2009; Rellan-Alvarez et al. 2009; Larbi et al. 2010; Kiczka-Cyriac 2011).

In leafs, Fe from xylem sap is probably unloaded by FRD3 (ferric reductase defective) citrate-Fe(III) transporter, as Roschzttardtz et al. (2011) suggest, and then taken up by cells with YSL proteins. Brüggemann et al. (1993) proposed Fe(III) reduction preceding uptake into leaf mesophyll cells. Since leaf ferric chelate reductase transforming Fe(III) to Fe(II) favors Fe-Cit complex as a substrate, it is possible that this reduction occurs at the cell surface and subsequently promotes Fe-NA formation (Conte and Walker Elsbeth 2011). It is also possible that ZRT–IRT like proteins or Natural Resistance-Associated Macrophage Protein1 family assist in Fe(II) uptake to leaf cells, and then in cells, Fe(II) binds to NA for further translocation (Roschzttardtz et al. 2011). Another Fe transformation is required before it is sequestered in vacuoles in form of Fe(III)-ferritin complex (Briat et al. 2007). Strategy II plants are able to limit Fe transformations, because they can translocate Fe(III) complexed with phytosiderophores in xylem and other apoplastic spaces. However, this alternative transport route is activated mainly under Fe deficiency conditions, which stimulate phytosiderophores synthesis (Kiczka-Cyriac 2011; Guelke-Stelling and von Blanckenburg 2012).

Evidence of iron trafficking in leaf blades in form of complexes with nicotianamine came from the experiments on triple and quadruple loss-of-function *Arabidopsis* mutants targeted towards gene encoding nicotianamine synthase (*NAS*). Unless grown under Fe deficiency or Ni supply, triple mutants *nas4x-1* appeared almost normal during vegetative stage. Upon transition to reproductive stage, they showed an interveinal leaf chlorosis and

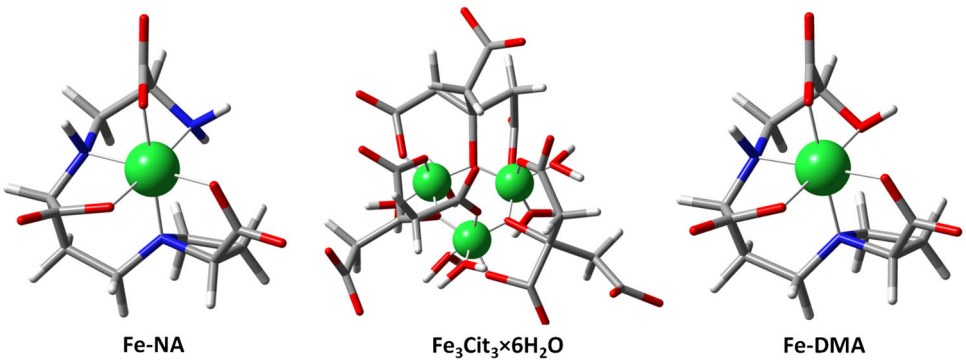

Fig. 3 Examples of the most common Fe^{3+} complexes in plants. *Green label* Fe^{3+}, *grey label* carbon, *red label* oxygen, *blue label* nitrogen and *white label* hydrogen atoms. Generated with Avogadro program (UFF optimization with conjugate gradient) and GaussView 5.0 (color figure online)

increased Fe content in leaves. Fe uptake in root of *nas4x-1* plants was up-regulated at this stage, most likely because intercostal leaf areas with mesophyll cells could not acquire Fe in sufficient amounts and stimulated root Fe uptake (Schuler and Bauer 2011). Such a stimulation could be obtained by microRNAs transport in phloem from Fe-deficient mesophyll cells to roots (Buhtz et al. 2010; Sheible et al. 2011; Kehr 2012). Quadruple mutant *nas4x-2* was severely affected: leaf chlorosis was present even during its early vegetative stage with Fe accumulation in vascular system and impaired transport of Fe to mesophyll (Schuler and Bauer 2011).

Fe was transported in phloem mostly in form of a ferric ion Fe^{3+} (45.2 μM), while ferrous ion Fe^{2+} accounted for only 3.8 % of the total Fe concentration (1.5 μM), according to the generic phloem model by Harris et al. (Schmidke et al. 1999; Harris et al. 2012). Two forms of Fe formed different complexes: 99 % of Fe^{2+} was chelated with NA, while almost 70 % of Fe^{3+} was bound to glutamic acid, almost 21 % to nicotianamine and 9.9 % to citric acid (Fig. 4a). Assumption that most iron in phloem sap is present in the form of a ferric ion in Harris model came from works of Schmidke et al. (1999) and Maas et al. (1988). However, phloem is supplied with Fe remobilized from leaf vacuoles: during this remobilization, Fe(III) stored in vacuoles is reduced to Fe(II) to ensure chelation with NA and subsequent trafficking towards phloem (Briat et al. 2007). Emergence of Fe(III) in phloem rather than Fe(II) would have to be preceded with another step of oxidation; studies of Fe tracing by isotope fractionation indicate that after remobilization from vacuoles, iron is not subjected to ligand exchange or oxidation/reduction reaction (Kiczka-Cyriac 2011; Guelke-Stelling and von Blanckenburg 2012). Therefore, ferrous ion could be accounted for dominant form of iron in phloem. Ferric ion could be transported in phloem bound mainly to ITP (iron transport protein, a dehydrin) identified only in castor bean *Ricinus communis* (Morrissey and Guerinot 2009).

Evidence suggests that iron can be also transported to embryo and pollen through apoplastic space, if cells lack symplastic connection. In this case, Fe is present in the apoplast in form of complexes with citrate and malate ($Fe(III)_3Cit_2Mal_2$, $Fe(III)_3Cit_3Mal$, $Fe(III)Cit_2$) (Roschzttardtz et al. 2011; Grillet et al. 2013). Interestingly, Grillet et al. (2013) showed that before uptake to embryo, Fe(III) was reduced to Fe(II) not enzymatically by ferric reductase, but chemically by ascorbate. Presence of this ascorbate reduction system was shown in two dicotyledonous plants: *Pisum sativum* and *Arabidopsis thaliana* (Grillet et al. 2013).

Translocation of micro- and macronutrients

After metals are acquired from soil, symplastic transport leads them to the vascular bundles. There, transporting proteins load micronutrients to xylem and a long-distance transport begins. Xylem loading is most probably mediated by HMA transporters: HMA2/4 for Zn^{2+}; HMA5 for Cu^{2+} (Hussain et al. 2004; Hanikenne et al. 2008; Kobayashi et al. 2008; Wong and Cobbett 2009; Siemianowski et al. 2011; Deng et al. 2013). Apart from HMA transporters, there is evidence that PCR2, a cysteine-rich protein localized to the PM and expressed in vascular tissue of shoots and root elongation zone, can export metals to xylem. Yeasts expressing *Arabidopsis PCR2* gene export Zn from cells, while *pcr2 Arabidopsis* mutants accumulate higher levels of Zn in roots (Song et al. 2010). Transporters loading Mn^{2+} to xylem are yet unknown (Williams and

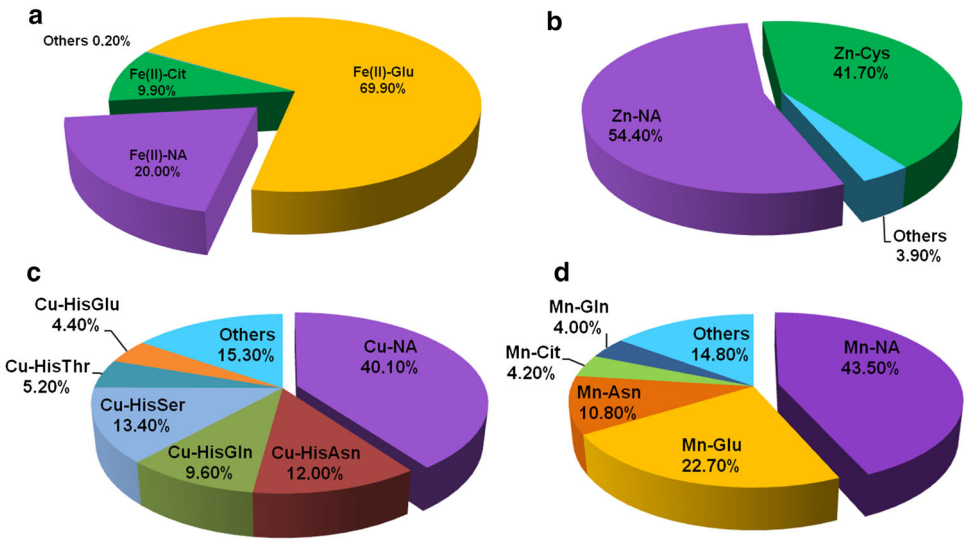

Fig. 4 Graphical representation of the calculated ligand pool composition for complexes formed with trace metals in phloem sap. Speciation model was computed with program ECCLES and involved the analysis of complexes formed with iron(II) (**a**), zinc (**b**), copper (**c**) and manganese (**d**) in plants under normal conditions. Iron(III) (bound in 99 % to nicotianamine) is not represented. Data according to Harris et al. (2012)

Pittman 2010). Calcium enters xylem mostly through the apoplastic route (White 2001; Gilliham et al. 2011).

In silico simulations of xylem sap predicted that Zn^{2+} and Mn^{2+} ions are bound by citrate and malate, from 70 to 90 % and 30 to 60 %, respectively (White et al. 1981). However, other experiments observed that Zn^{2+} can be transported as a free-hydrated ion, with a smaller portion coordinated with LMWOA (Salt et al. 1999). Copper is transported in xylem as a free ion (around 30 %) or bound to amino acids and nicotianamine (Curie et al. 2009; Irtelli et al. 2009). Copper excess stimulated directly proportional increase in histidine content in xylem sap of *Brassica carinata*, while copper starvation induced concentration of NA (Irtelli et al. 2009). In xylem of graminaceous plants, phytosiderophore DMA can replace NA in complexes with Cu^{2+}: studies on Cu speciation in xylem sap of rice (*Oryza sativa*) identified Cu(II)-DMA as a dominant complex. This may suggest that previously described OsYSL16 transporter loading Fe(III)-DMA could be also involved in trafficking Cu(II)-DMA complexes to xylem (Ando et al. 2012; Kakei et al. 2012).

Nickel uptake and distribution in plants have been reviewed by Yusuf et al. (2011). In xylem sap, Ni was primarily believed to be complexed by histidine, as shown in the Ni hyperaccumulators *Alyssum lesbiacum* and *T. caerulescens* (Krämer et al. 1996; Morel et al. 2009). However, recent studies show that in xylem sap of another Ni-accumulating *Alyssum* species, *A. sepyllifolium*, grown under field conditions, most of the Ni occurs as a free-hydrated cation (about 70 %) or forms complexes with citric acid (18 %). Other carboxylic acids (oxalate, malate, malonate, aspartate) complexed altogether <13 %, while histidine did not play a role in Ni translocation in xylem at all (Alves et al. 2011).

Calculated model of metals speciation (Fig. 4) in phloem sap implied a high degree of complexation of all trace metals, with Mn^{2+} having highest free ion concentration accounting for 1.16 % of total Mn. Macronutrients Ca^{2+} and Mg^{2+} were much less chelated: 22 % of Ca and 16 % of Mg were present as a hydrated ions. Nicotianamine was the most important ligand for Mn^{2+}, Cu^{2+} and Zn^{2+} and bound around 44, 40 and 54 % of each ion, respectively (Fig. 4b–d). Other significant complexes present in phloem were as follows: Zn-cysteine (41 %), mixed-ligand species of Cu(His)(L) (60 %, where L is most likely Asn, Gln or Ser) and Mn-glutamate (23 %). Macronutrients Ca^{2+} and Mg^{2+} bound 72 % of the citrate. Removal of these two metal ions from speciation model increased Mn^{2+} and Fe^{3+} chelation with citrate, having no impact on the speciation of other ions. Glutathione (GSH, tripeptide glutamyl-cysteinyl-glycine), a possible chelating agent for Zn^{2+} had been removed from the calculations for no or insignificant binding to Zn (results of two simulations with different sets of binding constants) (Harris et al. 2012). Not included in the model, nickel is most likely transported in phloem in form of complexes with nicotianamine or histidine (Chen et al. 2009).

Transport of non-essential metals

Non-essential metals are transported in plants apoplastically through transporters for essential metals or via ion channels. Xylem unloading of these metals occurs probably through plasma membrane-localized transporters of cells associated with xylem, mimicking the process occurring at the soil-root interface. Subsequently, TMs could be transported further into leaf blade in form of NA complexes through symplastic pathway.

Lead in plants is transported apoplastically or through non-selective cation channels (Pourrut et al. 2013). In xylem, Pb is probably bound by citrate: a positive correlation between Pb and citrate concentrations has been found in two species varying in shoot lead accumulation, *Sesuvium portulacastrum* and *B. juncea* grown with a supplementation of Pb. Interestingly, higher toleration to Pb in *S. portulacastrum* in comparison to *B. juncea* may be a result of higher concentration of citric acid in xylem and, therefore, higher translocation rate (Ghnaya et al. 2013). Lead may also form complexes with histidine. Due to low mobility of Pb in plants, only a small portion of this metal reaches above-ground tissue, thus transport in phloem is probably very limited (Pourrut et al. 2013).

As(V) has a close similarity to phosphate (Pi), which allows it to be transported to xylem via the high-affinity Pi transport system. As(III), on the other hand, is transported in monocotous rice to xylem by the Si/arsenite effluxer Lsi2; dicotous transporter is still unknown (Catarecha et al. 2007; Verbruggen et al. 2009b). As(III) transporter in dicots most likely belongs to the NIP family (nodulin-26 like intrinsic protein) of aquaporins (Verbruggen et al. 2009b). As(III) was found to be dominant form in xylem sap of cucumber and the following concentration order was observed: As(III) > As(V) > dimethylarsinic acid (Mihucz et al. 2005). Recent study in barley (*Hordeum vulgare*) showed that a silicon transporter *HvLsi6* is localized to the plasma membrane at parenchyma cells of vascular bundles and (during reproductive stage) in nodes. This data obtained by Yamaji et al. (2012) suggested a role of HvLsi6 in Si xylem unloading in leaf blade and sheath along with intervascular transfer of Si in nodes. Considering homologous transporter OsLsi2 has a similarity level of 82 % in amino acids sequence and is able to mediate efflux of As, HvLsi6 might play a role in xylem unloading of As(III) in graminaceous plants (Ma 2009; Yamaji et al. 2012).

Cadmium is transported through Fe^{2+}, Ca^{2+} and Zn^{2+} transporters and channels *in planta* (including: ZIP family, Nramp family, LCT1 channels) (Gallego et al. 2012). In xylem sap, cadmium may be bound by citrate, as have been shown by Zorrig et al. (2010) in lettuce (*Lactuca sativa*). Interestingly, administration of Cd increased translocation of both Cd and Zn in lettuce shoots (Zorrig et al. 2010). Low-affinity cation transporter OsLCT1 has been found to load cadmium to phloem sap (Uraguchi et al. 2011).

Phloem transport of non-essential metals was not accounted for in speciation model of Harris et al. (2012). Glutathione and glutathione derivatives, phytochelatins (PCs), which bind strongly to Cd, Hg, As(III) are probable ligands for non-essential TMs in phloem sap (Mendoza-Cozatl et al. 2011). Phytochelatins complexed with Cd had been identified in *Brassica napus* phloem. Data suggested that also GSH-Cd complexes contribute to the long-distance Cd movement in phloem (Mendoza-Cozatl et al. 2008; Kato et al. 2010). Although PCs have a strong affinity for Zn, they appear in phloem sap only after Cd treatment, which would suggest that under normal conditions, Zn is not complexed with PCs (Harris et al. 2012). Mendoza-Cozatl et al. (2011) proposed that thiol-conjugates in phloem of *Arabidopsis* are transported towards different sink tissues: only GSH-Cd complexes are detected in seeds, while PC-Cd conjugates are most likely sequestered in root vacuoles, subsequently after uptake from phloem by ABCC1 and ABCC2 transporters.

Interestingly, TMs can be also loaded to phloem directly from xylem. This shortcut route for xylem-to-phloem movement had been identified in rice with the use of radioisotope-labeled tracers. Such tracers enable the discrimination between newly absorbed elements and elements present in plant at the beginning of the experiment (Fujimaki et al. 2010; Kobayashi et al. 2013). The accumulation pattern of cadmium uptake and translocation in rice suggest that at the nodes, cadmium can be transferred xylem-to-phloem without being unloaded into leaf blades (Fujimaki et al. 2010). Surprisingly, when Cd tracer had been applied to vascular bundles of xylem in a crown root, images showed both upward (towards new leaf sink tissue) and downward Cd transport, towards tissue below the connection point of treated crown root. This implies not only direct transfer of Cd to phloem, but also immediate redirection of part of Cd from upward movement in xylem towards root sink tissue (Kobayashi et al. 2013).

Concluding remarks

Vascular plants favor complexation of trace metals with carboxylic acids to amino acids, probably because N compounds are much more valuable to plants than C and O compounds. Usually, a part of total metal content in xylem sap and phloem sap is present as non-chelated hydrated ions. Apparition of metals bound to peptides (DMA or NA) in vascular bundles most likely coincides with increased trace metals demand and uptake under metal deficiency, which stimulate action of YSL transporters and synthesis of phytosiderophores. Fe trafficking in plants is still not fully understood, especially transformations from Fe(III) to Fe(II) in different tissues and vice versa need to be explored with greater attention.

Source-to-sink transport in phloem had been strongly undervalued in terms of trace metals mobilization from leaves. Almost all experiments conducted for improvement of root-to-shoot TMs translocation focus only on enhancement of upwards movement for better phytoextraction or biofortification. This point of view neglects plants natural mechanisms of protection of phytosynthetically active tissues from toxic effects of excessive TMs accumulation. Plants ability to direct metals from leaves back to roots can be partially responsible for decreased TMs accumulation in shoots after every effort had been made to induce translocation to aerial tissues. Signals (e.g., small RNAs) transmitted from overloaded leaves can further impair TMs uptake in roots. Therefore, in the future, an attempt should be made to thoroughly assess how much TM translocation towards shoot is decreased due to upturn movement in phloem. Whether this is a significant issue for efficient phytoextraction or a process mainly with no real impact is a question to be answered.

Author contribution Authors contributed equally to the paper. Metal complexes were optimized and modeled by MS. Drawings were prepared by AK.

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