Biometals (2013) 26:623–632
DOI 10.1007/s10534-013-9643-1

Nicotianamine is a major player in plant Zn homeostasis

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Received: 31 May 2013 / Accepted: 3 June 2013 / Published online: 18 June 2013

Abstract Nicotianamine (NA) is among the most studied plant metal chelators. A large body of evidence supports its crucial role for Fe distribution in plants and as a precursor of phytosiderophore synthesis in grasses. NA forms stable complexes in vitro not only with Fe(II) and Fe(III) but also with various other divalent metal cations including Zn(II). Early observations indicated a possible contribution of NA to Zn trafficking in plants. Numerous studies on transgenic monocot and dicot plants with modulated NA levels have since then reported Zn accumulation phenotypes. *NAS* genes were shown to represent promising targets for biofortification efforts. For instance, NA was found to bind Zn in rice grains in a form bioavailable for humans. Recently, additional strong support for the existence of Zn–NA complexes *in planta* has been obtained in rice, *Arabidopsis thaliana* and the Zn hyperaccumulating plant *A. halleri*. We review the evidence for a role of NA in the intercellular and long-distance transport of Zn in plants and discuss open questions.

Keywords Plant metal homeostasis · Metal hyperaccumulation · Zn homeostasis · Zn speciation · Biofortification · *Arabidopsis halleri*

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Introduction

Zn needs to be distributed to a myriad of Zn-requiring proteins. During evolution Zn has with increasing availability in an oxidizing atmosphere been recruited for more and more functions in biological systems (Dupont et al. 2006). This is reflected by the estimated rise in the proportion of Zn-binding proteins from 5–6 % of the proteome in bacteria and archaea to about 9 % in eukaryotes (Andreini et al. 2006). Zn is a cofactor for a large number of enzymes in all six enzyme commission (EC) classes, most prominently among hydrolases (EC3) (Andreini et al. 2008). More than 300 enzyme families are Zn-dependent (Coleman 1992). The increase in the relative number of Zn-proteins in eukaryotes is largely attributable to an expanded usage of Zn in regulatory proteins such as transcription factors. This inspired the hypothesis that the bioavailability of Zn was a limiting factor in the evolution of eukaryotes with their higher demand for DNA-binding proteins (Dupont et al. 2010).

Low-molecular-weight chelators are essential for the necessary intra- and inter-cellular trafficking of Zn.

In accordance with the Irving–Williams series (Irving and Williams 1948) Zn is strongly preferred over other micronutrients except copper for interaction with N-, O- and S-containing organic ligands (Colvin et al. 2010). Thus, unwanted interactions of Zn with proteins and other components of biological systems need to be suppressed through tight control over the availability of free intracellular Zn. For eukaryotic cells, results from a multitude of experimental approaches converged in the estimate that cytosolic labile Zn lies in the picomolar range (Colvin et al. 2010), meaning essentially the absence of free Zn ions and a sharp contrast to the cellular Zn quota of 0.1–0.5 mM, which the large number of Zn-requiring sites translates into (Eide 2006). Effective buffering maintains free cytosolic Zn within the narrow range that ensures sufficient supply to Zn proteins while preventing unspecific interactions. Zn buffering as well as "Zn muffling", i.e. the modulation of non-steady state changes in cytosolic free Zn ions (Colvin et al. 2010), are dependent not only on the function of Zn-binding proteins and Zn transporters but also on high-affinity low-molecular-weight chelators. In mammalian cells glutathione (GSH) takes part in cytosolic Zn buffering and muffling (Hogstrand et al. 2009).

A second function of low-molecular-weight chelators in Zn homeostasis is the facilitation of long-distance transport. In plants, Zn is taken up by roots from the surrounding soil. Following uptake, which is possibly assisted by chelator-dependent mobilization, Zn has to reach vascular tissues for the translocation to Zn sites in leaves, stems, flowers and seeds. Xylem and phloem have to be loaded and unloaded. Furthermore, there is exchange between these tissues. Besides GSH plant cells synthesize other Zn chelators including phytochelatins (PCs), GSH-derived metal binding peptides, and nicotianamine (NA) (Sinclair and Krämer 2012). The latter has been mostly investigated in the context of Fe nutrition (for reviews see Curie et al. 2009; Scholz et al. 1992; Schuler and Bauer 2011). Here we discuss the evidence for an important role of NA in plant Zn homeostasis, with emphasis on most recent findings.

Discovery and prevalence of the metal chelator nicotianamine

NA was first isolated as a new amino acid from leaves of tobacco (Noma et al. 1971). Its structure was determined following isolation from beech nuts (Kristensen and Larsen 1974). A little later NA was found to represent the "normalizing factor" that restores wild-type Fe distribution in leaves of the tomato mutant *chloronerva* (Budesinsky et al. 1980). A large body of literature has since then established NA as a major metal chelator in plants. Guided by the obvious intercostal chlorosis phenotype of the *chloronerva* mutant, which is due to the mislocalization of Fe, NA has been ascertained as a crucial factor for the trafficking of Fe in plants (Curie et al. 2009; Schuler and Bauer 2011; Stephan et al. 1996). Early investigations had indicated that NA is present not only in all vascular plants, but also in mosses and certain fungi such as basidiomycetes (Rudolph et al. 1985). Thus, it was hypothesized that NA function goes beyond long-distance transport of Fe. In grasses NA serves in addition as precursor for the synthesis of mugineic acid (MA) phytosiderophores (Shojima et al. 1990).

The enzyme responsible for the condensation of three molecules S-adenosyl-methionine (SAM) to form NA, nicotianamine synthase (NAS), was purified from barley (Herbik et al. 1999; Higuchi et al. 1999). In parallel, the gene defective in *chloronerva* was isolated and found to encode an NAS (Ling et al. 1999). With this it became possible to infer the distribution of NA in nature from genomic data. For instance, a functional NAS was found in the ascomycete *Neurospora crassa* (Trampczynska et al. 2006). Based on currently available sequence data we can see that *NAS* genes are apparently ubiquitous among higher plants. In addition they are found in mosses (*Physcomitrella patens*) but not in algae. Thus, one could speculate that *NAS* genes were important for the evolution of land plants. However, the *Selaginella moellendorffii* genome (www.phytozome.net/selaginella) apparently does not carry an *NAS* gene. Outside the plant kingdom *NAS* genes are found in archaea, bacteria and ascomycetes (Fig. 1). All these organisms appear to possess only a single copy while in higher plants small gene families arose. Interestingly, they remained intronless.

NA as a metal chelator

Starting with the discovery that NA rescues the *chloronerva* mutant phenotype, metal chelating capacities of NA were investigated in vitro. The first reports

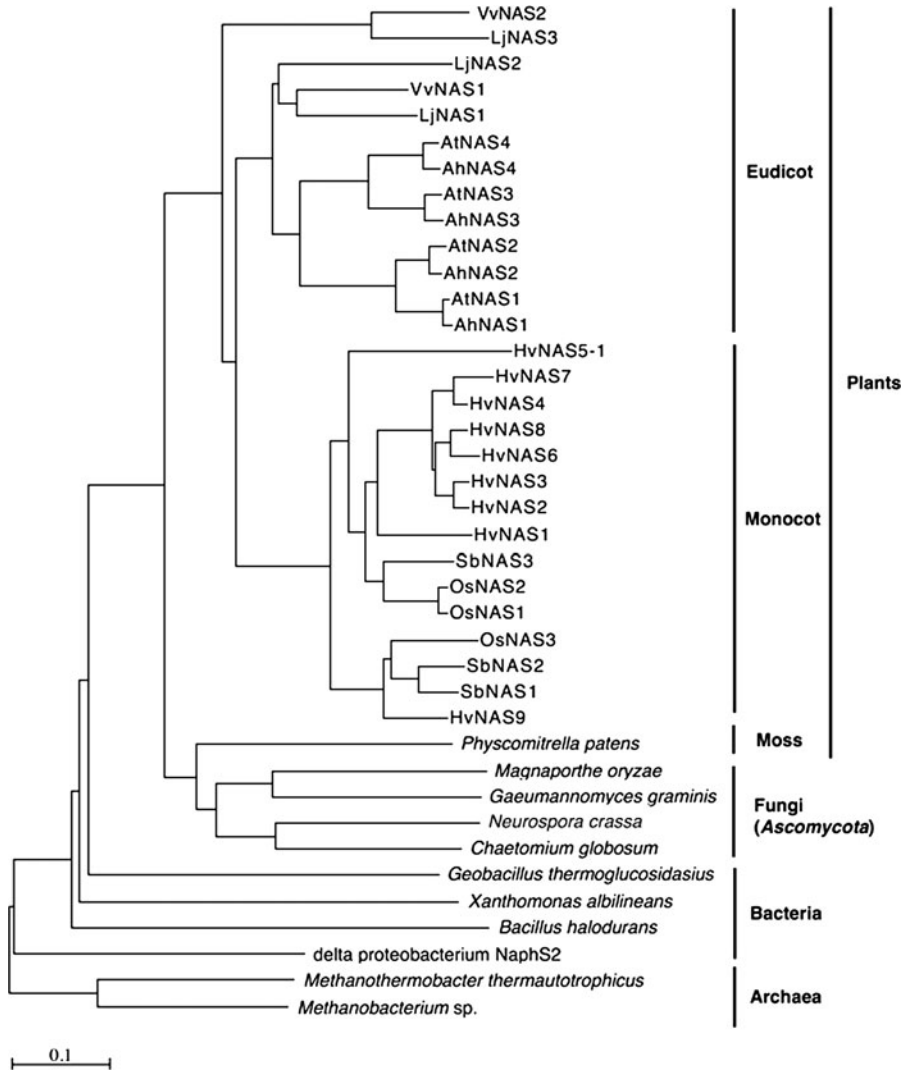

Fig. 1 Phylogenetic tree of NAS proteins in representative prokaryotes and eukaryotes. Phylogenetic analysis was performed by the distance-based neighbor-joining method with CLUSTAL W. The tree was visualized with NJPLOT software. Aligned sequences (amino acid residues 33–276 of AtNAS1) were used to generate the phylogenetic tree. The *scale bar* of 0.1 is equal to 10 % sequence divergence. The accession numbers of the sequences are shown in parenthesis: *Methanobacterium sp.* (KQ53444.1), *Methanothermobacter thermautotrophicus* (WP_010876313.1), delta proteobacterium NaphS2 (WP_006421039.1), *Bacillus halodurans* (WP_010898230.1), *Xanthomonas albilineans* (WP_012916945.1), *Geobacillus thermoglucosidasius* (YP_004588391.1), *Chaetomium globosum* (EAQ85681.1), *Neurospora crassa* (XP_958379.1), *Gaeumannomyces graminis* (EJT72278.1), *Magnaporthe oryzae* (XP_003719353.1), *Physcomitrella patens* (EDQ52909.1), *OsNAS1* (BAF11809.1), *OsNAS2* (BAF11808.1), *OsNAS3* (BAF22621.1), *SbNAS1* (C5WR27), *SbNAS2* (C5X6C8), *SbNAS3* (Sb01g037480), *HvNAS1* (BAA74580.1), *HvNAS2* (BAA74582.1), *HvNAS3* (BAA74581.1), *HvNAS4* (BAA74583.1), *HvNAS5-1* (AB011267.1), *HvNAS6* (BAA74586.1), *HvNAS7* (BAA74587.1), *HvNAS8* (AAD32650.1), *HvNAS9* (AAD32651.1), *AtNAS1* (AED90808.1), *AtNAS2* (AED96719.1), *AtNAS3* (AEE28417.1), *AtNAS4* (AEE33394.1), *AhNAS1* (AFH08365.1), *AhNAS2* (AFH08366.1), *AhNAS3* (CAE45015.1), *AhNAS4* (CCD74500.1), *LjNAS1* (BAH22562.1), *LjNAS2* (BAH22563.1), *LjNAS3* (chr3.LjT36L22.160.r2.a), *VvNAS1* (F6I4S7), *VvNAS2* (A5BHX9). Os: *Oryza sativa* (rice), Sb: *Sorghum bicolor* (sorghum), Hv: *Hordeum vulgare ssp. vulgare* (barley), At: *Arabidopsis thaliana*, Ah: *Arabidopsis halleri*, Lj: *Lotus japonicus* (a wild legume), Vv: *Vitis vinifera* (grape)

demonstrated the formation of stable complexes with Fe(III) and Cu(II) (Budesinsky et al. 1980). Subsequent more extensive studies determined the following log stability constants for complexes with divalent transition metal cations: Mn(II), 8.8; Fe(II), 12.1/12.8; Zn(II), 14.6/15.4; Ni(II), 16.1; Cu(II), 18.6 (Benes et al. 1983) (the

second value for Fe(II) and Zn(II) was later reported by Anderegg and Ripperger 1989). At pH > 6 these complexes were calculated to exist practically undissociated in aqueous solution suggesting their possible occurrence in the cytosol and the phloem (Stephan et al. 1996). Unequivocal detection of NA-metal complexes by direct infusion electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was more recently used to study the nature and stability of NA complexes with the physiologically relevant metal cations across a pH range of 2.5–8.5 (Rellan-Alvarez et al. 2008). Mn(II)–NA, Fe(II)–NA, Fe(III)–NA, Zn(II)–NA, Ni(II)–NA and Cu(II)–NA were all completely formed at alkaline pH. Differences were apparent around neutral and at acidic pH. For instance, while Mn(II)-NA only started to form at pH > 6.2, Fe(II)–NA and Zn(II)–NA were completely formed at pH 6.2. Ni(II)–NA and Cu(II)–NA were detected even at pH values of 4 and lower. Interestingly, in metal-exchange experiments at acidic (5.5) and slightly alkaline pH (7.5) to approximate conditions in xylem and phloem, respectively, addition of Zn(II) to Fe(II)–NA complexes resulted in effective competition yet a stronger formation of Zn(II)–NA under acidic conditions. Possibly this reflects pH effects on the kinetics of the exchange reaction. Taken together these speciation data suggest that *in planta* Zn(II)–NA complexes can exist in cytosol and phloem as well as in the xylem (Rellan-Alvarez et al. 2008).

Early observations indicating a role of NA in Zn chelation

Phytosiderophores of the MA type are derived from and structurally similar to NA. Therefore, observations implicating phytosiderophores in Zn chelation can be interpreted as early indirect evidence for an involvement also of NA in plant Zn trafficking. Indeed it was found that wheat and other grasses show enhanced release of phytosiderophores under Zn deficiency (Cakmak et al. 1994). Moreover, variation in this process correlated with Zn efficiency among wheat genotypes suggesting a contribution of phytosiderophores to Zn mobilization in the soil (Cakmak et al. 1996). Correspondingly, it was later found that transcript abundance of *NAS* and other MA biosynthesis genes is higher in barley roots under Zn-deplete conditions. The contribution of MA to Zn adsorption from soil particles could be confirmed by directly demonstrating Zn–MA uptake through barley roots (Suzuki et al. 2006). Zn–phytosiderophore uptake had previously been shown for maize (von Wiren et al. 1996). In contrast, the contribution of MAs to Zn nutrition in rice appears to lie rather in the facilitation of Zn translocation (Bashir et al. 2012). Secretion of MAs by roots of Zn deficient rice plants is reduced while MA synthesis is transcriptionally activated. Furthermore, Zn–deoxymugineic acid (DMA) enhances Zn root-to-shoot translocation rather than root Zn uptake (Suzuki et al. 2008).

When the accumulation of Zn and other micronutrients was studied in wild-type tomato and *chloronerva*, only small effects were seen with the exception of a strongly reduced root-to-shoot translocation of Cu (Pich et al. 1994). Shoot Zn levels appeared largely unaffected by the presence or absence of NA. However, roots of *chloronerva* plants accumulated more Zn than wild type.

Modulations of plant NA concentrations and their effects on Zn distribution

Cloning of *NAS* genes (and other MA biosynthesis genes) enabled the engineering of NA levels, i.e. the generation (i) of NA-deficient plants other than *chloronerva*, and (ii) of NA-overproducing plants. Tobacco plants free of detectable NA were obtained via the overexpression of a barley NA aminotransferase (NAAT), which catalyzes the conversion of NA to an intermediate in MA biosynthesis (Takahashi et al. 2003). NAAT overexpressors phenocopied the interveinal chlorosis of the *chloronerva* mutant concomitant with aberrant distribution of Fe in leaves. Leaf concentrations were significantly reduced for Fe, Cu and Zn. Conversely, HvNAS-overexpressing tobacco plants accumulated more Fe and Zn in leaves, flowers and seeds. These findings led the authors to speculate about a role of NA in intracellular Zn transfer (Takahashi et al. 2003). Enhanced leaf Zn accumulation was independently confirmed in 35::*AtNAS* plants (Douchkov et al. 2005). Additional experiments with HvNAS-overexpressing tobacco plants grown on serpentine soil revealed stronger Zn accumulation in stems and seeds (Kim et al. 2005).

Several studies were performed with rice plants overexpressing *NAS* genes. They were motivated by the concept of biofortification (Mayer et al. 2008; Palmgren et al. 2008; Zhao and McGrath 2009). The micronutrient intake of a large proportion of the world's population is too low. Fe and Zn deficiency in humans are widespread and among the most prevalent deficiencies. According to WHO estimates about two billion people are affected by insufficient Zn intake and Zn deficiency accounts for ca. 2.9 % of worldwide loss of healthy life years (WHO 2002). Thus, the potential health benefits of increasing the micronutrient density of major crops such as cereals are huge.

Activation-tagged rice plants with elevated expression of *OsNAS3* or *OsNAS2* and correspondingly higher NA concentrations were found to accumulate more Zn in leaves and seeds (Lee et al. 2009, 2011). Increases were about twofold for *OsNAS3* lines and nearly threefold for *OsNAS2* lines specifically in the endosperm. These effects were attributed to higher NA levels and enhanced phytosiderophore secretion. Speciation analysis demonstrated the existence of a new pool of low-molecular-weight Zn species in seeds of *OsNAS2*-overexpressing rice plants with NA and DMA being the dominant ligands. Importantly, Zn chelated by NA and DMA is more readily bioavailable after consumption as was demonstrated in feeding studies with Zn-deficient mice (Lee et al. 2011). Thus, engineering NA synthesis in cereals has great Zn (and Fe) biofortification potential.

OsNAS3-overexpressing rice plants accumulated more Fe in their roots, leaves and seeds, too (Lee et al. 2009). Comparable observations were reported for rice lines overexpressing *A. thaliana NAS1* plus the Fe storage protein ferritin. Zn and Fe brown grain concentrations were 1.5–2-fold elevated, respectively (Wirth et al. 2009). The combination of ferritin and NAS overexpression with that of a Fe–NA transporter (OsYSL12) yielded field-harvested rice grain with 1.6-fold higher Zn concentrations (Masuda et al. 2012). When the association between NA levels and Zn accumulation was analyzed in grains across large populations of wild-type and transgenic rice lines overexpressing one of the three rice *NAS* genes, a clear positive correlation was found (Johnson et al. 2011). Increases in polished grains due to elevated Fe and Zn accumulation in the endosperm again demonstrated considerable biofortification promise.

Recent evidence for the contribution of NA to Zn trafficking in plants

The findings for *NAS*-overexpressing rice plants strongly suggest a contribution of Zn–NA complexes to the mobility and storage of Zn. Indeed, a speciation analysis of rice phloem sap via size-exclusion and anion-exchange chromatography separated Zn and Fe fractions. The respective co-eluting ligands were identified by ESI-TOF-MS as NA for Zn and DMA for Fe (Nishiyama et al. 2012).

Using a similar approach, Zn–NA complexes were detected also in roots of the Zn hyperaccumulator *A. halleri* (Deinlein et al. 2012). About 15 plant taxa possess the remarkable ability to accumulate Zn in their aboveground tissues to levels >100-fold higher than normally found in plants—or in fact any other organism in nature (Krämer 2010). A series of comparative transcriptome studies showed that in *A. halleri* and the other hyperaccumulating model species *Noccaea caerulescens* several metal homeostasis genes are constitutively higher expressed when compared to the non-hyperaccumulating relative *A. thaliana*, leading to the hypothesis that hyperaccumulation evolved through alterations in the regulation of metal transporters and metal chelator synthesis (Hanikenne and Nouet 2011; Krämer 2010; Verbruggen et al. 2009). Among the genes showing the largest expression differences between *A. halleri* and *A. thaliana* are *NAS* genes (Becher et al. 2004; Talke et al. 2006; Weber et al. 2004). Similar results were reported for *N. caerulescens* (van de Mortel et al. 2006). Constitutively high *AhNAS2* transcript levels in *A. halleri* roots are associated with elevated NA concentrations (Weber et al. 2004). This is a species-wide trait as shown by analyses of *A. halleri* populations from natural sites in Germany and Poland that exhibit different levels of soil Zn ranging from non-contaminated to heavily contaminated (Deinlein et al. 2012). The low-molecular-weight Zn fraction extractable from *A. halleri* roots co-elutes with NA, GSH and PCs. Suppression of *AhNAS2* expression by RNAi resulted in reduced root NA concentrations which correlated with a significant reduction of root-to-shoot Zn translocation (Deinlein et al. 2012). Size exclusion chromatography coupled to ICP-MS indicated a shift in Zn buffering from NA to thiols in *AhNAS2*-suppressed plants. Cultivation on native *A. halleri* soils demonstrated a contribution of root NA to Zn

mobility. Root NA concentrations correlated positively with leaf Zn accumulation on non-contaminated soil (Fig. 2). Thus, AhNAS2 is besides AhHMA4 (Hanikenne et al. 2008) the second Zn hyperaccumulation factor identified to date.

Careful analysis of an *A. thaliana nas* mutant and the identification of a new component influencing cytosolic NA availability provided further insights. The genome of *A. thaliana* contains four *NAS* isoforms that originated from a single ancestral *NAS* gene. *AtNAS1* and *AtNAS4* are expressed both in roots and in shoots whereas *AtNAS2* is exclusively expressed in root tissue and *AtNAS3* only in leaves (Klatte et al. 2009). Through a series of crosses between T-DNA insertion lines and their offspring a collection of double, triple and finally quadruple mutants was generated. Line *nas4x-2* is completely devoid of NA and showed severe symptoms such as leaf interveinal chlorosis and sterility (Schuler et al. 2012). Zn concentrations of leaves and flowers were reduced compared to wild type plants. This is consistent with the role of NA in root-to-shoot translocation of Zn in *A. halleri*. Transcript abundance

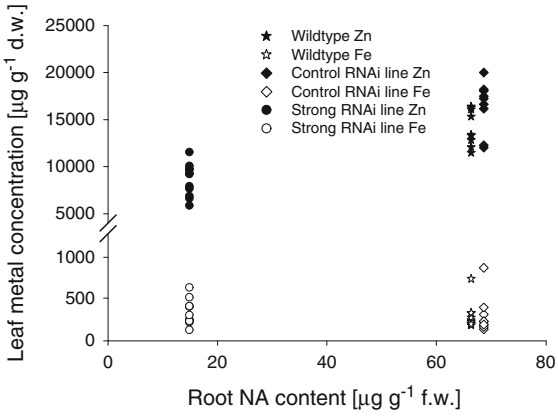

Fig. 2 Root NA concentrations are positively correlated with leaf Zn accumulation in the Zn hyperaccumulator *A. halleri*. *AhNAS2* is strongly overexpressed in *A. halleri* roots and concomitantly NA concentrations are higher relative to *A. thaliana*. A set of *AhNAS2*-RNAi lines differing in the degree of *AhNAS2* suppression was generated and characterized. Shown here are data derived from experiments with these lines (Deinlein et al. 2012). NA concentrations of wild type and RNAi plants were determined in hydroponic culture. Plants were cultivated on native *A. halleri* soil from a non-contaminated site. Zn and Fe accumulation in leaves was determined. While Fe levels are not affected by root NA concentrations, Zn accumulation is reduced in *AhNAS2*-suppressed plants indicating a contribution of root NA to root-to-shoot translocation of Zn even under these near-natural conditions

of *ZIP4*, an established Zn deficiency marker, was higher in young and mature leaves as well as in flowers of *nas4x-2* plants. Sterility was demonstrated to be caused by a lack of Fe and Zn, possibly because NA-mediated delivery of Fe and Zn to the growing pollen tube is defective (Schuler et al. 2012).

Zinc-induced Facilitator 1 (ZIF1) is a vacuolar membrane protein of the major facilitator superfamily. It was originally identified in a forward genetic screen as being important for *A. thaliana* basal Zn tolerance (Haydon and Cobbett 2007). More recently it was shown to mediate vacuolar sequestration of NA (Haydon et al. 2012). Yeast experiments indicated that ZIF is not able to transport Zn–NA complexes or Zn alone since no rescue of mutants defective in vacuolar sequestration of Zn was achieved by co-expressing *ZIF1* and an *NAS* gene. In plants overexpressing ZIF1 the ratio between cytosolic and vacuolar NA was shifted towards compartmentalization in the vacuole. As a consequence, more Zn was trapped in root cell vacuoles resulting in reduced root-to-shoot transport of Zn and the induction of transcriptional Zn deficiency responses in shoots. Thus, cytosolic NA was again demonstrated to enhance Zn mobility in the root symplasm and translocation to the shoot.

Open questions

Taken together there is now convincing cumulative evidence for a major role of NA as a Zn ligand in plants. The findings derived from the modulation of NA levels in tobacco, rice, *A. thaliana* and the Zn hyperaccumulator *A. halleri* converge in the hypothesis that NA significantly contributes to intercellular and long-distance transport of Zn. However, the exact mechanisms of NA-mediated Zn trafficking and sequestration are far from understood.

The *in planta* existence of Zn–NA complexes at the neutral to slightly alkaline pH of the cytosol and the phloem is now well-documented. Do these complexes exist at the acidic pH of the vacuole and the xylem as well? In vitro data (Rellan-Alvarez et al. 2008) and the sequestration of NA and Zn in root vacuoles of ZIF-overexpressing *A. thaliana* plants (Haydon et al. 2012) support this assumption.

NA was demonstrated to mediate root-to-shoot translocation of Zn. Most likely this effect is

associated with the facilitation of xylem loading. Zn buffering by cytosolic NA can suppress sequestration in root vacuoles and thereby enhance symplastic mobility of NA. Unknown is whether this occurs in all cells of the root or only in specific cell types (Fig. 3). In fact it appears highly likely that NA availability for cytosolic Zn buffering is tightly controlled in a cell-specific manner. For instance, ZIF1 expression—and thereby NA sequestration—is controlled by the transcription factor POPEYE, which is thought to mediate a pericycle-specific Fe deficiency response (Long et al. 2010). Cellular resolution of the analyses will be required to dissect the network of metal chelator and transporter activities that ensures homeostasis of metals such as Zn, Fe, Cu, or Mn, whose trafficking pathways are at least partly shared.

Does NA play in addition a direct role in the loading of the xylem, for instance via the interaction with Zn efflux systems such as HMA4, the main component of xylem loading in *A. halleri* (Hanikenne et al. 2008)? Is NA loaded into the xylem as a binding partner for Zn (Fig. 3)? To date, only Ni–NA complexes have been detected in xylem sap (Mari et al. 2006). Connected with these questions is a possible crosstalk between low-molecular-weight metal chelators. Compensatory up-regulation of synthesis was clearly demonstrated for citrate and NA to maintain Fe transport when deficiency of one of the two chelators occurs (Schuler et al. 2012). Data for *A. halleri* plants with reduced root NA indicated an up-regulation of thiol Zn chelation, suggesting the possibility of a similar crosstalk between Zn ligands (Deinlein et al. 2012).

Transport of NA and Zn–NA across the plasma membrane and organellar membranes is only partially understood. Transporters with homology to ZIF1 are present in rice. ENA1 mediates NA efflux when expressed in Xenopus oocytes (Nozoye et al. 2011) and has recently been depicted as an NA efflux transporter with biofortification potential in rice (Schroeder et al. 2013). NA efflux from xylem parenchyma cells into the xylem could be important if NA has a direct role in xylem translocation of Zn.

Candidates for the transport of Zn–NA complexes are proteins of the YSL family (Curie et al. 2009). However, it is not yet clear if they accept Zn–NA complexes as substrates. In rice, YSL proteins are considered likely candidates for Zn–NA and Zn–DMA transport but direct evidence is lacking (Bashir et al. 2012). The same applies to dicot plants (Sinclair and Krämer 2012).

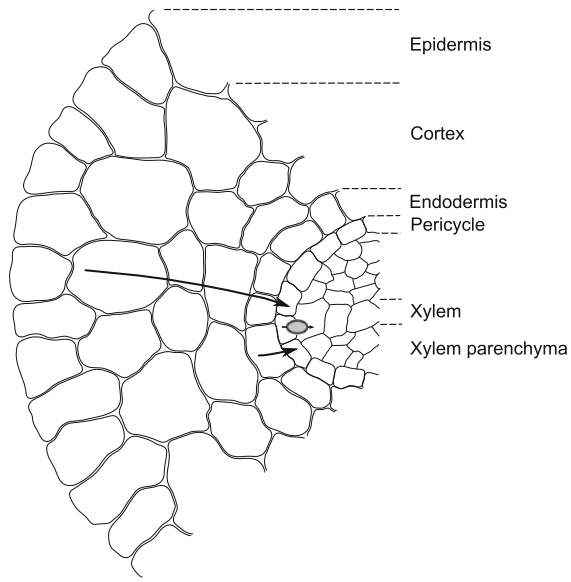

Fig. 3 Possible mechanisms underlying the facilitation of Zn root-to-shoot translocation by NA. Shown is a schematic cross-section drawn after a micrograph of an *A. halleri* root (Hanikenne et al. 2008). Three different scenarios are illustrated. The *black arrows* indicate NA-dependent symplastic mobility of Zn either across the whole root (Hanikenne and Nouet 2011) or only in specific cell types such as the endodermis and pericycle. The *grey symbol* represents loading of either Zn–NA complexes or NA into the xylem

With respect to the role of NA in Zn hyperaccumulation it will be important to ask whether stronger *NAS* expression is sufficient to explain elevated NA accumulation. Alternatively, the synthesis of the precursor SAM might be regulated differently, too. SAM synthetases are among the hyperaccumulation candidate genes in *A. halleri* (Talke et al. 2006). Also, it will be interesting to investigate the evolutionary history of *NAS* genes. Many metal homeostasis genes in hyperaccumulators show copy number expansion (Talke et al. 2006).

Acknowledgments Work in the authors' laboratory is supported by the Deutsche Forschungsgemeinschaft. U.D. and S.U. gratefully acknowledge postdoctoral fellowships from the

Deutsche Akademische Austauschdienst and the Japanese Society for the Promotion of Science, respectively.

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