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RESEARCH PAPER

Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis

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Abstract

A better understanding of the role of the *Arabidopsis* ZIP family of micronutrient transporters is necessary in order to advance our understanding of plant Zn, Fe, Mn, and Cu homeostasis. In the current study, the 11 *Arabidopsis* ZIP family members not yet well characterized were first screened for their ability to complement four yeast mutants defective in Zn, Fe, Mn, or Cu uptake. Six of the *Arabidopsis ZIP* genes complemented a yeast Zn uptake-deficient mutant, one was able partially to complement a yeast Fe uptake-deficient mutant, six *ZIP* family members complemented an Mn uptake-deficient mutant, and none complemented the Cu uptake-deficient mutant. *AtZIP1* and *AtZIP2* were then chosen for further study, as the preliminary yeast and *in planta* analysis suggested they both may be root Zn and Mn transporters. In yeast, *AtZIP1* and *AtZIP2* both complemented the Zn and Mn uptake mutants, suggesting that they both may transport Zn and/or Mn. Expression of both genes is localized to the root stele, and *AtZIP1* expression was also found in the leaf vasculature. It was also found that AtZIP1 is a vacuolar transporter, while AtZIP2 is localized to the plasma membrane. Functional studies with *Arabidopsis AtZIP1* and *AtZIP2* T-DNA knockout lines suggest that both transporters play a role in Mn (and possibly Zn) translocation from the root to the shoot. AtZIP1 may play a role in remobilizing Mn from the vacuole to the cytoplasm in root stellar cells, and may contribute to radial movement to the xylem parenchyma. AtZIP2, on the other hand, may mediate Mn (and possibly Zn) uptake into root stellar cells, and thus also may contribute to Mn/Zn movement in the stele to the xylem parenchyma, for subsequent xylem loading and transport to the shoot.

Key words: *Arabidopsis*, AtZIP1, AtZIP2, Mn transport, Zip transporters, Zn transport.

Introduction

The ZIP family of membrane transporters has been shown to play a role in the transport of four essential micronutrients: Zn, Fe, Mn, and Cu (Eide *et al*., 1996; Grotz *et al*., 1998; Pence *et al*., 2000; Wintz *et al*., 2003; Cohen *et al*., 2004; Pedas *et al*., 2008; Lin *et al*., 2009). ZIP, which stands for *Z*RT-*I*RT-like *P*roteins, was named for the two founding members, the high affinity yeast plasma membrane Zn uptake transporter, ZRT1, and the high affinity *Arabidopsis thaliana* plasma membrane Fe uptake transporter, IRT1 (Eide *et al*., 1996; Zhao and Eide, 1996). Subsequent studies of other plant ZIP family members demonstrated that not all the members of the ZIP family are involved in plasma membrane micronutrient uptake. An example of this in plants is *AtIRT2*, which was recently shown to play a role in Fe homeostasis by transporting Fe into endomembrane vesicles, and is not involved in cellular uptake as originally hypothesized (Vert *et al*., 2009).

ZIP family members have also been shown to transport heavy metals such as Cd, and hence the ZIP family may also play a significant role in how various heavy metals, both essential and toxic, are taken up and translocated throughout the plant (Guerinot, 2000; Pence *et al*., 2000; Rogers *et al*., 2000). A better understanding of the roles and functions of each of the

15 members of the *Arabidopsis* ZIP family should lead to new insights into micronutrient/heavy metal homeostasis. Certainly a primary goal of such an effort should be to identify the metals that each ZIP family member transports. Other important features of metal transporters that were focused on in this study are the membrane localization for ZIP transporters, whether they transport metals into or out of a specific organelle, and the regulation of ZIP gene expression by changes in plant micronutrient (Zn, Mn, Fe, or Cu) status. Gaining a better understanding of the *Arabidopsis* ZIP family as a whole should also help us better understand micronutrient nutrition in other biological systems as well, as the ZIP family of transport proteins is found in all branches of life, including plants, fungi, animals, and protists (Guerinot, 2000).

To date, only a limited number of members of the ZIP family have been characterized in plants (primarily in *Arabidopsis*) with regards to both their transport capabilities and their role *in planta*. Currently, there is a fairly detailed understanding of the roles and functions for three *Arabidopsis* ZIP transporters, AtIRT1, AtIRT2, and AtIRT3, with AtIRT1 being by far the most well studied based on its seminal role in root Fe uptake and transport (Eide *et al*., 1996; Rogers *et al*., 2000; Vert *et al*., 2001, 2002, 2009; Connolly *et al*., 2002; Lin *et al*., 2009). Very little or nothing is known about the function of the other 12 *Arabidopsis* ZIPs. Hence in this study, the aim was to characterize broadly the remaining ZIP family members to begin to gain insights into what metals they transport, and then two of these 11 ZIPs, AtZIP1 and AtZIP2, were functionally characterized in more detail for their possible roles in root Zn and Mn transport and homeostasis.

Materials and methods

Cloning of the Arabidopsis *ZIP family genes*

AtZIP2, *ZIP7*, and *ZIP11* were requested from RIKEN from a scan of full-length clones (www.brc.riken.jp). *ZIP3* was graciously given to us by Dr Guerinot, *AtZIP1*, *5*, *6*, *8*, *9*, *10*, and *12* were cloned using full-length primers and cDNA from root and shoot tissue from Zn-deficient and Zn-replete *Arabidopsis* plants (Supplementary Table S1 available at *JXB* online).

Protein predictions

The translated AtZIP proteins were analysed using the online site, WoLF pSORT (http://psort.ims.u-tokyo.ac.jp), to estimate their predicted membrane localization. In addition, the AtZIP proteins were queried against the SubCellular Proteomic Database (SUBA; http://www.plantenergy.uwa.edu.au), for predicted subcellular localization. To estimate the secondary protein structure, including the number of predicted transmembrane domains, the translated proteins were analysed using the Mobyle, a bioinformatics analysis tool (von Heijne, 1992) (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred).

Relative expression of ZIP family genes in Arabidopsis

Arabidopsis ZIP gene expression data were summarized from the AtGeneExpress database for global *Arabidopsis* gene expression through different developmental stages that was compiled by Schmid *et al*. (2005). The AtGeneExpress database was mined for the expression of 10 ZIP family members (At3g12750, At5g59520, At2g32270, At1g05300, At2g30080, At2g04032, At4g33020, At1g31260, At1g55910, and At5g62160) at day 7, 15, and 21 in both roots and shoots. The data are publically available at http://jsp.weigelworld.org/.

Yeast studies

Four different yeast mutant strains were used in these studies: the Zn uptake-defective mutant *zrt1/zrt2Δ* (*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1*::LEU2 *zrt2*::HIS3), the Fe uptake-defective mutant *fet3/fet4Δ* (*MATa can1 his3 leu2 trp1 ura3 fet3*::HIS3 *fet4*::LEU2), the Mn uptake-defective mutant *smf1Δ* (SLY8; *MATa ura3 lys2 ade2 trp1 his3 leu2 smf1*::HIS3), and the Cu uptake-defective mutant *ctr1/ctr3Δ* (*MATa ctr1::ura3::Kanr ctr3::*TRP1 *his3 lys2-802* CUP1r). Each strain was maintained on YPD until introduction of either the gene of interest or the empty vector. Cultures of each yeast mutant strain containing one of the genes of interest were grown in liquid SC-URA to an optical density (OD) of 1 and then serially diluted 10-, 100-, and 1000-fold. Each dilution was plated out onto the specific restrictive media for that mutant. For the *zrt1/zrt2Δ* mutant, the restrictive medium contained SC-URA plus 1 mM EDTA and 500 μM $ZnCl_2$ for Zn-limiting growth conditions (Pence *et al*., 2000). For *smf1Δ*, the medium was SC-URA containing 20 mM EGTA for Mn-limiting conditions (Supek *et al*., 1997). The *ctr1/ctr3Δ* mutant was assayed for growth on YPE medium (10 g of yeast extract and 20 g of peptone per litre, and 10% ethanol) for Cu-limiting conditions (Pena *et al*., 2000). For the *fet3/fet4Δ* mutant, cells were grown on SC-URA (pH 4.0) containing 80 μM bathophenanthroline disulphonate (BPS) which produced Fe-limiting growth conditions (Eide *et al*., 1996).

Quantitative RT–PCR

For quantitative PCR (qPCR), *Arabidopsis* Col-0 plants were grown on a modified Johnson's solution containing 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 0.1 μM $NiSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, and 2 mM MES (pH 5.5). For all metal treatments, plants were grown for 2 weeks on this medium and then switched to the same nutrient solution except that Zn, Cu, Mn, or Fe was omitted, and the plants were grown for an additional 7 d. Plants were then harvested, snap-frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. Total RNA was isolated using the Plant RNeasy RNA mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Transcript levels were measured using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) with the primer pairs GCATGCGGGTCATGTTCAC and CAACTCGGTCGAACCATGTG for *AtZIP1* and GTTTGTAGCGGCTGGGAGTAA and GTCATCA TCCTCCCTCGACTCT for *AtZIP2*. A total of 25 ng of purified cDNA was used in each reaction to compare transcript levels across treatments. 18S was used as an internal reference and was amplified using the primer pair CGCTATTGGAGCTGGAATTACC and AATCCCT TAACGAGGATCCATTG to normalize across treatments and species. A 1 ng alquot of cDNA was used in these reactions since expression levels were high. Quantitative real-time reverse transcription–PCR (RT–PCR) was performed using an ABI 7500 real-time PCR system and SYBR Green kit (Applied Biosystems). PCR conditions used were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, and 60 °C for 1 min. A dissociation curve was performed after each of the two biological replicates to ensure only one product was being amplified.

Protein localization

AtZIP1 and *AtZIP2* were cloned into the pSAT1 enhanced green fluorescent protein (eGFP) expression vector in-frame with the C-terminus of eGFP using the *Kpn*I site, and transiently expressed in freshly isolated protoplasts from 3- to 4-week-old *Arabidopsis* seedlings according to Sheen *et al*. (2001). Confocal images were taken 15–18 h post-transfection of the *Arabidopsis* protoplasts. Images were taken on a Leica TCS-SP5 confocal microscope (Leica Microsystems, Exton, PA, USA) with a ×63magnification, numerical aperture 1.2, water immersion objective. The eGFP was excited with a blue argon ion laser (488 nm), and the emitted fluorescence was collected between 505 nm and 545 nm.

Promoter localization

To study the tissue-specific expression of both *AtZIP1* and *AtZIP2*, 1 kb of promoter sequence upstream of each start codon was cloned in front

of a β-glucuronidase (GUS) reporter using the pGPTV:BAR vector and incorporated into *A. thaliana* ecotype Columbia via transformation with *Agrobacterium tumefaciens*, using the floral dip method for stable transformation. Primers used for amplification are listed in Supplementary Table S1 at *JXB* online. Transformed plants were selected after growth on soil using BASTA, and surviving seedlings were grown to seed. T_2 seeds were then assayed for GUS activity by growing them for 10–14 d on a modified Johnson's solution (composition listed above in the Quantitative RT–PCR section) under Zn-, Fe-, Mn-, or Cu-limiting conditions. *AtZIP1*p::GUS or *AtZIP2*p::GUS plants were then assayed for GUS activity in a solution containing 1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) β-D-glucuronic acid in 50 mM Na_2HPO_4, pH 7.0, and 0.1% Triton X-100 for ~4 h. The reaction was stopped by removing the GUS solution and replacing it with 95% ethanol. At least 10 independent transformation events for both ZIP1 and ZIP2 were tested for expression of the GUS reporter and, in each transgenic line, the image for the promoter::GUS reporter was similar.

Phenotypic analysis of AtZIP1 *and* AtZIP2 *T-DNA knockout lines*

Seeds corresponding to the *AtZIP1* (At3g12750) and *AtZIP2* (At5g59520) genomic regions were requested from the ABRC (Alonso *et al*., 2003). Genotyping of individual plants was done using one of the two primers listed in Supplementary Table S1 at *JXB* online, with one primer anchored in the open reading frame of each ZIP member to allow for the possibility of the T-DNA insertion being incorporated in either direction. The primer GCTGTTGCCCGTCTCACTGGTG was used for the T-DNA insert. PCR conditions were 95 °C for 30 s; 55 °C for 30 s; 72 °C for 4 min; 35 cycles were run for all lines tested. The PCR product was then purified using a Qiagen PCR clean up kit (Qiagen) and sequenced with both the primers listed above. To verify the lack of *AtZIP1* transcript in the *AtZIP1* T-DNA line, the 5' portion of the *AtZIP1* transcript was assayed for using the primer pair AAAAAGGATTCCGGCGTTACAA and CCCAATCTCAAGCACCTGCGACACTAT; the 3' end of the gene was studied using the primer pair TCGCAGGTGCTTGAGATTGGGATA GTT and AGATAAGCAGCCAAGTGAAGCCAGAGA. To verify the lack of *AtZIP2* transcript in the *AtZIP2* T-DNA line, the 5' portion of the gene was assayed with the primer pair GCGACGAAGAAGAG GAGACCAACCAG and TATCTCACGACGTCCTTCCTCTTTCAC, and the 3' end of the gene was studied using the primer pair, AAGAG GAAGGACGTCGTGAGATAAAGA and TAACCGCAACGTACAC AAAAACTCCAC.

Growth conditions and determination of mineral content for T-DNA knockout lines

Nutrient-replete T-DNA lines were grown on 1× Murashige and Skoog (MS) medium plus 1% sucrose. To provide Zn-deficient or high Zn plants, either Zn was omitted from the medium or $ZnSO_4$ was added to give a final Zn concentration of 300 μM. To alter the plant Mn status, plants were grown on 1× MS medium where either the Mn was omitted or $MnSO_4$ was added to give a final concentration of 250 μM. For inductively coupled plasma atomic emission spectroscopy (ICP-AES) determination of plant Zn content, plants were grown on 1× MS+1% sucrose with a total Zn concentration of 300 μM. For ICP determination of plant Mn content, plants were grown on 1× MS+1% sucrose with a total Mn concentration of 250 μM. Following growth for 7 d, plants were harvested and metals adsorbed in the root cell wall were desorbed in 5 mM $CaCl_2$ solution for 15 min using well established protocols in our lab (Hart *et al*., 1998*a*, *b*), and then rinsed in 18 MΩ water. Roots and shoots were separated, dried, weighed, and analysed for Zn and Mn content using ICP-AES.

Results

Isolating the 11 remaining Arabidopsis *ZIP family members*

In an attempt to characterize the 11 remaining uncharacterized *Arabidopsis* ZIP family members functionally, public repositories were searched for full-length cDNA clones of the *Arabidopsis ZIP* genes. Only *ZIP2*, *ZIP7*, and *ZIP11* were found as full-length cDNA clones in the RIKEN database. *AtZIP3* was obtained from the Guerinot Lab, which they originally used in the research reported in Grotz *et al*. (1998). To clone the remaining eight members of the *Arabidopsis* ZIP family, primers were designed based on the predicted open reading frame listed in TAIR, and the ZIP genes were cloned from RNA isolated from roots and shoots of plants grown under Zn-replete and Zn-deficient conditions. Both Zn-replete and Zn-deficient conditions were used to increase the chance that a given ZIP transporters was expressed. The DNA sequences for all isolated genes were as listed in TAIR except *ZIP6* and *ZIP8*. The version of *AtZIP6* used here harbours a point mutation which changes amino acid 65 from an isoleucine to a methionine. This mutation is in the beginning of the second predicted transmembrane domain. The version of *AtZIP8* which was isolated was significantly different from the reference gene with regards to the predicted genomic structure presented on TAIR, in that the last predicted intron failed to be removed, which led to a truncation in the predicted protein, causing the loss of the last two transmembrane domains. The experiments were not successful in isolating any other versions of *ZIP8* despite repeated efforts to clone this gene from either the roots or shoots of *Arabidopsis* plants grown under control and Zn-, Fe-, Mn-, or Cu-limiting conditions. Otherwise all other ZIP family members were as predicted by the gene models in TAIR.

Yeast complementation

To determine the metal specificities for transport of the proteins encoded by these 11 ZIP genes, each gene was tested for its ability to complement one of four different yeast metal uptake mutants. The four different yeast lines consisted of mutants defective in Zn uptake (*zrt1/zrt2Δ*), Cu uptake (*ctr1/ctr3Δ*), Fe uptake (*fet3/fet4Δ*), or Mn uptake (*smf1Δ*). Of the 11 different genes tested, six ZIP genes, *ZIP1*, *ZIP2, ZIP3*, *ZIP7*, *ZIP11*, and *ZIP12*, were able to complement the *zrt1/zrt2Δ* yeast mutant fully or partially under Zn-limiting conditions (Fig. 1). Six *AtZIP* genes were also able to complement the Mn uptake mutant *smf1Δ* fully or partially under Mn-limiting conditions; these genes are *ZIP1*, *ZIP2*, *ZIP5*, *ZIP6*, *ZIP7*, and *ZIP9*. None of the 11 genes tested was able to complement the Cu uptake mutant, *ctr1/ctr3Δ*, and only *ZIP7* was able to complement the Fe uptake mutant, *fet3/fet4Δ* partially. Of the 11 genes tested, only *ZIP8* failed to complement any of the four uptake mutants.

From these yeast complementation studies, it was determined that, potentially, ZIP7 could transport Zn, Mn, and Fe, ZIP1 and ZIP2 could transport Zn and Mn, ZIP3, ZIP11, and ZIP12 could transport Zn, and ZIP5, ZIP6, and ZIP9 could transport Mn.

Relative expression of ZIP family members

To begin to better understand the role of the ZIP family of transporters in plants, the expression of 10 of the 11 plant ZIP genes studied here was compiled from a global gene expression map for *Arabidopsis* development (Schmid *et al*., 2005), and is summarized in Table 1 for 7-, 15-, and 21-day-old plants. For

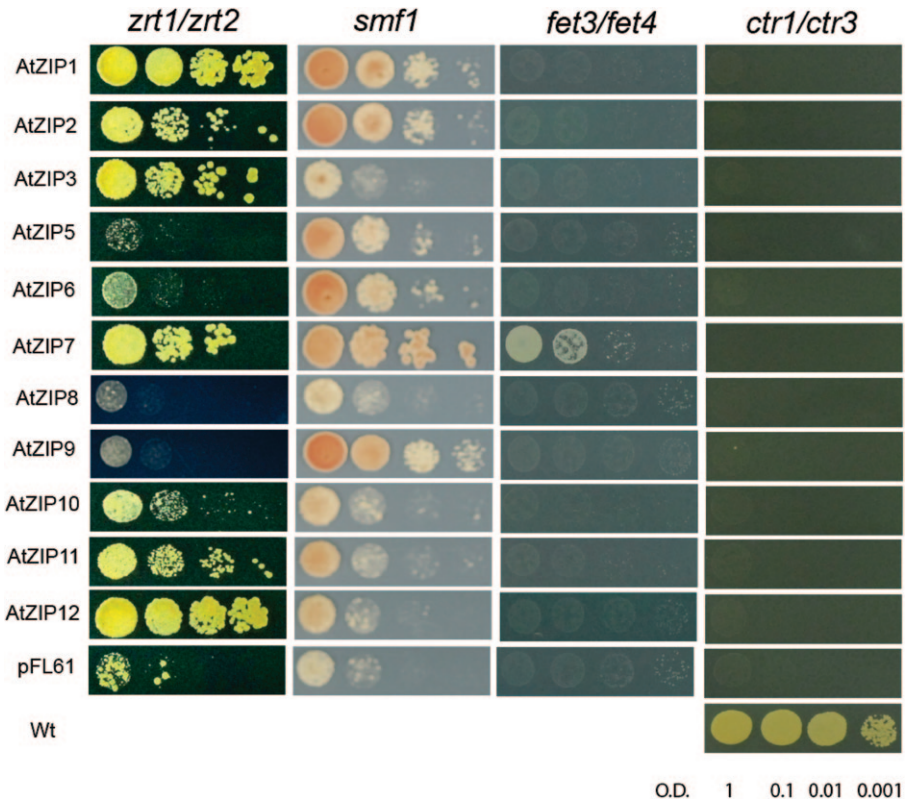

Fig. 1. Complementation of yeast metal uptake mutants with *AtZIP* genes. Complementation of yeast mutants defective in the uptake of Zn (*zrt1/zrt2*Δ), Mn (*smf1*Δ), Fe (*fet3/fet4*Δ), or Cu (*ctr1/ctr3*Δ) expressing any one of the 11 *AtZIP* genes studied, or the empty vector, pFL61, as a control. Each gene was analysed for its ability to confer growth of yeast mutants defective in the transport of Zn, Mn, Fe, or Cu on media low in each of these mineral nutrients. Complementation is shown after 48–72 h of growth on selective media containing the limiting micronutrient of interest. Each spot represents a 1:10 dilution of the culture starting with an OD of 1 on the far left (10-, 100-, and 100-fold dilutions). (This figure is available in colour at *JXB* online.)

the study of Schmid *et al*. (2005), all of the ZIPs studied here were analysed for expression, except *AtZIP8*, which is not on the Affymetrix array used for that study. ZIP gene expression in both roots and shoots is presented in Table 1 to determine if there is an organ-/tissue-specific bias in gene expression for specific ZIPs. From this analysis, it was found that ZIP1, ZIP2, ZIP3, ZIP5, and ZIP6 are all expressed at higher levels in the roots than in the shoots. Also ZIP1, ZIP2, ZIP3, and ZIP5 increase in relative expression in the roots as the plant gets older. The ZIP genes that showed significantly higher expression in the shoots are ZIP7 and, to a lesser extent, ZIP11, which showed high shoot expression at day 7 in the shoots but then its expression decreased as the plant aged. ZIP9, ZIP10, and ZIP12 showed relatively equal expression in both roots and shoots, and did not show any difference in expression as the plant aged.

It was beyond the scope of this study to characterize all 11 ZIP genes in detail. Thus for the subsequent more detailed research, it was decided to focus on two ZIP transporters, AtZIP1 and AtZIP2. These two ZIP transporters were two of the three ZIPs (along with ZIP7) that appear to mediate both Zn and Mn uptake, which was of interest, especially since very little is known about plant Mn transporters in particular. Also, it was decided to focus on candidate root metal uptake transporters for this more detailed study, and ZIP1 and ZIP2 were two of four ZIPs (along with ZIP3 and ZIP6) that are expressed more strongly in roots.

Table 1. *Relative expression of 10 members of the ZIP family of transporters in* Arabidopsis *in 7-, 15-, and 21-day-old plants compiled from the AtGenExpress database* (Schmid *et al*., 2005)
Listed are the relative expression of 10 *Arabidopsis* ZIP family members in both root and shoot (leaf) tissue. Relative expression values are in relation to expression of internal standards on the Affymetrix ATH1 arrays used for the study.

	Root (day 7)	Root (day 15)	Root (day 21)	Shoot (day 7)	Shoot (day 15)	Shoot (day 21)
ZIP1	45	93	126	33	28	18
ZIP2	122	1702	2394	7	10	7
ZIP3	149	305	434	12	12	12
ZIP5	6	38	44	7	10	10
ZIP6	117	95	113	43	42	20
ZIP7	10	9	8	112	52	225
ZIP9	7	18	7	7	7	7
ZIP10	9	5	4	5	5	5
ZIP11	42	146	171	150	148	52
ZIP12	26	23	23	27	26	22

Investigation of AtZIP1 *and* AtZIP2 *expression in response to different metal treatments*

The relative transcript levels of *AtZIP1* and *AtZIP2* were quantified in *Arabidopsis* plants grown under either nutrient-replete conditions, Zn deficiency, Fe deficiency, Cu deficiency, or Mn deficiency. In nutrient-replete plants, *AtZIP1* was found to be expressed ~50% more in the roots than in the shoots when expression was normalized to either 18S or actin2 transcript levels (actin data not shown). *AtZIP1* transcript abundance in roots was affected most by Zn deficiency, which significantly increased transcript abundance >4-fold relative to roots of replete-grown plants (Fig. 2A). Expression of *AtZIP1* was also increased in the roots by Fe deficiency, which resulted in a 2.5-fold increase in *AtZIP1* expression; however, Cu and Mn deficiency had no significant effects on *AtZIP1* transcript levels. Increased shoot expression of *AtZIP1* was also seen in response to Mn deficiency, which increased transcript levels ~80% relative to levels of *AtZIP1* expression in shoots of replete-grown plants (Fig. 2B). Cu and Fe deficiency had no effect on *AtZIP1* expression, and Zn deficiency significantly reduced *AtZIP1* expression by ~50% relative to expression in shoots of replete-grown plants.

AtZIP2 was found to be expressed ~10-fold more in the roots than the shoots of nutrient-replete plants (data not shown). In roots, *AtZIP2* expression was decreased in response to Fe deficiency, while Zn, Mn, and Cu deficiency had no effect on expression (Fig. 3A). Shoot expression of *AtZIP2* was found to be very low, and this low level of expression was decreased by ~60–70% in response to Mn and Fe deficiency (Fig. 3B). *AtZIP2* expression was not altered by Zn or Cu deficiency.

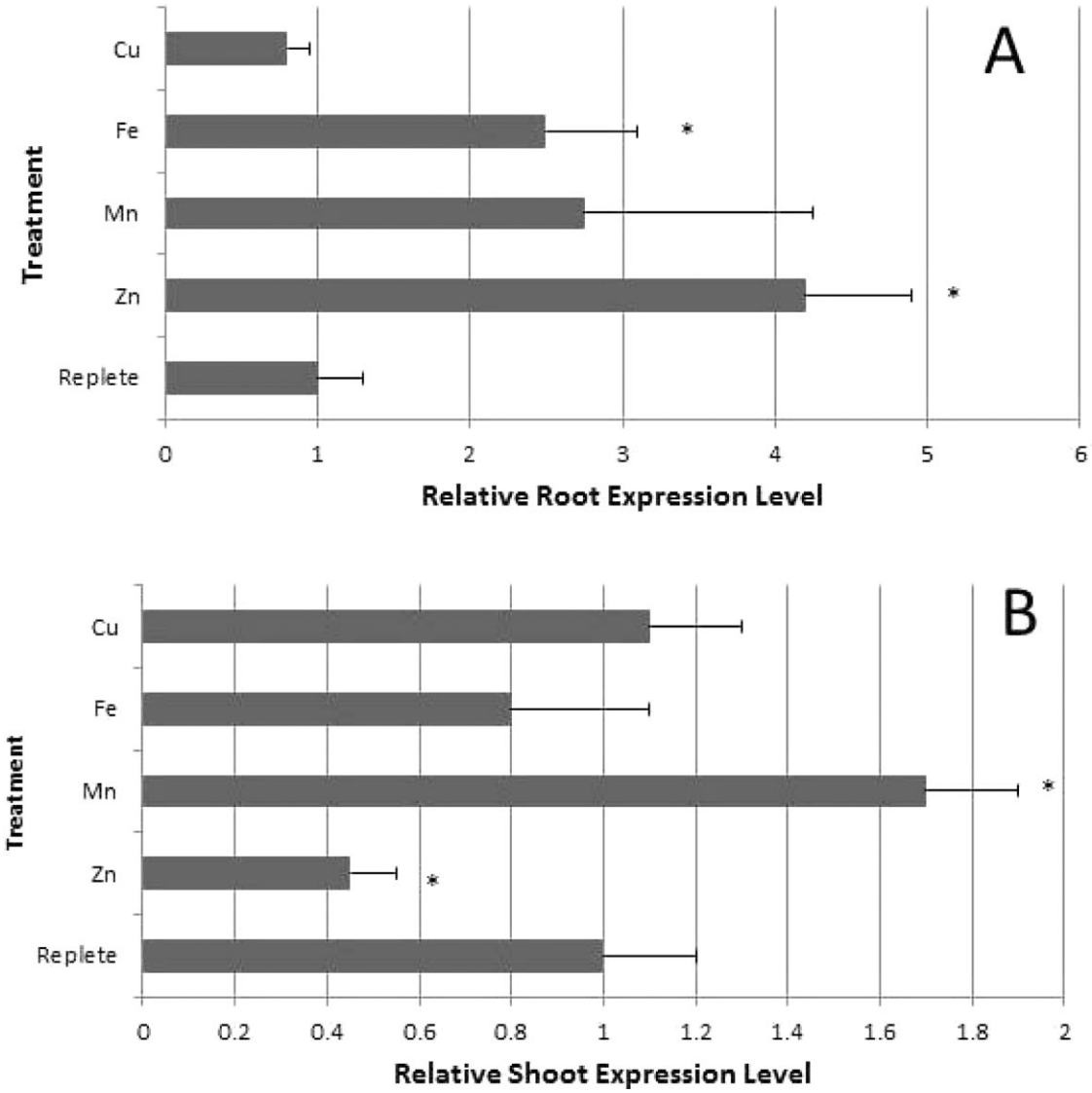

Fig. 2. Relative transcript levels of *AtZIP1*. Quantitative real-time PCR analysis of *AtZIP1* expression in: (A) roots or (B) shoots of *A. thaliana* plants grown on nutrient-replete, –Zn, –Mn, –Fe, or –Cu nutrient solution. The average transcript levels ±SD are presented for four technical replicates. *AtZIP1* expression was normalized to 18S levels for differences in expression among different treatments. Expression under nutrient-replete conditions was set to 1 as the frame of reference within each experiment. Asterisks indicate significant differences relative to the nutrient-replete treatment ($P < 0.05$).

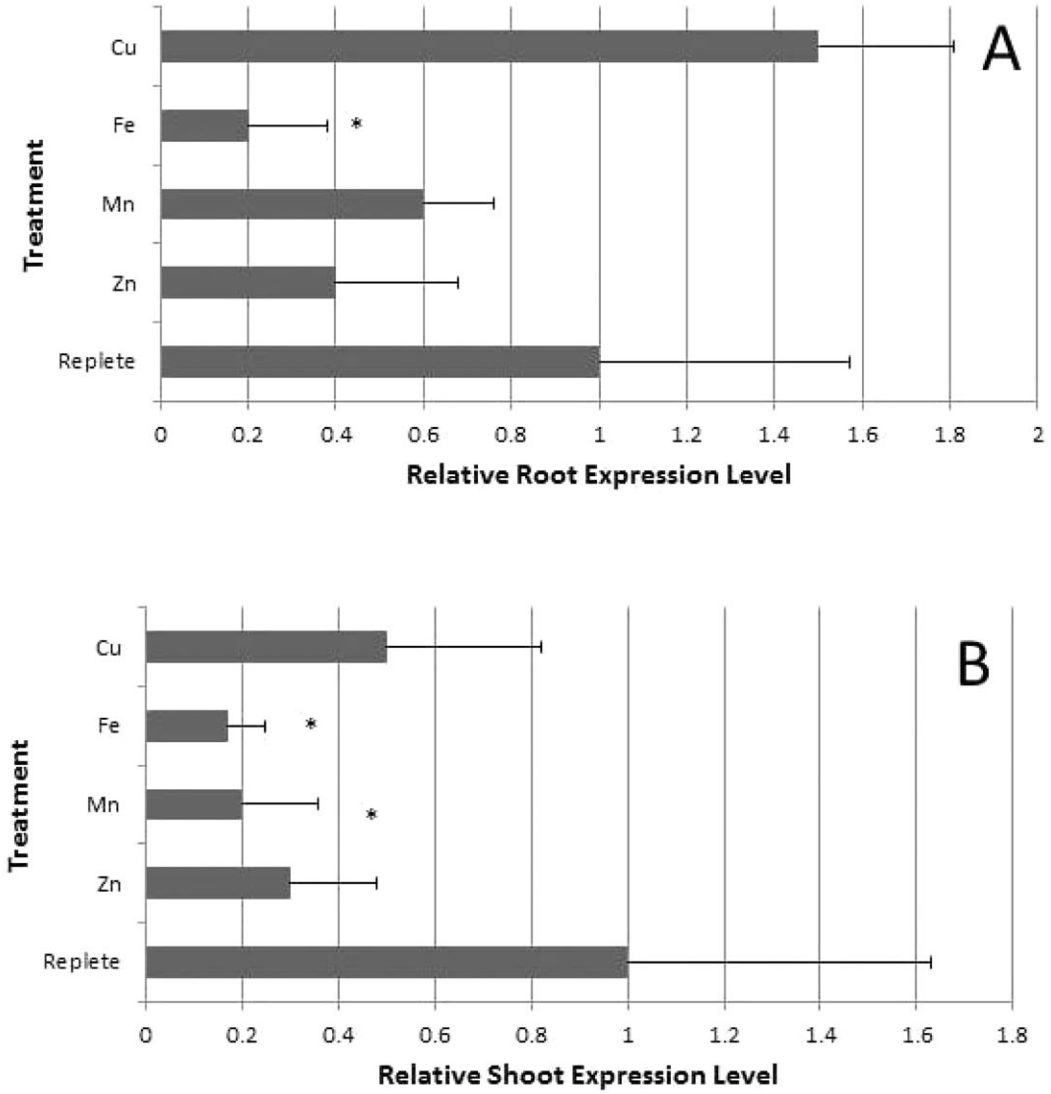

Fig. 3. Relative transcript levels of *AtZIP2*. Quantitative real-time PCR analysis of *AtZIP2* expression in: (A) roots or (B) shoots of *A. thaliana* plants grown on nutrient-replete, –Zn, –Mn, –Fe, or –Cu nutrient solutions. The average transcript levels ±SD are presented for four technical replicates. *AtZIP2* expression was normalized to 18S levels for differences in expression among different treatments. Expression under nutrient-replete conditions was set to 1 as the frame of reference within each experiment. Asterisks indicate significant differences relative to the replete treatment $P < 0.05$).

Localization of the AtZIP1 and AtZIP2 protein

Both AtZIP1 and AtZIP2 were cloned in-frame with an N-terminal eGFP and transiently expressed in *Arabidopsis* protoplasts. The eGFP:ZIP1 fusion protein localized to the vacuole (Fig. 4A–D). The arrow in Fig. 4 indicates the characteristic invaginations associated with a vacuolar localization. The image for expression of the eGFP:ZIP2 fusion was consistent with a plasma membrane localization (Fig. 4E–H).

Tissue-specific expression

To study the tissue-specific expression of both *AtZIP1* and *AtZIP2*, 1 kb of promoter sequence upstream of each start codon was cloned in front of a GUS reporter gene and stably transformed into *Arabidopsis* plants. Plants harbouring the *AtZIP1*p::GUS reporter were grown under nutrient-replete conditions as well as in response to Zn, Fe, Mn, and Cu deficiency. Analysis of T_3 homozygous lines showed that *AtZIP1* is predominantly expressed in the root stele and in the shoot vasculature (Fig. 5A–F). It was only possible to see *AtZIP1*::GUS expression in seedlings grown under Zn deficiency conditions. In either nutrient-replete plants or plants subjected to Fe, Mn, or Cu deficiency, GUS staining was seen, indicating that Zn deficiency induced the greatest increase in *AtZIP1* expression, which is consistent with what was seen from the qPCR data in Fig. 2A, at least for expression in roots.

*AtZIP2*p::GUS lines were also studied under the same conditions as for *AtZIP1*, and *AtZIP2* was found to be expressed at lower levels than *AtZIP1*, with the strongest expression occurring under

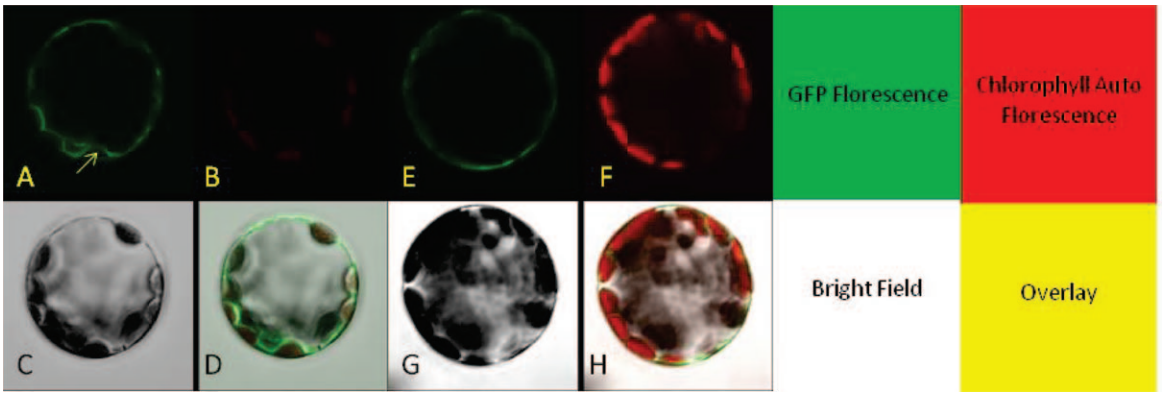

Fig. 4. Subcellular localization of eGFP:AtZIP1 and eGFP:AtZIP2. Both AtZIP1:eGFP and AtZIP2:eGFP fusion proteins were transiently expressed in *Arabidopsis* protoplasts for 16 h. Each set of four panels consists of the GFP–AtZIP image (upper left), chlorophyll fluorescence (upper right), bright field image (lower left), and a combined image of the three channels (lower right). (A–D) eGFP:AtZIP1; (E–H) eGFP:AtZIP2.

nutrient-replete conditions. Like *AtZIP1*, *AtZIP2* is expressed most strongly in the root stele. As one moves from the younger to more mature root regions, strong stelar *AtZIP2* expression was seen in the primary root elongation zone, with lower expression in the stele of lateral roots (Fig. 5G–K). Then, particularly in the primary root, *AtZIP2* stelar expression increases in more mature regions of the root, especially close to the root–shoot junction (Fig. 5I, K). No expression of the *AtZIP2*p::GUS reporter could be seen in the shoots.

Phenotypic analysis of AtZIP1 *and* AtZIP2 *T-DNA knockout lines*

To better understand the role of *AtZIP1* and *AtZIP2* in Zn and Mn nutrition, seeds of plants harbouring projected T-DNA insertions in these two genes were obtained from the ABRC. Two independent homozygous lines were isolated with a disruption in an exon of *AtZIP1*. Conformation of the insertions identified two distinct T-DNA lines for *AtZIP1* and one for *AtZIP2* with T-DNA insertions in an exon. As depicted in Fig. 6A, the T-DNA insertions for the two *AtZIP1* T-DNA lines are located towards the 3' end of the gene. One of these lines, SALK_023634, results in a predicted truncated AtZIP1 protein that has lost the last two transmembrane domains; the SALK_109897 line is predicted to generate a truncated protein that has lost half of the eighth transmembrane domain. As mentioned above, only one T-DNA line could be found that had an insertion located in an AtZIP2 exon. The T-DNA line (SALK_094937) with a T-DNA insertion located in the *AtZIP2* coding region is predicted to encode a protein that has lost the last three transmembrane domains (Fig. 6B). To confirm that the lines were true knockouts and not knockdown lines, primers were designed for both the 5' and 3' ends of the *AtZIP1* and *AtZIP2* transcripts. As seen in Fig. 6, only partial transcripts could be obtained for both *AtZIP1* and *AtZIP2*; no full-length transcripts were found for any of the three T-DNA lines tested.

When the T-DNA *Arabidopsis* lines were grown on nutrient-replete media, no obvious phenotype was observed for the three lines. Subsequently, plants were grown first on either Zn-deficient (–Zn) or high Zn (300 μM Zn) media, and root tolerance (relative root growth) was quantified for each T-DNA knockout line. There was no difference observed in relative root growth for either *AtZIP1* T-DNA knockout line compared with Col-0 in response to Zn deficiency or high Zn growth conditions (Fig. 7A). However, the *AtZIP2* T-DNA knockout line exhibited significant responses to growth under both Zn growth conditions compared with Col-0 plants, showing increased tolerance to Zn deficiency and increased sensitivity to growth on high Zn (Fig. 7A).

The T-DNA lines were also assayed for root and shoot Zn accumulation in response to growth for 2 weeks on high Zn (300 μM Zn). ICP analysis of root and shoot Zn content for the T-DNA lines showed that roots of *AtZIP2* knockout plants accumulated significantly more Zn than roots of Col-0 plants (Fig. 7B), which is consistent with the increased sensitivity to high Zn seen in Fig. 7A. It should be noted that the relatively high root Zn concentrations (1000–2000 ppm) are due in part to Zn^{2+} binding to negatively charged cation exchange sites in the cell walls that is not totally removed by any desorption protocol including the standard protocol (15 min desorption in 5 mM $CaCl_2$; Hart *et al.*, 1998*a*, *b*). When micronutrient metal uptake in roots was first studied, detailed desorption studies were conducted and for the divalent cations Zn^{2+}, Cu^{2+}, Mn^{2+}, and Cd^{2+} it was found that no desorption protocol removed all of the cell wall-bound metal. The only way this could be accomplished was with longer desorption times that also allowed significant efflux of the absorbed metal from the cytoplasm. Hence the 15 min desorption period using 5 mM $CaCl_2$ (the 5 mM Ca^{2+} relatively effectively desorbs other metal cations bound to the cell wall) is a compromise in that it is the longest desorption period which can be run without allowing for significant loss of Zn or Mn ions that have been previously absorbed into the root symplasm. The T-DNA lines were also grown on Mn-deficient (–Mn) and high Mn (250 μM) conditions; both the *AtZIP1* and *AtZIP2* knockout lines were more sensitive to Mn deficiency compared with Col-0 plants, with the *AtZIP1* T-DNA lines exhibiting considerably greater sensitivity to Mn deficiency (Fig. 8A). When high Mn stress was imposed, *AtZIP2* lines were somewhat more tolerant to high Mn compared

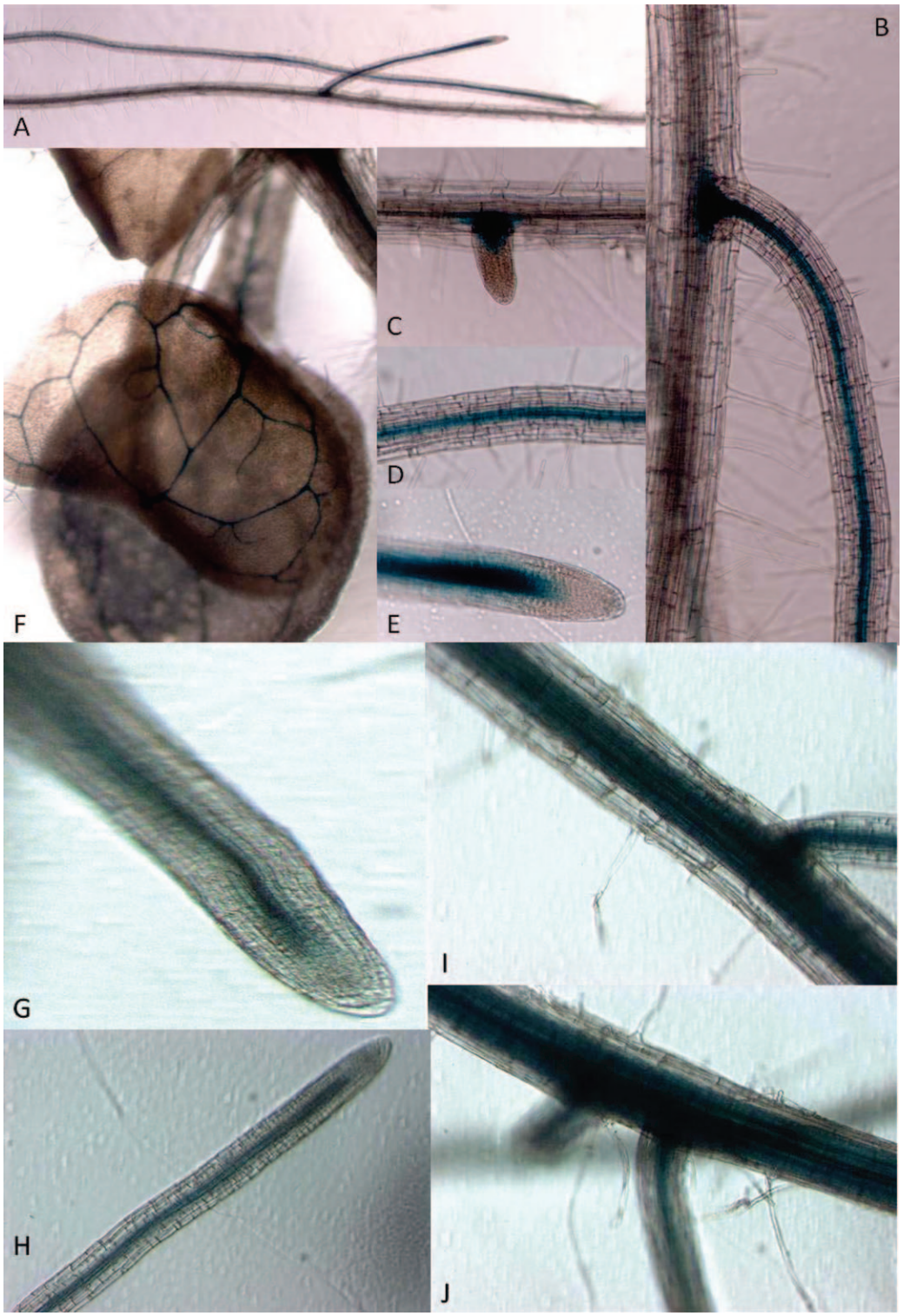

Fig. 5. Tissue localization of *AtZIP1 and AtZIP2* expression in transgenic *Arabidopsis*. *Arabidopsis* seedlings were transformed with either 1 kb of the *AtZIP1* promoter (A–F) or 1 kb of the *AtZIP2* promoter (G–K), driving a GUS reporter. The seedlings were grown for 3 weeks on a modified Johnson's solution without Zn for analysis of *AtZIP1* expression, or replete modified Johnson's solution for analysis of *AtZIP2* expression. These were the only plant nutrient conditions for which significant expression of each *ZIP* gene could be visualized via GUS staining. AtZIP1p::GUS expression in: (A) the root system on 14-day-old plants at low magnification; (B) lateral root from the primary root; (C) young emerging lateral root; (D) mature portion of a lateral root; (E) tip of a lateral root; (F) *Arabidopsis* leaf showing expression localized to the vasculature. AtZIP2p::GUS expression in: (G) a young lateral root; (H) the same young lateral root at lower magnification; (I) mature region of the tap root; (J) tap root near the root–shoot junction. At least 10 independent transformation events for both ZIP1 and ZIP2 were tested for expression of the GUS reporter, and, in each transgenic line, the image for the promoter::GUS reporter was similar.

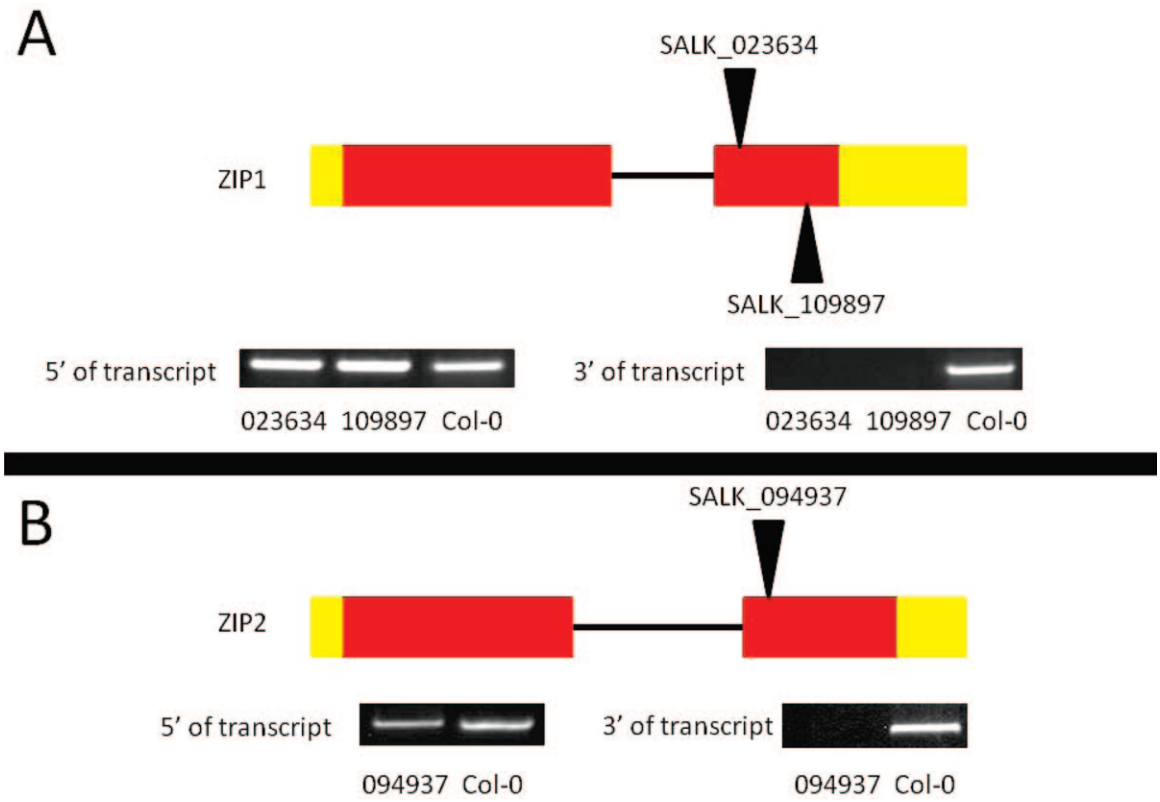

Fig. 6. T-DNA insertion lines. Diagrammatic model depicting the genomic structure of (A) *AtZIP1* and (B) *AtZIP2* along with the location of the T-DNA insertions. Grey boxes represent either the 5'- or 3'-untranslated region, black boxes represent an exon, the black line represents an intron, and the black triangle is the relative location of the T-DNA insertion. Below the *AtZIP1* and *AtZIP2* genomic models in A and B are the results of qRT–PCR for each gene in the respective T-DNA lines, showing that using primers from the 5' end of the gene yielded a product, while qRT–PCR with primers for the 3' end of each gene did not. (This figure is available in colour at *JXB* online.)

with Col-0 plants (based on root growth), whereas *AtZIP1* lines showed no difference in tolerance to high Mn relative to Col-0 plants.

With regards to root and shoot Mn accumulation in response to growth on high Mn, both T-DNA knockout lines exhibited much greater root Mn accumulation compared with Col-0 plants (6- to 7-fold greater), but no differences in shoot Mn accumulation were seen (Fig. 8B).

Because root Fe uptake can sometimes interact and interfere with root Zn uptake, Fe accumulation was also looked at in the two knockout lines under the same replete, high and –Zn, and high and –Mn growth conditions. It was found that there were no differences in root and shoot Fe accumulation between the *AtZIP1* and *AtZIP2* knockout lines and the Col-0 plants grown under all three nutrient regimes, as seen for nutrient-replete plants in Supplementary Fig. S1 at *JXB* online (data for high Zn/–Zn and high Mn/–Mn grown plants are not shown for the sake of clarity).

Discussion

The ZIP family has been implicated in the transport of the essential micronutrients/heavy metals Fe, Zn, Mn, and Cu (Eide *et al*., 1996; Grotz *et al*., 1998; Pence *et al*., 2000; Wintz *et al*., 2003; Cohen *et al*., 2004; Pedas *et al*., 2008; Lin *et al*., 2009). When the remaining 11 members of the *ZIP* family which to date have not been characterized were tested for their ability to complement the various yeast uptake mutants, six of the ZIPs were able to transport Zn, six were able to transport Mn, and one ZIP was able to transport Fe. Ten of the 11 were able to complement at least one mutant, three were able to complement two different mutants and one transporter gene, *ZIP7*, was able to complement the Zn-, Fe-, and Mn-deficient uptake mutants. Only *ZIP8* failed to complement any of the yeast transport mutants. This may be due to: (i) the alternative transcript for *ZIP8* repeatedly isolated in this study possibly encoding a non-functional protein; (ii) possible mislocalization of the ZIP8 protein in yeast to an internal membrane; or (iii) the possibility that *ZIP8* may be a pseudogene. It was not possible to isolate the predicted *ZIP8* sequence listed on TAIR, but it should be noted that one of the two introns was edited out in the ZIP8 sequence which was isolated here, and most probably represents a real transcript. Also, the lack of the predicted *ZIP8* mature transcript may suggest that if *ZIP8* is a true gene, it must be expressed at very low levels or may only be expressed under specific conditions or in a tissue not studied herein. This inability to clone *ZIP8* was not entirely surprising as there are no listed *ZIP8* expressed sequence tags in the TAIR database; the only evidence that ZIP8 is expressed is two peptide matches.

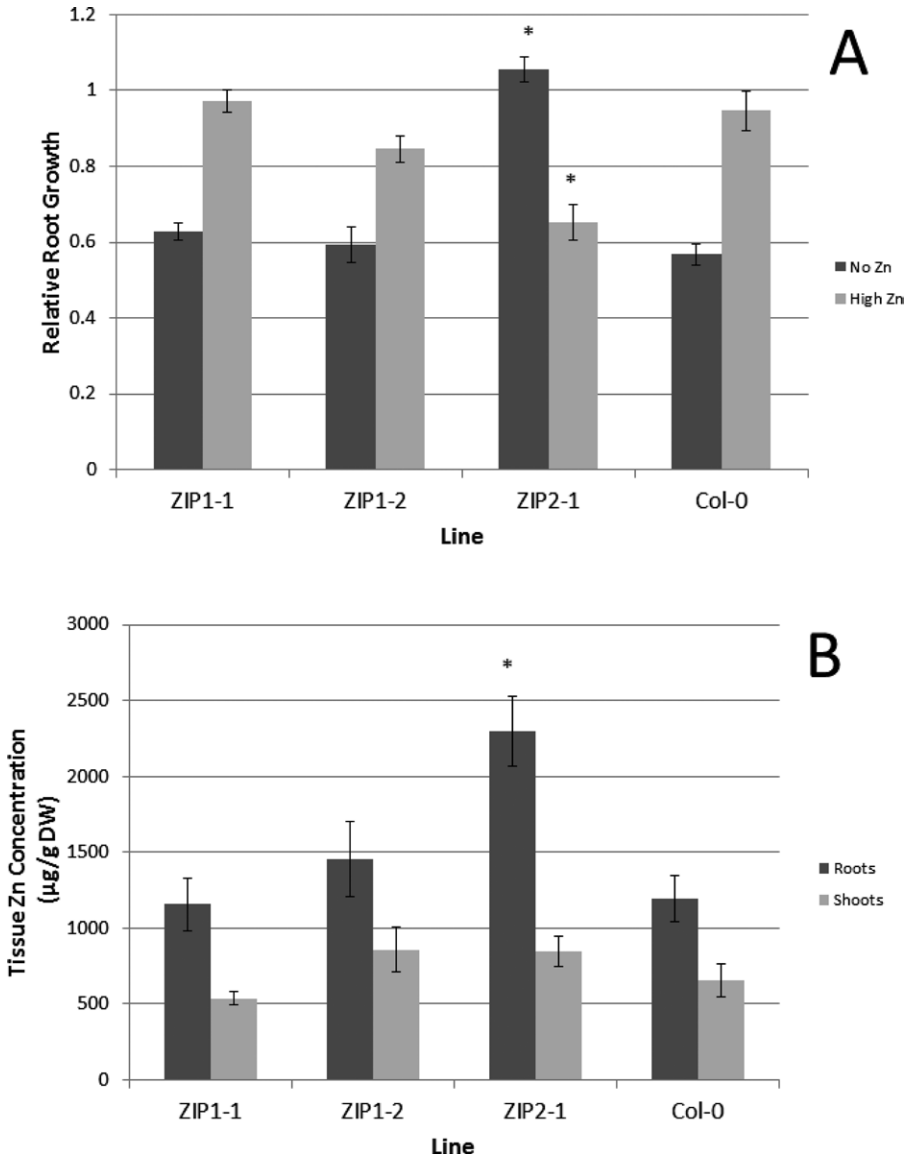

Fig. 7. Tolerance to Zn deficiency and high Zn, as well as Zn accumulation in *AtZIP1* and *AtZIP2* T-DNA knockout lines. (A) Tolerance to Zn deficiency (–Zn nutrient solution) and high Zn (nutrient solution +300 µM Zn) based on relative root growth (root growth on high Zn or – Zn media divided by root growth in nutrient-replete medium) for *AtZIP1* and *AtZIP2* T-DNA insertions lines. Plants were grown for 10 d on either nutrient-replete medium (MS with 1% sucrose), high Zn medium (MS with 1% sucrose and a final Zn concentration of 300 µM $ZnSO_4$), or Zn-deficient medium (MS with 1% sucrose and Zn omitted). Data are the means ±SE for 50 plants ($n > 50$). Significance was determined using ANOVA with Tukey's post-hoc test. An asterisk indicates a significant difference relative to Col-0 ($P < 0.05$). Shown are data from one representative experiment using 50 plants. Experiments were run at least twice with the same results. (B) Root and shoot Zn accumulation determined via ICP-AES for 2-week-old *AtZIP1* or *AtZIP2* T-DNA insertion lines and Col-0 plants grown on MS nutrient solution+1% sucrose and 300 µM Zn. Significance was determined using ANOVA and Tukey's post-hoc test ($P < 0.05$).

AtZIP1 transcript levels increase in the roots as the *Arabidopsis* plant ages, and its expression decreases in the shoot during the same developmental time sequence. A*tZIP1* transcript levels were higher in the roots under both Zn deficiency (4-fold increase) and Fe deficiency (2.5-fold increase) relative to expression in nutrient-replete roots, while no significant changes in *AtZIP1* expression were seen in response to the other micronutrient deficiencies. This increase in relative transcript levels under Zn deficiency in the roots is similar to what was seen when *AtZIP1* was first cloned by Grotz *et al*. (1998). In the shoots, Zn deficiency resulted in reduced *AtZIP1* transcript abundance, while Mn deficiency resulted in increased expression levels of *AtZIP1*. A region that is very similar in sequence to the zinc-responsive element identified in the *AtZIP4* promoter (Assunção *et al*., 2010) was found in the *AtZIP1* promoter, although it differs by 2 bp from the *AtZIP4* ZRE; this element is located ~700 bp upstream of the start codon in the *AtZIP1* promoter. Assunção *et al*. (2010) showed that the lack of expression of the transcription factors driving Zn responsiveness in the ZIPs (bZIP19 and bZIP23) did lead to altered *AtZIP1* expression and thus *AtZIP1*

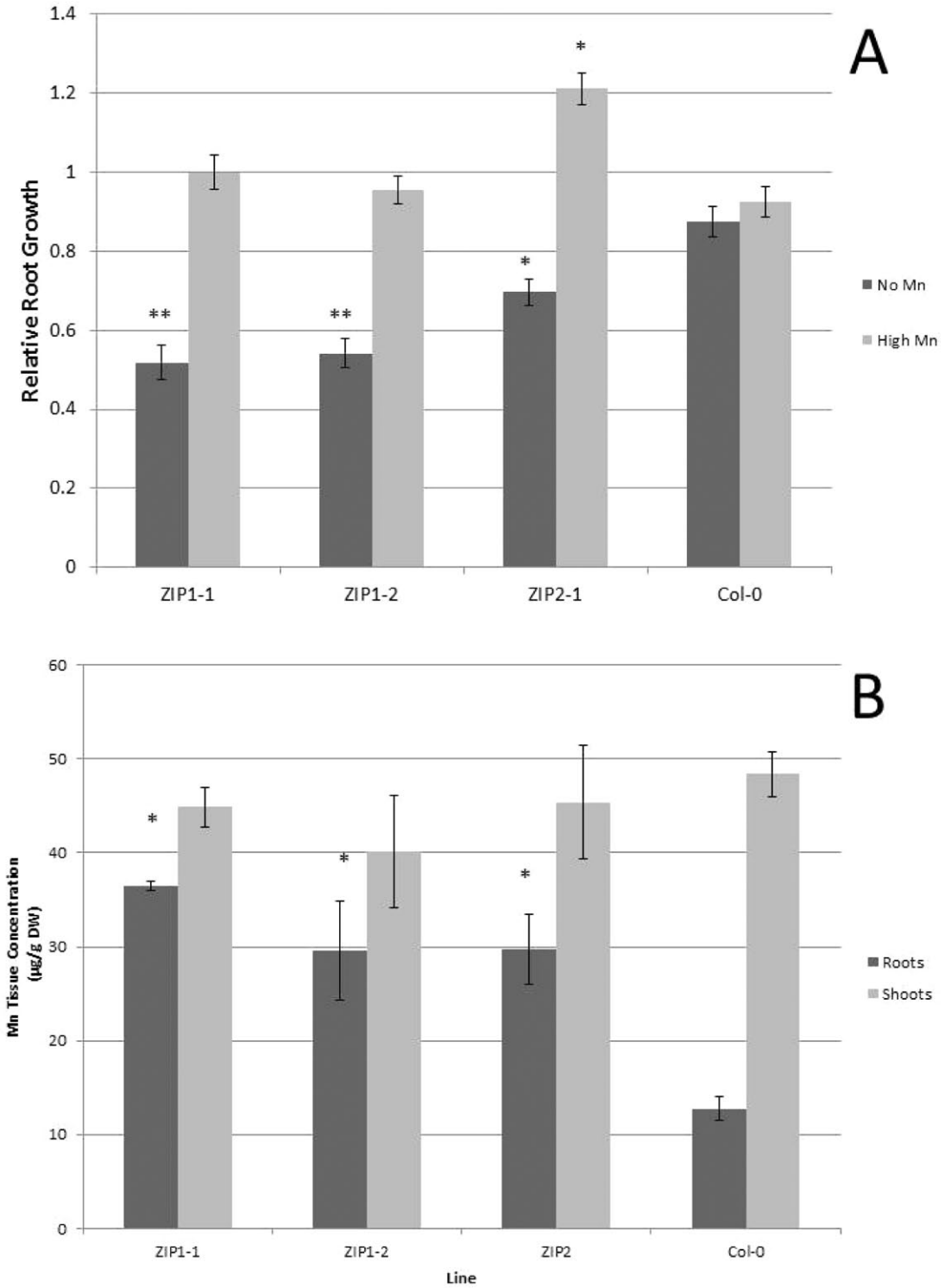

Fig. 8. Tolerance to Mn deficiency and high Mn, as well as Mn accumulation in *AtZIP1* and *AtZIP2* T-DNA knockout lines. (A) Tolerance to Mn deficiency (–Mn nutrient solution) and high Mn (nutrient solution +250 µM Mn) based on relative root growth (root growth on high Mn or – Mn media divided by root growth in nutrient-replete medium) for *AtZIP1* and *AtZIP2* T-DNA insertions lines Plants were grown for 10 d on either nutrient-replete medium (MS with 1% sucrose), high Mn medium (MS with 1% sucrose and a final Mn concentration of 250 µM Mn), or Mn-deficient medium (MS with 1% sucrose and Mn omitted). Data are the means ±SE error for 50 plants ($n > 50$). Significance was determined using ANOVA and Tukey's post-hoc test. An asterisk indicates a significant difference relative to Col-0 plants ($P < 0.05$). Shown are data from one representative experiment using 50 plants. Experiments were run at least twice with the same results. (B) Root and shoot Mn accumulation determined via ICP-AES for 2-week-old *AtZIP1* or *AtZIP2* T-DNA insertion lines and Col-0 plants grown on MS nutrient solution+1% sucrose and 250 µM Mn. Significance was determined using ANOVA and Tukey's post-hoc test ($P < 0.05$).

may be an additional target of the two bZIP proteins. This may also suggest that a somewhat broader promoter sequence is recognized by the bZIP transcription factors in controlling Zn homeostasis in plants.

Root and shoot *AtZIP2* transcript abundance was decreased in response to Zn, Fe, and Mn deficiency. The *AtZIP2* promoter does not appear to harbour any ZRE elements similar to those identified by Assunção *et al*. (2010), which would explain the lack of activation of *AtZIP2* expression under Zn-limiting conditions. However, the observed lower transcript levels under Zn and Mn deficiency suggest that *AtZIP2* is not a primary transporter involved in Zn and Mn uptake from the soil under Zn- and Mn-limiting conditions. Also, the *AtZIP2* expression pattern fits well with what was presented for the same gene in the Atgenexpress database for global gene expression using the Affymetrix ATX array, which also showed ~10-fold higher transcript levels in the roots relative to the shoots, and that older plants exhibit much higher expression than is seen in younger plants (Table 1). *AtZIP2* also appears to be one of the most highly expressed ZIP genes in *Arabidopsis*, but only in mature roots.

To better understand the role that both *AtZIP1* and *AtZIP2* have in Zn homeostasis in plants, plant growth and Zn accumulation were quantified in *AtZIP1* and *AtZIP2* T-DNA insertion lines in plants grown under both high and low Zn conditions. From these findings presented in Fig. 7, which were a lack of an effect of knocking out *AtZIP1* on tolerance and tissue accumulation in response to low or high Zn, it appears that AtZIP1 probably does not play a major role in Zn uptake. Possibly because of its vacuolar localization, it could play a role in remobilizing Zn from the vacuole. Several members of the ZIP family in animals and yeast have been shown to be involved in transporting Zn from the vacuole to the cytoplasm (MacDiarmid *et al*., 2000; Liuzzi and Cousins, 2004). The localization to the vacuole along with the lack of a phenotype when knocked out under high or low Zn conditions suggests that either *AtZIP1* plays a very specific role in Zn homeostasis, or there may be redundancy with other ZIP family members or other Zn transporters in the genome. However, the increased sensitivity to low Mn and the increased accumulation of Mn in roots in the *AtZIP1* knockout suggests that AtZIP1 probably plays a role in remobilizing Mn from the vacuole into the cytosol (Fig. 8A, B). In the current study, the tissue-specific expression of *AtZIP1* in the stele along with the *in planta* data suggests that AtZIP1 plays a role in helping to remobilize Mn for translocation to the shoot when the plant is responding to low Mn status.

It was somewhat surprising to find that AtZIP1 is localized to the vacuole in *Arabidopsis* but was still able to complement a yeast mutant defective in plasma membrane Zn uptake. This is not the first time a plant micronutrient transporter complemented yeast mutants defective in plasma membrane metal uptake but were found to reside in an endomembrane in plants. The ZIP family member, IRT2, was found in an initial study to complement yeast plasma membrane Fe and Zn uptake-defective mutants (Vert *et al*., 2001). In a subsequent publication, AtIRT2 was found to localize to endomembrane vesicles in *Arabidopsis* (Vert *et al*., 2009). Also, AtNramp3 and AtNramp4 were both shown to complement the *fet3/fet4* Fe uptake mutant (Thomine *et al*., 2000), but were later found to localize to the vacuole in plants and are involved in the efflux of Fe out of the vacuole (Lanquar *et al*., 2005). There are two possible explanations for the ability of the ZIPs that localize to plant endomembranes to complement yeast plama membrane transporter mutants. It is possible in yeast, particularly for AtZIP1, that it mediates vacuolar Zn transport from the vacuole to the cytoplasm when the yeast are grown on low Zn, and this provides enough cytoplasmic Zn to promote yeast growth on the low Zn media. Alternatively, it may be that in yeast, the transporters and particularly AtZIP2, which appears to be a Mn/Zn uptake transporter, are mislocalized to the yeast plasma membrane and mediate Mn and Zn uptake into the cell.

AtZIP2 most probably plays a role in Mn (and possibly Zn) transport into the root vasculature for translocation to the shoot and not root Mn uptake from the soil, based on its high root expression in the stele, as well as the phenotypes observed for the T-DNA *AtZIP2* knockout line. The T-DNA insertion line lacking *AtZIP2* expression was more tolerant to high Mn and less tolerant to low Mn, which is consistent with AtZIP2 playing a role in ultimately providing Mn to the shoot. The suggestion is that this transporter mediates Mn uptake into the cells of the root stele, which assists in providing Mn to the xylem parenchyma, where other transporters such as AtHMA2/4 mediate xylem loading of the metal for transport to the shoot in the transpiration stream. This would also be consistent with the increased root Mn (and Zn) accumulation in this knockout line resulting from a lack of or reduced transport into the root stele, and ultimately into the xylem and up to the shoot. This could maintain root Zn and Mn at high levels. It is also unlikely that AtZIP2 plays a role in root Zn uptake from the soil, given the tissue in which ZIP2 is expressed. When previous findings had shown that AtZIP2 was a high affinity Zn uptake transporter with an appropriately low K_m for Zn uptake from the soil, it did seem like a logical candidate for this role (Grotz *et al*., 1998). However, the data presented in the current study support a role for AtZIP2 in metal transport into the stele which presumably promotes the accumulation of the metal in xylem parenchyma. This is supported by the observation that root expression of *AtZIP2* under Zn deficiency is reduced compared with nutrient-replete conditions. It is likely that expression of a major root Zn uptake transporter should be up-regulated under Zn deficiency conditions, similar to IRT1 being up-regulated by Fe deficiency. This lower level of expression in the roots under deficiency conditions again suggests a role for ZIP2 in the transport of Zn and Mn to or into the xylem parenchyma, which would be associated with higher gene expression under conditions of sufficient Zn or Mn to help facilitate the movement of the metals to the xylem for transport to the shoots. *AtZIP2* expression was higher in more mature regions of the root and lower in the growing root tip within the regions of cell division and differentiation. The literature is lacking in definitive studies quantifying which regions of the root contribute most to Zn translocation to the shoot. However, it is generally accepted, as shown in Shi *et al*. (2009), that Zn (or another divalent cation) uptake for translocation to the shoot is greater in the mature regions of the root. Future studies mapping which portions of the roots are the most active in Zn uptake from the soil as well as how Zn moves throughout the root, when compared with the cell- and tissue-specific expression of these transporters, will be useful in determining which root micronutrient transporters are involved in root Zn uptake from the soil.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Root and shoot Fe accumulation determined via ICP-AES for 2-week-old *AtZIP1* or *AtZIP2* T-DNA insertion lines and Col-0 plants grown on MS nutrient solution+1% sucrose.

Table S1. A list of primers used to amplify the predicted open reading frames of 11 *AtZIP* family members and to isolate the promoters of *AtZIP1* and *AtZIP2*.

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