

Arabidopsis thaliana transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency

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Zinc is an essential micronutrient for all living organisms. When facing a shortage in zinc supply, plants adapt by enhancing the zinc uptake capacity. The molecular regulators controlling this adaptation are not known. We present the identification of two closely related members of the *Arabidopsis thaliana* basic-region leucine zipper (bZIP) transcription factor gene family, *bZIP19* and *bZIP23*, that regulate the adaptation to low zinc supply. They were identified, in a yeast-one-hybrid screening, to associate to promoter regions of the zinc deficiency-induced *ZIP4* gene of the Zrt- and Irt-related protein (ZIP) family of metal transporters. Although mutation of only one of the *bZIP* genes hardly affects plants, we show that the *bzip19 bzip23* double mutant is hypersensitive to zinc deficiency. Unlike the wild type, the *bzip19 bzip23* mutant is unable to induce the expression of a small set of genes that constitutes the primary response to zinc deficiency, comprising additional ZIP metal transporter genes. This set of target genes is characterized by the presence of one or more copies of a 10-bp imperfect palindrome in their promoter region, to which both bZIP proteins can bind. The bZIP19 and bZIP23 transcription factors, their target genes, and the characteristic *cis* zinc deficiency response elements they can bind to are conserved in higher plants. These findings are a significant step forward to unravel the molecular mechanism of zinc homeostasis in plants, allowing the improvement of zinc bio-fortification to alleviate human nutrition problems and phytoremediation strategies to clean contaminated soils.

biofortification | zinc homeostasis regulation | plant nutrition | abiotic stress | adaptation

Zinc is an essential cofactor for many transcription factors, protein interaction domains, and enzymes in plants (1). Plants are thought to control zinc homeostasis by using a tightly regulated network of zinc status sensors and signal transducers controlling the coordinated expression of proteins involved in zinc acquisition from soil, mobilization between organs and tissues, and sequestration within cellular compartments (2). Although candidate genes for the required proteins such as zinc transporters and chelator biosynthesizing enzymes are found, no regulator of such network was ever identified in plants.

Zinc influx facilitators, members of the ZIP family of metal transporters, are thought to play a major role in zinc uptake in plants (3). In *Arabidopsis* there are 15 ZIP genes (4), with *ZIP1*, *ZIP2*, *ZIP3*, and *IRT3* functionally characterized as zinc uptake transporters (3, 5). Gene expression analysis has shown that approximately half of the ZIP genes are induced in response to zinc deficiency (3, 5–8). The *ZIP4* gene in particular is strongly induced upon shortage in zinc supply (3, 6–8).

We focused on the promoter of this zinc-deficiency-responsive gene as the starting point for unraveling the regulation of the zinc homeostasis network in plants. By using DNA fragments of the zinc-deficiency-responsive *Arabidopsis ZIP4* gene promoter as bait in a yeast-one-hybrid assay, we have identified two transcription factors, which constitute an essential regulatory function for the ad-

aptation of plants to zinc deficiency, and which are unique among known transcription factors of the plant zinc homeostasis network.

Results

Complementation Analysis and Zinc-Deficiency Response of the *Arabidopsis ZIP4* Gene. The *ZIP4* gene is induced upon zinc deficiency and resembles known zinc transporters. To show its function as a zinc transporter we used it to complement the *Saccharomyces cerevisiae zrt1zrt2* mutant, which is defective in high- and low-affinity zinc uptake transporters causing an increased zinc requirement (9). Complementation was effective in a drop spotting assay and in liquid culture (Fig. 1*A* and *B*), supporting the conclusions that *ZIP4* mediates zinc uptake. The analysis of *ZIP4* transcript levels by quantitative RT-PCR (qPCR) on 3-week-old *Arabidopsis* seedlings grown on agar plates with and without zinc supply (Fig. 1*C*) shows a very strong induction under zinc-deficient conditions, in agreement with previous reports (3, 6–8). We cloned the promoter of the *ZIP4* gene upstream of the GUS reporter gene and transformed it to *Arabidopsis*. GUS expression was apparent only when transgenic plants were grown on zinc-deficient medium, in full analogy to endogenous *ZIP4* gene expression (Fig. 1*D*).

Yeast-One-Hybrid Screening and Identification of *bZIP19* and *bZIP23*.

To identify transcription factors controlling *ZIP4* expression, we used six overlapping *ZIP4* promoter fragments as baits in a yeast-one-hybrid assay. In addition we used three tandem repeats of a 10-bp palindrome motif as bait. This motif is present in two copies close to the predicted transcription start in the *ZIP4* promoter (Fig. 2*A*). Only when screening with baits containing two copies of the 10-bp palindrome or the three tandem repeats (Fig. 2*A*; fragments E, F, and G), several clones of *bZIP19* (At4g35040) and *bZIP23* (At2g16770) were identified (Table S1). These are two genes of the basic region/leucine zipper motif (bZIP) family of transcription factors. *Arabidopsis* contains 75 members of the bZIP family, divided over 10 groups based on sequence similarities (10). All bZIP proteins contain a characteristic

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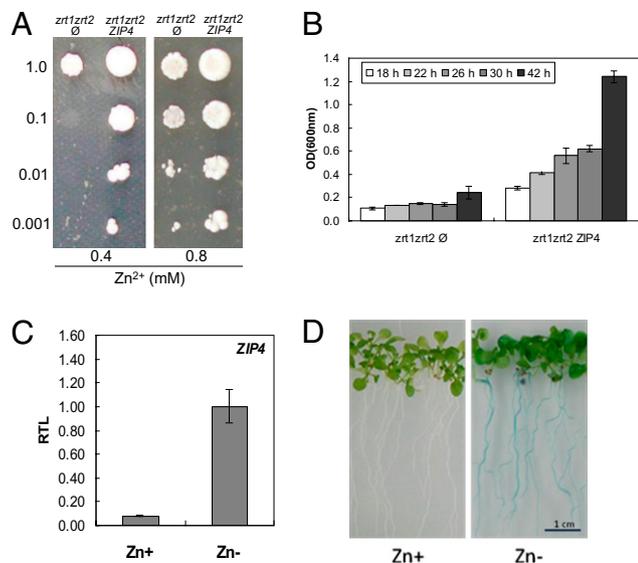


Fig. 1. Gene expression and zinc transport of the *Arabidopsis* ZIP4 zinc transporter. (A) Growth of *zrt1zrt2* *S. cerevisiae* cells carrying either the empty vector (*zrt1zrt2* \emptyset) or expressing ZIP4 (*zrt1zrt2* ZIP4) was assayed by spotting serial dilutions of cells (OD_{600} is shown on the left) on SD-URA selective medium with 0.4 or 0.8 mM $ZnCl_2$. (B) OD measurements at the indicated time-intervals of *zrt1zrt2* \emptyset and *zrt1zrt2* ZIP4 on SD-URA selective liquid medium with 0.4 mM $ZnCl_2$ (mean \pm SEM). (C) Relative transcript levels (RTL) of ZIP4 in 3-week-old *Arabidopsis* seedlings grown in MS medium, with 30 μ M $ZnSO_4$ (Zn+) or without (Zn-) (mean \pm SEM). (D) GUS expression analysis in roots of *Arabidopsis* 3-week-old seedlings, stably transformed with a ZIP4promoter::GUS construct, grown in MS medium, with 30 μ M $ZnSO_4$ (Zn+) or without (Zn-).

and highly conserved basic domain, which binds DNA, and a leucine zipper dimerization motif (11, 12). bZIPs can form homo- and/or heterodimers, which bind DNA in a sequence-specific manner and are capable of binding short palindromic or pseudopalindromic target sequences (13). Plant bZIPs are important for the regulation of pathogen defense, environmental signaling, and development. However, so far no function is assigned to approximately two-thirds of the bZIP members (10). Among these are the *bZIP19* and *bZIP23* genes, which belong to bZIP group F (10). This group contains a third member, *bZIP24* (At3g51960), not identified in the yeast-one-hybrid assay, which was recently found to be a regulator of salt response (14). The *bZIP19* and *bZIP23* predicted protein sequences share 69% of amino acid sequence identity, and each shares 28% and 32%, respectively, with *bZIP24*. All three members of the F group contain two characteristic histidine-rich motifs (Fig. S1A). Based on Genevestigator (www.genevestigator.com) assembled microarray expression data, *bZIP19*, *bZIP23*, and *bZIP24* are expressed at low levels in all examined tissues (Fig. S2A–C).

To investigate the involvement of these three *bZIP* genes in controlling adaptation of plants to low zinc supply, we analyzed their transcript levels by qPCR in 3-week-old wild-type seedlings grown on agar plates at normal and high zinc concentrations and without zinc supply. Expression of both *bZIP19* and *bZIP23* was approximately two times higher when no zinc was supplied compared with normal zinc supply. *bZIP19* was slightly higher expressed than *bZIP23* (Fig. 2B). Expression of *bZIP24* was lower than expression of the other two and not obviously affected by the zinc status of the medium. Therefore, we hypothesized that only *bZIP19* and *bZIP23* are involved in the regulation of the response to zinc deficiency in *Arabidopsis*. If so, single or double mutants for these genes should be impaired in a proper zinc deficiency response.

Double T-DNA Insertion Mutant *m19m23* Is Hypersensitive to Zinc Deficiency. To determine their mutant phenotypes, we obtained

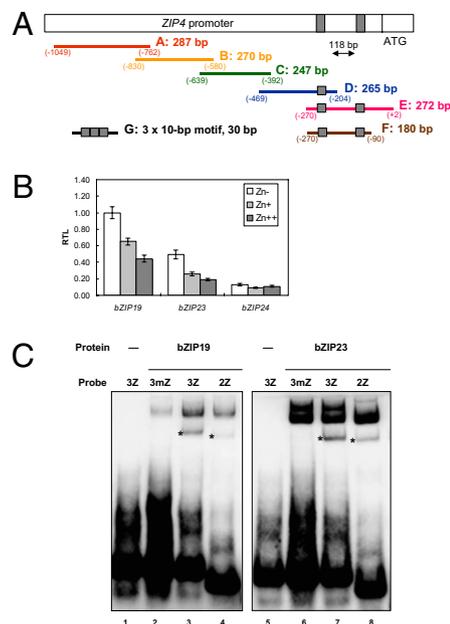


Fig. 2. Yeast-one-hybrid baits, F group *bZIP* gene expression, and EMSA with *bZIP19* and *bZIP23*. (A) Schematic diagram of the bait fragments (A–G) used to construct the reporter vectors in the yeast-one-hybrid assay. The gray box represents a 10-bp palindromic motif. (B) Relative transcript levels (RTL) of *bZIP19*, *bZIP23*, and *bZIP24* in 3-week-old *Arabidopsis* seedlings grown in MS medium, without (Zn-), with 30 μ M (Zn+) or with 300 μ M $ZnSO_4$ (Zn++) (mean \pm SEM). (C) EMSA show that in vitro translated *bZIP19* and *bZIP23* protein can specifically bind to three tandem repeats of the 10-bp palindromic motif (3Z; 3 x ZDRE; lanes 3 and 7), corresponding to bait G in A, and to two tandem repeats (2Z; lanes 7 and 8), causing the bound fragments to migrate slower through the gel (*) than the labeled free probes found at the bottom of the gel. The observed shift (*) does not occur when using a three-tandem-repeat fragment of a mutated element (3mZ; lanes 2 and 6). A control assay, in which the empty vector was used for in vitro translation, does not show the band shift (lanes 1 and 5), indicating specific binding of *bZIP19* and *bZIP23* to the ZDRE probes.

homozygous T-DNA insertion lines for the *bZIP19* and *bZIP23* genes (respectively named *m19* and *m23*), which were devoid of full-length *bZIP19* or *bZIP23* transcript (Fig. S2D and E). We crossed both single mutants to generate a homozygous double mutant line (*m19m23*). Wild-type plants and single or double mutants grown on soil did not show any obvious phenotypic differences (Fig. S3); however, when seedlings were grown on zinc-deficient agar media, only the double mutant line showed a zinc-deficiency hypersensitive phenotype (Fig. 3A), visible as very poor growth and strong chlorosis. To exclude in vitro effects during tissue culture, we grew plants for a longer time on hydroponics medium. Four-week-old double mutant plants growing at low zinc supply showed a strong growth reduction compared to wild-type plants, as determined by dry weight comparison (Fig. 3B and C). These plants also had a decreased zinc uptake, with only 58% and 73% of the respective shoot and root zinc concentration of wild-type plants (Fig. 3C). Under these conditions, also the *m19* single mutant showed reduction in growth and decreased zinc uptake when compared with the wild type (Fig. 3B). When growing in zinc-sufficient or zinc-excess media, we did not see differences between the mutants and the wild type (Fig. 3A and B), neither for zinc concentration nor for dry weight (Fig. S4A and B). Also when plants were grown on potting mix, containing Zn as trace element, there was no obvious morphological difference between wild-type and mutant plants, including plant fertility. To prove that mutations in *bZIP19* and *bZIP23* indeed caused the phenotype of the *m19m23* double mutant we expressed either the *bZIP19* or the *bZIP23* cDNA, each under control of the

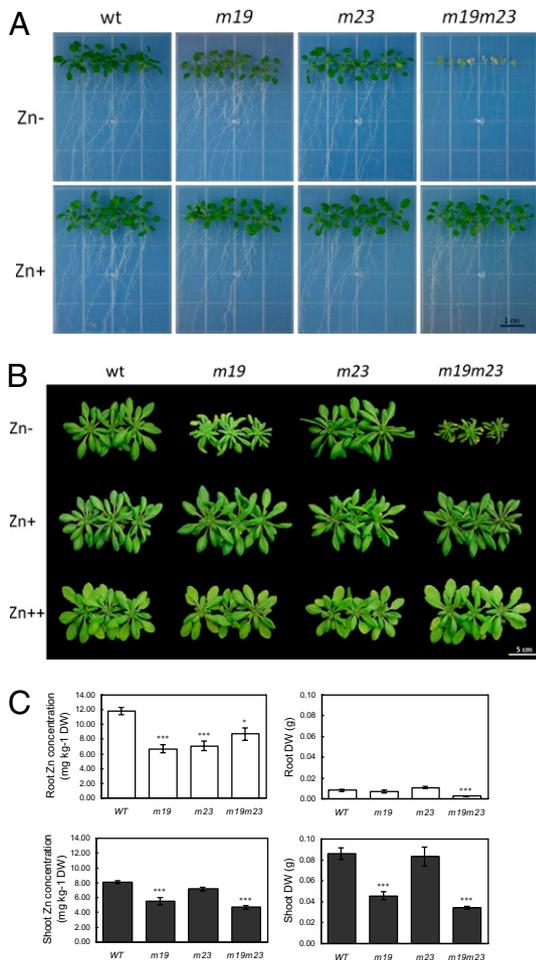


Fig. 3. The double T-DNA insertion mutant *m19m23* is hypersensitive to zinc deficiency. (A) Effect of zinc deficiency on 3-week-old seedlings of *Arabidopsis* (WT), *bZIP19* T-DNA insertion mutants (*m19*), *bZIP23* T-DNA insertion mutants (*m23*), and double T-DNA insertion mutants (*m19m23*) grown in MS medium without (Zn-) and with 30 μ M ZnSO₄ (Zn+). (B) Effect of zinc supply on 4-week-old plants of *Arabidopsis* (WT), *m19*, *m23*, and *m19m23*, grown in hydroponics at 0.05 μ M (Zn-), 2 μ M (Zn+), and 25 μ M ZnSO₄ (Zn++). (C) Zinc concentration, in mg kg⁻¹ of dry weight, and dry weight (DW), in g, of shoots (gray bars) and roots (white bars) of 4-week-old WT, *m19*, *m23*, and *m19m23* plants, grown in hydroponics at 0.05 μ M ZnSO₄ (Zn-). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; representing significant differences of the mean in comparison with the WT mean (mean \pm SEM).

constitutive CaMV 35S promoter, in the double mutant. This expression fully complemented its zinc deficiency hypersensitive phenotype (Fig. 4A).

These findings show that *bZIP19* and *bZIP23* encode essential transcription factors that control the zinc deficiency response in *Arabidopsis*. They act redundantly, although *bZIP19* is only partially redundant. *bZIP* transcription factors generally act as dimers. Because they are partially redundant, *bZIP19* and *bZIP23* are unlikely to act as strict hetero(dimers), which is in line with previous predictions for *bZIP* proteins (15).

ZDRE Is Present in the Promoter of *Arabidopsis* Zinc-Deficiency-Responsive Genes. The DNA target sequence for binding *bZIP19* and *bZIP23* should be within the 10-bp imperfect palindrome present as two copies in the *ZIP4* promoter (Fig. 2A), because three tandem copies of this sequence were sufficient to identify both *bZIP* genes separately in the yeast-one-hybrid assay. To confirm that *bZIP19* and *bZIP23* specifically bind to this element, we performed an electro-

phoretic mobility shift assay (EMSA), using the three tandem copies of the 10-bp palindrome, as used in the yeast-one-hybrid assay. In addition we used two tandem copies of the palindrome and three tandem copies of a modified 10-bp palindrome in which the TCGA core has been mutated to TAGA. This analysis showed that both *bZIP19* and *bZIP23* can bind in vitro to three and two tandem copies of the 10-bp palindrome, but not to three tandem copies of a mutated version of the 10-bp palindrome (Fig. 2C). We therefore decided to refer to the consensus sequence of this unique *cis* element (RTGTTCGACAY) as the Zinc Deficiency Response Element (ZDRE). The ZDRE does not have the typical ACGT core as found in the A-box (TACGTA), C-box (GACGTC) or G-box (CACGTG) (10, 16, 17) DNA elements, to which plant *bZIPs* are known to preferentially bind. Although other consensus *bZIP* binding sites have been reported (18, 19), the ZDRE element identified in this study is not among them.

To further confirm that ZDRE is indeed the characteristic *cis* element to target genes for transcriptional control by *bZIP19/23*, we screened the promoters of 14 other *Arabidopsis* *ZIP* transporter genes for ZDREs. *ZIP1*, *ZIP3*, *ZIP4*, *ZIP5*, *ZIP9*, *ZIP12*, and *IRT3* contain one or two ZDRE copies in their promoters (allowing maximal two mismatches outside the core sequence), and these genes do not show the typical induction of expression in the *m19m23* double mutant under zinc-deficient conditions as we saw in the wild-type (Fig. 4B). Expression of *ZIP2*, which has no ZDRE sequence in its promoter and is not zinc deficiency-induced (3), is not affected when comparing mutant and wild-type (Fig. 4B).

To determine the effect of loss of *bZIP19/23* function on global gene expression we performed a microarray experiment comparing roots of 4-week-old *m19m23* double mutant plants with those of wild-type plants. Plants were grown hydroponically and treated in their last week with low zinc supply. Only 23 genes showed a statistically significant alteration of transcript levels exceeding a 1.5-fold difference threshold (Table 1). Most of them (16 genes) were down-regulated in the double mutant, including the mutated *bZIP19* gene. Among these, 11 genes are known to be induced in wild-type *Arabidopsis* roots upon zinc deficiency (3, 6–8) and nine genes contain one or more copies of the ZDRE (allowing one mismatch) in their promoter regions and are thus the likely direct targets of *bZIP19* and *bZIP23*, important for the primary zinc deficiency response, whereas the other genes represent a secondary effect. These findings confirm the important role of the *bZIP19/23* genes in controlling *Arabidopsis* zinc deficiency response. It also shows that the strong negative effect on growth and on zinc concentration of the *m19m23* mutant when grown under zinc deficiency (Fig. 3A–C) is largely explained by reduction in expression of a relatively small group of zinc homeostasis genes mainly involved in uptake and translocation of metals.

***bZIP19* and *bZIP23* and the ZDRE Motifs Are Conserved in the Plant Kingdom.** *bZIP* genes that are closely related to *Arabidopsis* *bZIP19* and *bZIP23* are found in several species, including rice, poplar, and soybean (respectively Os**bZIP48**, Ptr**bZIP38**/Ptr**bZIP39**, and Gmb**ZIP121**/Gmb**ZIP122**), but also in gymnosperms like *Picea glauca* and *Pinus taeda* (Pgb**ZIP4** and Ptb**ZIP12**, respectively) and lower plants like the Bryophyte *Physcomitrella patens* (Ppb**ZIP18** and Ppb**ZIP19**) (20) (Fig. S1B and C). In addition to conservation of the transcription factors, we checked for conservation of ZDREs in the promoter of target genes. In a bioinformatics analysis of the rice (*O. sativa*) and poplar (*P. trichocarpa*) genomes we determined: (i) the presence of the ZDRE motif in promoter regions; (ii) how many genes contain the motif; and (iii) whether the presence of the motif is conserved between apparent orthologs. As motif sequence we used ATGTTCGACA[C/G/T], which is a simplification of RTGTTCGACAY, assuming no bias for the orientation of the motif. The same motif search was used for *Arabidopsis* to verify the method. Promoter regions were defined as starting 1,000 bp upstream of the transcription start and including the 5' untranslated leader or 1,500 bp upstream of the translation start when the transcription start was

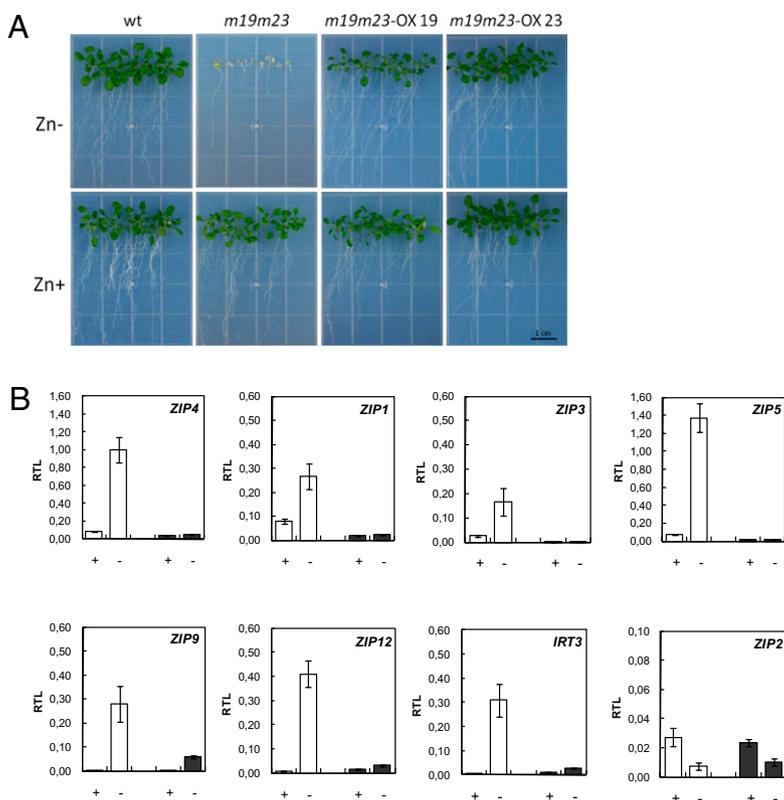


Fig. 4. Complementation study and expression analysis of putative target genes. (A) *Arabidopsis* wild-type plants (WT), double T-DNA insertion mutants (*m19m23*), and double mutants constitutively expressing either *bZIP19* (*m19m23-OX19*) or *bZIP23* (*m19m23-OX23*), grown for 3 weeks in MS medium without (Zn-) and with 30 μ M ZnSO₄ (Zn+). (B) Relative transcript levels (RTL) of *ZIP4*, *ZIP1*, *ZIP3*, *ZIP5*, *ZIP9*, *ZIP12*, *IRT3*, and *ZIP2* in 3-week-old wild-type seedlings of *Arabidopsis* (WT) (white bars) and *m19m23* double mutants (gray bars) grown in MS medium with 30 μ M ZnSO₄ (Zn+) or without (Zn-) (mean \pm SEM).

unknown. Using the search tool Vmatch (21), 141 genes were found for *Arabidopsis*, 219 for *O. sativa* and 115 for *P. trichocarpa* containing between one and three copies of the motif (without mismatches, except for the variation at the last nucleotide) (Dataset S1). Among this set of genes, the positions of the motif showed no preference relative to the transcription start. The number of genes with motifs identified in *Arabidopsis* is considerably larger than the number of genes we previously found to be differentially expressed in roots between the *m19m23* double mutant and wild-type, because it will contain functional and nonfunctional *cis* elements. The screen did identify six of the nine genes involved in zinc deficiency that all contain one or more copies of the ZDRE (Table 1) and identified *ZIP12* and *IRT3*, which we confirmed by qPCR to be nonresponding in the *m19m23* double mutant seedlings (Fig. 4B). The ZDREs in the other three genes (*ZIP1*, *ZIP3*, and *ZIP9*) contained one more mismatch compared with the search motif than we allowed for detection.

We subsequently determined which genes from *O. sativa* and *P. trichocarpa* could be orthologs of any of the *Arabidopsis* genes containing at least one motif in its promoter. This way, 43 rice and 53 poplar orthologs of *Arabidopsis* genes were found (Dataset S2), among them the *ZIP4* and *ZIP5* orthologs for rice and the *ZIP4*, *ZIP5*, *IRT3*, *NAS2*, and *NAS4* orthologs for poplar. Most of these genes contain 2 or 3 copies of the ZDRE in the promoter region, like their *Arabidopsis* orthologs. The conservation of the two bZIP transcription factors, the genes that respond to zinc deficiency, and the presence of the ZDRE transcription factor binding motif in their promoters strongly suggest that the regulatory functions of bZIP19 and bZIP23 have remained comparable over the course of evolution of higher plants before the separation of monocots and dicots and, perhaps, even in lower plants such as the moss *Physcomitrella patens*.

Discussion

We have shown that the *bZIP19* and *bZIP23* transcription factor function is essential for the response and adaptation of *Arabidopsis* to

low zinc supply. They represent unique zinc homeostasis regulators identified in plants. The identification of these transcription factors, as well as the ZDRE *cis* element they bind to and the target genes they regulate, constitutes an important step forward toward unraveling the regulation of the zinc homeostasis network in plants. Up to now, the best studied plant micronutrient homeostasis regulatory network is the iron deficiency response involving bHLH transcription factors such as the *Arabidopsis* *FIT*, *bHLH038*, *bHLH039*, *bHLH100*, and *bHLH101* genes (22–24), for which no transcription factor binding site has been found yet, and the rice *IDEF1* and *IDEF2* proteins—belonging to the ABI3/VP1 and NAC families of transcription factors—binding respectively to the IDE1 and IDE2 iron-deficiency-responsive elements (25, 26). The *Arabidopsis* bHLH transcription factors are strongly affected by iron status of the plant, whereas the rice transcription factors are not iron-regulated, more resembling the poor induction of *bZIP19* and *bZIP23* gene expression by zinc deficiency. There appears very little overlap between the iron and zinc deficiency responsive pathways. Only the *ZIP9* gene, which we find to be nonresponsive in the *m19m23* double mutant under zinc deficiency conditions (Table 1), is responsive to *FIT* under iron-deficient conditions (24).

The ZIP transporters are found in organisms at all phylogenetic levels including bacteria, fungi, plants, insects, and mammals and they play a major role in metal uptake in these organisms (27), but often their regulation is unknown. Only for the yeast *Saccharomyces cerevisiae* zinc responsive ZIP family, zinc transporters ZRT1 and ZRT2 are found to be regulated by the ZAP1 transcription activator, which binds to the zinc-responsive-element (ZRE) *cis* element (28). This way of transcriptional regulation appears to be specific for yeast, and ZAP1 orthologs have so far not been found in plants. The ZAP1/ZRE system of regulation does not resemble that of bZIP19/23 and the ZDRE. Instead it looks like plants have evolved their own zinc responsive regulation system controlling sufficient uptake of zinc in case of deficiency, depending on *bZIP19*- or *bZIP23*-like genes. Putative orthologs have been found in monocots and dicots,

Table 1. Differentially expressed genes detected by comparative microarray analysis of the root transcriptome of *Arabidopsis* wild-type and *m19m23* double mutant plants

Annotation	Gene model	FC (≥ 1.5)	A	Adjusted <i>P</i> value (BH)
Down-regulated				
bZIP transcription factor family protein	AT4G35040	-35.87	7.31	9.27E-08
ZIP3 (ZINC TRANSPORTER 3 PRECURSOR)*	AT2G32270	-26.23	9.67	1.35E-09
phosphatidylinositol 3- and 4-kinase family protein/ ubiquitin family protein	AT5G24240	-8.43	8.20	3.57E-08
ZIP5 (ZINC TRANSPORTER 5 PRECURSOR)*	AT1G05300	-8.34	6.90	4.15E-07
ZIP4 (ZINC TRANSPORTER 4 PRECURSOR)*	AT1G10970	-7.19	7.68	9.27E-08
ZIP9 (ZINC TRANSPORTER 9 PRECURSOR)*	AT4G33020	-3.23	6.90	4.41E-04
ZIP1 (ZINC TRANSPORTER 1 PRECURSOR)*	AT3G12750	-3.21	7.33	1.10E-05
nicotianamine synthase, putative (NAS2)*	AT5G56080	-3.02	9.24	1.28E-02
ATPAP27/PAP27 (purple acid phosphatase 27)	AT5G50400	-2.51	9.79	1.10E-05
nicotianamine synthase, putative (NAS4)*	AT1G56430	-2.46	7.68	1.98E-03
ATARP9 (ACTIN-RELATED PROTEIN 9)	AT5G43500	-2.33	7.28	3.16E-05
FRD3 (FERRIC REDUCTASE DEFECTIVE 3)	AT3G08040	-1.78	8.61	1.98E-03
prolyl oligopeptidase, putative/prolyl endopeptidase, putative/postproline cleaving enzyme, putative*	AT1G20380	-1.77	8.28	6.20E-03
WR3 (WOUND-RESPONSIVE 3)	AT5G50200	-1.60	11.63	2.48E-02
ZIP10 (ZINC TRANSPORTER 10 PRECURSOR)*	AT1G31260	-1.60	6.30	3.09E-02
similar to unknown protein [<i>Arabidopsis thaliana</i>] (TAIR:AT1G61260.1)	AT4G04990	-1.59	8.41	4.22E-03
Up-regulated				
LAC2 (laccase 2)	AT2G29130	1.82	7.01	1.82E-02
ANR1; DNA binding / transcription factor	AT2G14210	1.71	8.90	2.85E-02
similar to unknown protein [<i>Arabidopsis thaliana</i>] (TAIR:AT1G21670.1)	AT1G21680	1.57	8.98	2.85E-02
ATEBP/ERF72/RAP2.3 (RELATED TO AP2 3)	AT3G16770	1.55	9.68	4.18E-02
COBL2 (COBRA-LIKE PROTEIN 2 PRECURSOR)	AT3G29810	1.54	7.40	3.42E-02
kelch repeat-containing F-box family protein	AT1G80440	1.52	11.05	9.48E-03
ATPSK2 (PHYTOSULFOKINE 2 PRECURSOR)	AT2G22860	1.52	9.20	4.24E-02

*Indicates genes that contain one or more copies of the ZDRE in their promoter region.

Roots of 4-week-old plants grown in hydroponics and exposed in their last week to low zinc supply (0.05 μM ZnSO_4) were used. Fold change (FC) ≥ 1.5 and adjusted *P* values (Benjamini-Hochberg, BH) ≤ 0.05 were used as cutoffs. Average expression values (log₂ scale) of the gene models in the data set are indicated as A.

but also in gymnosperms and lower plants like *Physcomitrella patens* (20). These genes can be distinguished from putative paralogues resembling the *bZIP24* gene of *Arabidopsis* (Fig. S1), which, like *bZIP19* and *bZIP23*, belongs to group F of bZIP genes (10), and which has recently been identified as a regulator of salt stress response (14). Putative bZIP19/23 orthologs are markedly different from bZIP24 orthologs in their first 50 N-terminal amino acids, in the first of two conserved histidine-rich regions, which characterize the group F bZIP proteins, and in the region after the bZIP domain.

Such conservation of the zinc deficiency response regulatory network within the plant kingdom is interesting from an evolutionary perspective to understand the adaptation to extremely high zinc exposures that some plant species have evolved. It could also open up many new possibilities for the development of zinc bio-fortification strategies. Zinc deficiency afflicts up to 40% of the world's human population, mainly in developing countries where people depend on cereal-rich diets for sustenance (29, 30). Bio-fortification of crops with zinc, using plant breeding and other genetic technologies, offers a sustainable solution to this global problem (31, 32). Achieving a plant-based solution to alleviate zinc deficiency has thus far been hampered by insufficient knowledge on the mechanisms and regulation of the zinc homeostasis network in plants (32, 33). The identification of these transcription factors to regulate the zinc deficiency response may alter this for the better, as well as promote the development of zinc deficiency tolerant crops and of metal hyperaccumulator plants for phytoremediation of contaminated soil or water.

Methods

Plant Growth. *Arabidopsis* ecotype Columbia (Col-0) was used in all experiments. Plants were grown in climate chambers with 16 h light at 22 °C, 8 h at

20 °C, 120 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, and 50% relative humidity. Previous to germination, seeds had a 3-day stratification treatment in a cold room at 4 °C in the dark to promote uniform germination. For genetic analysis and transformation, plants were grown in pots with peat. For the plate-based assay, seeds were surface-sterilized by using chlorine vapor-phase seed sterilization and sown on plates with MS media (Duchefa Biochemie) supplemented with 1% sucrose and adjusted to pH 5.8. The MS media was prepared either without zinc (Zn-, zinc-deficient media), with 30 μM ZnSO_4 (Zn+, zinc-sufficient media) or with 300 μM ZnSO_4 (Zn++, zinc-excess media). For the hydroponically grown plants, seeds were sown on 0.55% agar-filled tubes and grown on a modified half-strength Hoagland's nutrient solution (34) prepared with either 0.05 μM (Zn-), 2 μM ZnSO_4 (Zn+), or 25 μM ZnSO_4 (Zn++). Zinc-sufficient (Zn+) and -excess (Zn++) media correspond to different zinc concentrations in agar-based media and hydroponics due to differences in zinc bioavailability. The hydroponic system consisted of 8-L-capacity containers (46 × 31 × 8 cm), with a nontranslucent 3-mm-thick plastic lid containing holes for placing agar-filled tubes in a 9 × 5 format. The nutrient solution was replaced once in the first week and twice in the weeks thereafter.

Construction of Reporter Vectors for the Yeast-One-Hybrid. The bait sequence in six reporter vectors (named A to F) were PCR-amplified fragments of the *ZIP4* promoter. These fragments covered the full promoter, starting 1,049 bp upstream of the start codon and had an overlap between fragments of 60–80 bp. The fragments were amplified from *Arabidopsis* genomic DNA by using proofreading polymerase (Pfu native; Stratagene), with PCR conditions as recommended by the manufacturer, and using primers with 5'-overhangs compatible with EcoRI/SacI (Table S2). The fragments were intermediately cloned into the pCR-Blunt II-TOPO vector (Invitrogen) according to the manufacturer's recommendations. The TOPO vector with each of the baits was digested with EcoRI/SacI, and the bait fragment was extracted from agarose gel (Qiagen Gel Extraction kit). The bait of reporter vector G consisted of a trimer of the following motif: ATGTCGACAT/C. Two antiparallel oligonucleotides, one representing the sense strand and the other its anti-sense complement strand, and containing 5'-overhangs compatible with

EcoRI/SacI, were synthesized (Table S2). Each oligonucleotide strand (0.1 μ g) were mixed in 10 μ l of 50 mM NaCl, annealed by heating at 70 °C for 5 min, and slowly cooled down to room temperature. The digested, PCR-derived baits (A to F) and the annealed oligonucleotide (G) were cloned into pHisI, previously digested with EcoRI/SacI according to the manufacturer's recommendations. Each reporter vector was confirmed by digestion analysis and sequencing.

Yeast-One-Hybrid Screening. The cDNA expression library was constructed with mRNA from *Arabidopsis* inflorescences and subsequently used to construct a Gateway compatible cDNA entry library by using of the CloneMiner cDNA library Construction Kit (Invitrogen). This cDNA entry library had a titer of 5×10^7 cfu·ml⁻¹ and was cloned into the pDEST22 vector (Invitrogen) via an LR recombination reaction by following the protocol provided by the manufacturer, yielding an expression library with a titer of 2×10^6 cfu·ml⁻¹. The reporter vectors were introduced into yeast strain PJ69-4A (35). For this purpose, yeast cells were transformed with digested (XhoI) linearized pHisI reporter vector by using a standard yeast transformation procedure (36). The empty pHisI vector, digested and undigested, and a nonintegrative reporter vector were used as controls. The cDNA expression library screening was performed by following the Large-Scale Yeast Transformation Protocol (PT3024-1; Clontech), which yielded a transformation efficiency of $5-9 \times 10^5$ cfu· μ g⁻¹ DNA. Screening with all of the reporter strains was performed on medium lacking His and in the presence of 20–40 mM 3-aminotriazole (3AT; the concentration was optimized for each reporter strain). Fifty-seven putative positive interactions were found (Table S3), and colony PCR was performed to amplify the cDNA inserts. Primers amplifying the regions adjacent to the *attB* recombination sites of pDEST22 vector were designed (5'-CGGTCCGAACCTCATAACAACCTC-3' and 5'-AGCCGACAACCTTGATTGGAGAC-3'). Amplification products of 47 putative positive colonies were sequenced, and the ones considered relevant are shown in Table S1. In total, 18 positive interactions (7 with *bZIP19* and 11 with *bZIP23*) were found when using the reporter vectors E, F, and G (Fig. 2A and Table S1). Positive colonies were

selected and the cDNA clone of the GAL4-AD library vector was isolated and sequenced.

Microarray Analysis. *Arabidopsis* wild-type (wt) and T-DNA insertion double mutant *m19m23* plants were grown in hydroponics medium as described above. They were grown for 3 weeks with normal zinc supply (Zn+, 2 μ M ZnSO₄) and 1 week with low zinc supply (Zn-, 0.05 μ M ZnSO₄) or normal zinc supply (Zn+). Roots of four plants per genotype and per treatment (Zn-/Zn+) were pooled in a two-biological-replica experiment, and RNA was extracted with the RNeasy plant RNA kit (Qiagen). Transcriptomes were analyzed by using 1 μ g of total RNA as starting material. Targets were prepared with the one-cycle cDNA synthesis kit followed by biotin-labeling with the IVT labeling kit (GeneChip One-cycle target labeling and control reagents; Affymetrix) and hybridized to the ATH1 gene chip for 16 h as recommended by the supplier (Gene expression analysis manual; Affymetrix). Raw data files were processed and quantile normalized in Bioconductor/R (37). Differential expression of each gene was tested for by applying an Empirical Bayes regularized *t* test (38). The *P* values were corrected for multiple testing by using the approach of Benjamini and Hochberg (39), providing control of the false discovery rate.

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