Supporting Information

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SI Methods

Yeast Complementation Experiment. A 1.3-kb full-length cDNA clone corresponding to the *Arabidopsis ZIP4* gene (APD09D10R; National Center for Biotechnology Information GenBank accession no. AV524735) was kindly provided by Kazusa DNA Research Institute (Kisarazu, Chiba, Japan). The open-reading frame of *ZIP4* was amplified with proofreading DNA polymerase (Pfu native; Stratagene), with PCR conditions as recommended by the manufacturer, and by using the primers 5′- GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACTCTTGTTCCCATGATC -3′ and 5′- GGGGACCACTTTGTACAAGAAAGCTGGGTATATTATTTGATTCTACAG -3′ containing Gateway recombination sites (underlined). The fragment was cloned into the entry vector pDONR207 (Invitrogen) by in vitro site-directed recombination before further recombination into the yeast expression vector pFL613 (1). The ATG start-codon of the cloned *ZIP4* cDNA was in frame with the pFL613 ATG start-codon and without stop-codons upstream. The construct was verified by sequencing. The *Saccharomyces cerevisiae zrt1zrt2* mutant, ZHY3, and its parental wild-type strain DY1457 were used for complementation studies (2). Yeast cells were grown on synthetic defined liquid media (SD) supplemented with auxotrophic requirements and 2% (wt/vol) glucose. Both yeast strains were transformed with the pFL613 empty vector, and *zrt1zrt2* was transformed with pFL613 containing *ZIP4*, using a standard yeast transformation procedure (3). *zrt1zrt2* complementation was tested by a drop spotting assay, spotting diluted cultures from a single colony of each transformant on selective SD-URA agar plates. The media was made zinc-limiting by adding EDTA (1 mM) and citrate (pH 4.2) (4) and supplemented with 0.4 (zinc-limiting media) or 0.8 mM $ZnCl_2$. Five colonies of each transformant were tested. *zrt1zrt2* complementation was also tested by measuring the OD_{600} in 5-mL cultures of the described SD-URA media with 0.4 mM $ZnCl_2$ inoculated with a 60-μL (0.8 mM $ZnCl_2$) single-colony overnight preculture at $OD_{600} = 1$. Three independent experiments were performed.

Electrophoretic Mobility Shift Assays. The coding sequences of bZIP19 and bZIP23 were PCR-amplified from full-length cDNA clones GSLTSIL54ZH09 and GSLTFB35ZE06 by using the following primers: bZIP19(F) 5′-taccatggaagacggtgagcttga-3′ with an NcoI restriction sit, bZIP19(R) 5′- gctctagatcaaactgctcttgatgc-3′ with an XbaI restriction site, bZIP23(F) 5′-taccatggacgacggtgagcttgagttt-3′ with an NcoI restriction sit, and bZIP23(R) 5′-gctctagatcaaactgctttcgctgc-3′ with an XbaI reatriction site (underlined). The PCR fragment was cloned into the pSPUTK in vitro translation vector (Strategene) upon NcoI-XbaI restriction. bZIP19 and bZIP23 proteins were in vitro translated by using the T_NT SPR High-Yield Wheat Germ Protein Expression System following the manufacturer's instructions (Promega). Biotin-labeled oligonucleotides and nonbiotin labeled complementary oligonucleotides were ordered (Invitrogen) to construct a three-tandem repeated ZDRE fragment (3Z), a mutated version of the three-tandem repeated ZDRE fragment (3mZ), and a two-tandem repeated ZDRE fragment (2Z) (Table S2). Primers were annealed by heating to 95 °C for 10 min in an annealing buffer [1 mM EDTA (pH 8), 10 mM Tris·HCl (pH 8), and 50 mM NaCl], and slowly cooling down to room temperature for 2 h.

In vitro translated proteins (2 μL, which is one-fifth of the in vitro translation mix) were incubated with 4 pmol biotin-labeled DNA probes in a binding solution [20 mM Tris·HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 0.25 μg/μl BSA, 1 mM DTT, and 0.25 μg/μl salmon sperm DNA] at 28 °C for 30 min. The DNA–protein complex was analyzed on a 5% negative PAGE (29:1 acrylamid:bisacrylamide) and run in 1× TBE buffer at 75 V for 1 h. After electrophoresis, the gel was blotted to Amersham Hybond-N^+ membrane (GE Healthcare). The signal was detected by using the Chemiluminescent Nucleic Acid Detection Module (Pierce) in a Genius:BOX Gel Documentation System (Westburg).

Identification of T-DNA Insertion Mutants. T-DNA SALK lines (5) were obtained from the Nottingham Arabidopsis Stock Centre. T-DNAs were found to be inserted 18 bp upstream of the *bZIP19* (At4g35040) start codon in *m19* (salk_144252), and 91 bp upstream of the *bZIP23* (At2g16770) start codon in *m23* (salk_045200). The T-DNA insertions were confirmed by PCR analysis with gene-specific primers (Left Primer and Right Primer) suggested by the Salk Institute Genomic Analysis Laboratory and a T-DNA border primer LBa1 (http://signal.salk.edu/tdnaprimers.2.html). Homozygous plants for each T-DNA insertion were selected. To obtain a double T-DNA insertion mutant, *m19m23*, F_2 progeny plants of a cross between homozygous *m19* and *m23* plants were selected by PCR analysis for homozygosity of each T-DNA insert.

Quantitative RT-PCR Analysis. Seedlings of *Arabidopsis* wild-type (wt) and homozygous T-DNA insertion mutants *m19*, *m23*, and *m19m23* grown for 3 weeks in MS medium at either Zn-, Zn+, or Zn++ conditions were harvested. Seedlings from a single plate, per genotype and per treatment, were pooled (6–8 seedlings) and homogenized in liquid nitrogen. For each genotype and treatment, seedlings were harvested from three to four different plates, representing independent experiments. Total RNA of the seedlings was extracted with an RNAeasy plant RNA kit (Qiagen) and treated with DNase to eliminate any genomic DNA (Fermentas). The kits were used according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA by using the iScript cDNA Synthesis Kit (Bio-Rad). Gene-specific primers for quantitative RT-PCR were designed according to genome sequence information for *Arabidopsis* (www.arabidopsis.org) and by using Vector NTI software (Invitrogen) (Table S4). Amplicon lengths were between 150 and 240 bp, and all primer combinations had at least 85% efficiency. The absence of genomic DNA was confirmed by performing a no-amplification control (without reverse transcriptase reaction) for every sample. For the PCR, 5 μL of a 100× dilution of the cDNAs, corresponding approximately to 2.5 ng of RNA, were used as template. In addition, the reaction contained 12.5 μL of iQ SYBR Green Supermix (Bio-Rad) and 5 pmol of forward and reverse primers (Invitrogen) in a total volume of 25 μL. The PCRs were performed in a 96-well plate with an iCycler thermal cycler and an iCycler iQ Real Time PCR System (Bio-Rad). The following standard thermal profile was used: 3 min at 95.0 °C, followed by 40 cycles of 15 s at 95.0 °C and 1 min at 60.0 °C. An 18S rRNA was used as an internal control to normalize the amount of template cDNA. Reactions were performed in 3–4 biological replicas and 2–6 technical replicas per biological replica for each genotype times treatment combination. Relative transcript levels (RTL) were calculated with the $2^{-\Delta\Delta CT}$ method (6).

Generation of Constructs. To generate overexpressor constructs for transformation of the *Arabidopsis* double T-DNA insertion mutant (*m19m23*), full-length cDNAs of *AtbZIP19* and *AtbZIP23* (clones GSLTSIL54ZH09 and GSLTFB35ZE06, respectively) were obtained from the Centre National de Resources Génomiques Végétales in the pCMV SPORT6 cloning vector, containing Gateway recombination sites. The cDNAs were cloned into the entry vector

pDONR207 (Invitrogen) by in vitro site-directed recombination, for further recombination into the overexpressor vector pGD625 (7). The constructs pCaMV35S::bZIP19 (OX19) and pCaMV35S::bZIP23 (OX23) were verified by digestion analysis and sequencing before transformation by electroporation into *Agrobacterium tumefaciens* strain AGL0. Subsequently, *m19m23* and *Arabidopsis* wild-type plants were transformed by floral dipping (8). Independent transformed lines were selected for a single insertion locus by antibiotic resistance and 3:1 segregation ratio of T2 seedlings. The overexpression of *bZIP19* or *bZIP23* was confirmed by RT-PCR. Five, respectively three independent lines of *m19m23*-OX19 and two of *m19m23*-OX23 were analyzed with 10 seedlings per line, grown in MS medium at either Zn- or Zn+ conditions, in two replicate plates per line. *Arabidopsis* wild-type and *m19m23* plants were used as controls.

Determination of Zinc Concentration. Roots and shoots of 4-week-old hydroponically grown *Arabidopsis* wild-type (wt), T-DNA insertion mutants *m19*, *m23*, and *m19m23*, were harvested, consisting of three plants per genotype for each of three treatments (Zn-, Zn+, Zn++) and two independent experiments. The root systems were desorbed with ice-cold 5 mM $PbNO_3$ for 30 min. Each root and shoot sample was wet-ashed in 2 mL of a 4:1 mixture of HNO_3 (65%) and HCl (37%), in Teflon bombs at 140 °C for 7 h. The concentration of zinc was determined by using flame atomic absorption spectrometry (Perkin-Elmer 1100B).

Statistics. Data analysis and statistics were done by using Microsoft Excel and SPSS 15.0 for Windows. Statistical analysis of zinc concentration and dry weight data, from *Arabidopsis* and mutant lines, was performed by one-way ANOVA followed by a post hoc Tukey test.

Bioinformatics Analysis. Plant proteins with similarity to bZIP19, bZIP23, and bZIP24 were identified by using the BLAST alignment tool (ref. 9; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the phylogenetic information from ref. 10. For alignment, the DNA sequences (name, GenBank accession number) used were as follows: GmbZIP121, DQ787070; GmbZIP122, DQ787054; OsbZIP48, NM_001065126; PgbZIP4, assembled from TA23040_3330 (http://plantta.jcvi.org/); PpbZIP18, BY964365; PpbZIP19, assembled from TA22536_3218 (http://plantta.jcvi.org/); PtbZIP12, assembled from TA5461_3352 (http://plantta.jcvi.org/); PtrbZIP38, XM_002305485; PtrbZIP39, XM_002313671. The predicted proteins sequences were aligned by using the AlignX module of Invitrogen Vector NTI vs. 10, which uses ClustalW. The same module was used to make a Neighbor Joining phylogenetic tree.

To assess the presence of the Zinc Deficiency Response Element (ZDRE) motif (ATGTCGACAT/C) in the promoter region of *Arabidopsis*, *Oryza sativa*, and *Populus trichocarpa* genes, FASTA files with all promoter regions of each plant species was prepared. The Arabidopsis sequence and annotation were retrieved from The Arabidopsis Information Resource (TAIR; www.arabidopsis.org) version 7. The sequence and annotation of rice (*Oryza sativa*) were retrieved from The Institute for Genomic Research (TIGR) Rice Database, version 5 (rice.plantbiology.msu.edu). The sequences and annotation of the poplar (*Populus trichocarpa*) genes were derived from the DOE Joint Genome Institute (JGI) database (genome.jgi-psf.org) version 1.1. If the translation start site was annotated, the extracted promoter region starts 1,000 bp upstream from the translation start. It the translation start was unknown the promoter region was determined to start a default of 1,500 bp upstream of the translation start. To ensure that motifs overlapping the translation site were recognized, the extracted region included up to the first nine bases of the coding sequence. The Vmatch (11) sequence similarity search tool was used to identify the presence of the ZDRE motif (without a mismatch) in the collected upstream regions. To identify potential orthology between genes from *Arabidopsis* and the two other plant species the BLAST (9) tool was used. For each species a FASTA file was created containing the sequences of all genes with a motif in the upstream region. The FASTA files of *O. sativa* and *P. trichocarpa* were compared by using BLAST against the *Arabidopsis* genes. Gene pairs were designated as candidate orthologs if they are at least 70% similar on nucleotide level.

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11. Kurtz S (2008) The Vmatch large scale sequence analysis software (University of Hamburg, Hamburg, Germany). Available at www.vmatch.de.

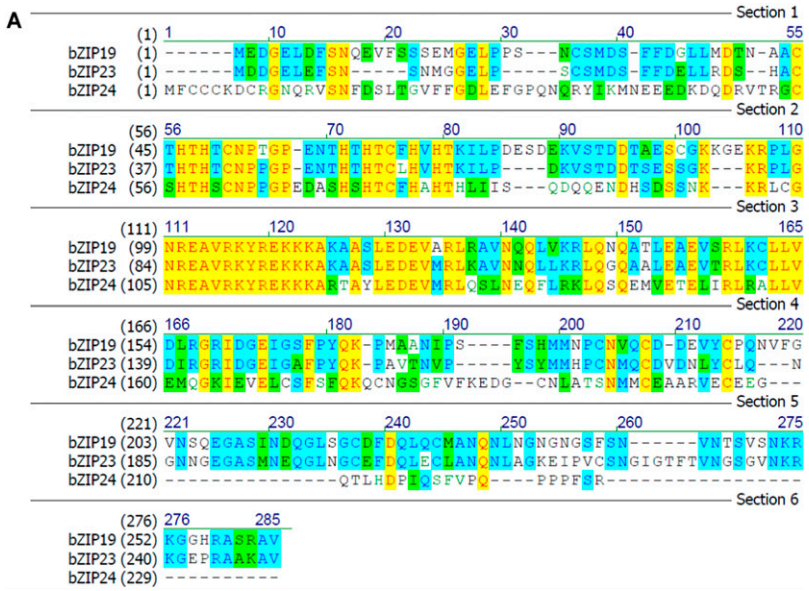

Fig. S1. (Continued)

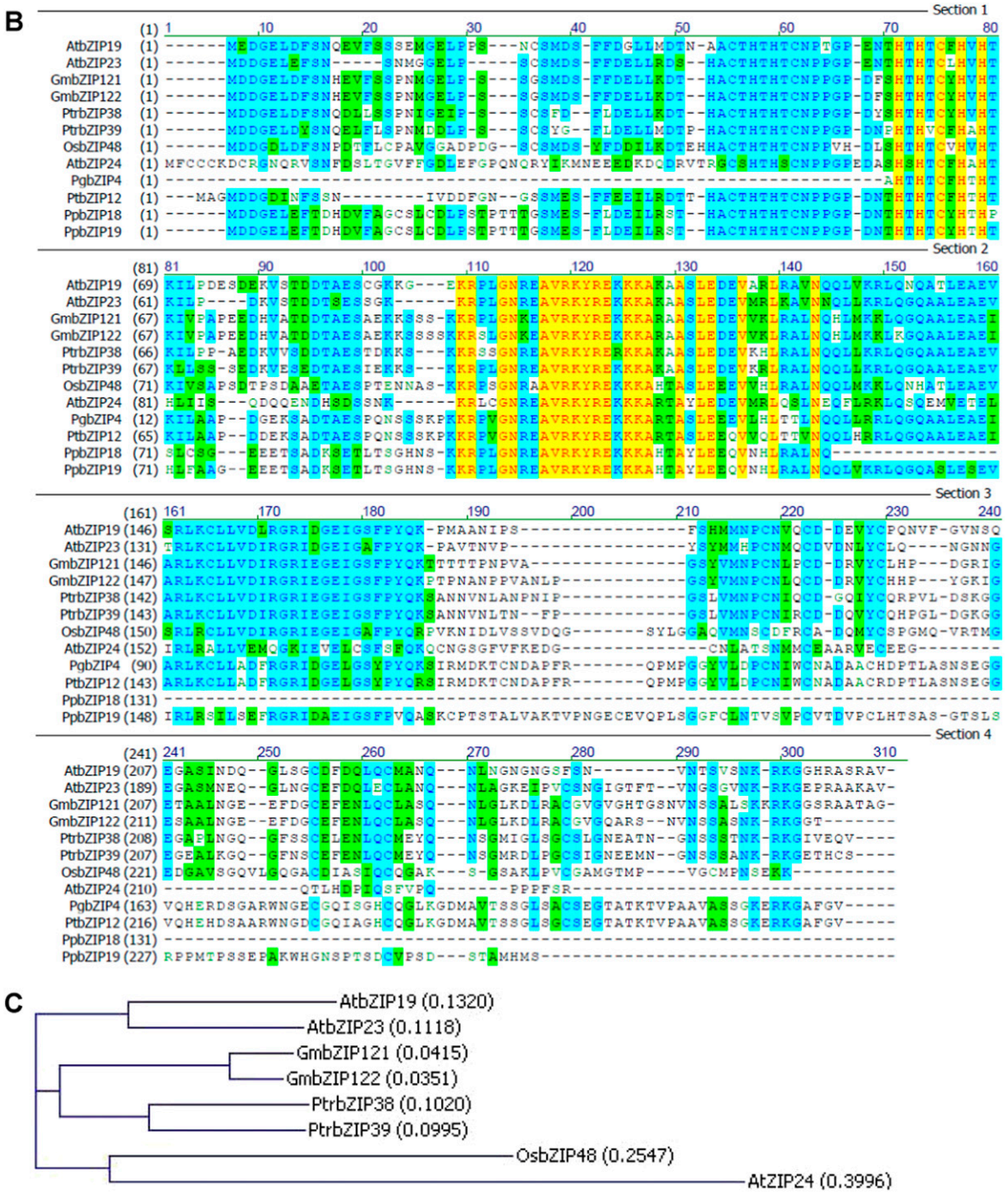

Fig. S1. (*A*) Amino acid sequence alignment of the *Arabidopsis* F group bZIP proteins, bZIP19, bZIP23, and bZIP24. Identical amino acids are indicated in red on a yellow background, amino acids that are conserved among two of three proteins are indicated in blue, blocks of similar amino acids are indicated in black on a green background and weakly similar amino acids are indicated in green. The conserved bZIP domain is represented by aa 106–137. Two conserved motifs rich in histidine residues are represented by aa 55–68 and 71–80. (*B*) Amino acid sequence alignment of plant F group bZIP proteins with more similarity to *Arabidopsis thaliana* (At) AtbZIP19 and AtbZIP23, than to AtbZIP24. Identical amino acids are indicated in red on a yellow background, amino acids that are conserved among two of three proteins are indicated in blue, blocks of similar amino acids are indicated in black on a green background and weakly similar amino acids are indicated in green. AtbZIP24 is distinguished from the other proteins by low similarity in the first 50 N-terminal amino acids and in a conserved region between positions 144–185. Species are indicated as follows: Gm, *Glycine max*; Ptr, *Populus trichocarpa*; Os, *Oryza sativa*; Pg, *Picea glauca*; Pt, *Pinus taeda*; Pp, *Physcomitrella patens*. (*C*) Neighbor joining tree, including distance values, showing the phylogenetic relationship between the Angiosperm F-group bZIP proteins from *B*.

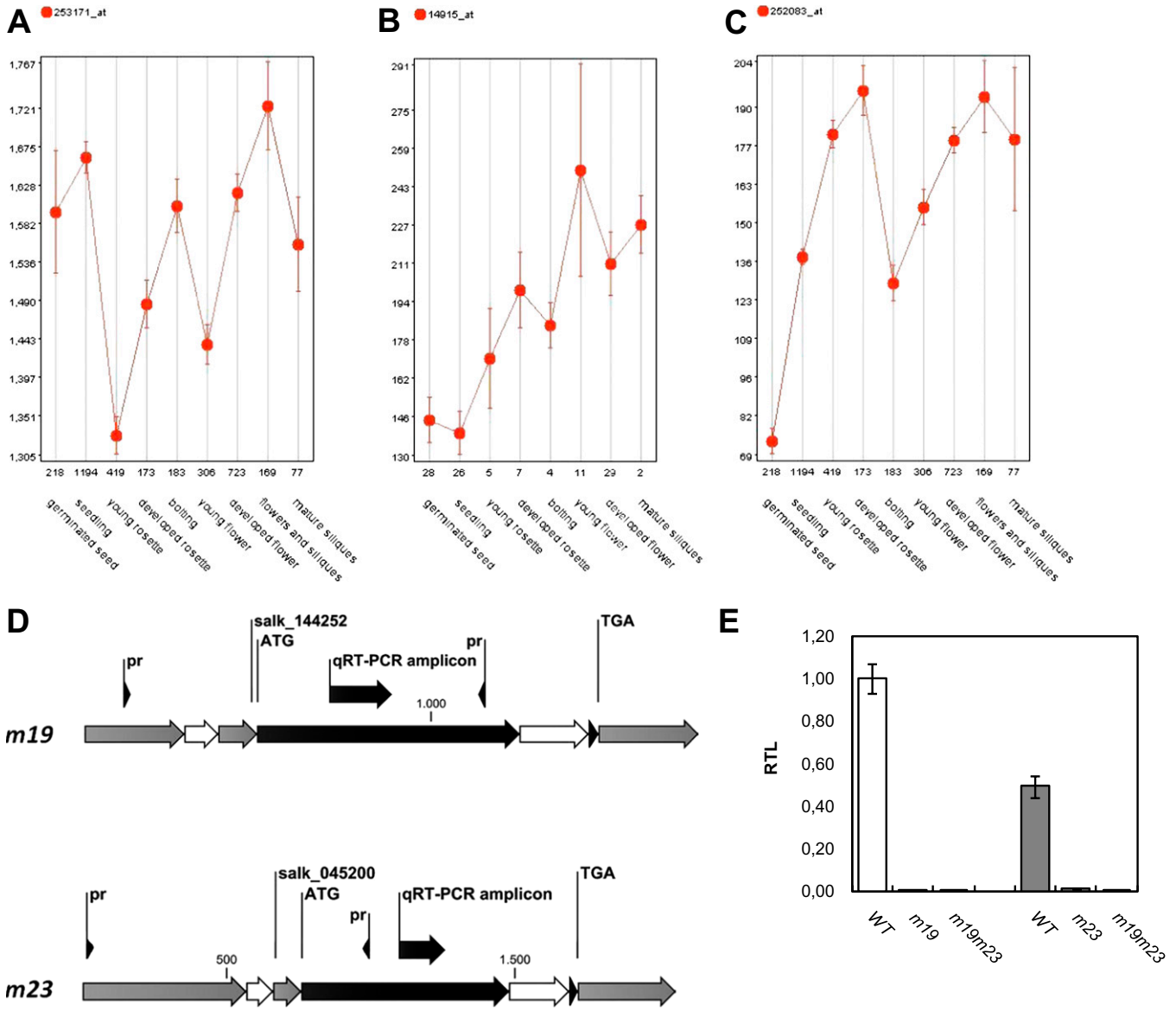

Fig. S2. Transcript abundance of *bZIP19* (*A*), *bZIP23* (*B*), and *bZIP24* (*C*) as assembled in Genevestigator (www.genevestigator.com), based on a collection of Affymetrix microarrays. For *bZIP19* and *bZIP24*, data of the ATH1 22K array are used; for *bZIP23*, data from the 8K array are used. Transcript levels are indicated on the *y* axis and different tissues are shown on the *x* axis. (*D*) Schematic drawing of the *bZIP19* mutant allele (*m19*) and the *bZIP23* mutant allele (*m23*). Exons (black), introns (white), and UTR (gray) are indicated as arrows in the scheme. The qRT-PCR amplicon is indicated as a black arrow above the sequence scheme and the correspondent primers are described in Table S2. The positions of the T-DNA insertion (salk_#), the ATG start codon, and TGA stop codon are shown. The primers used for genotyping the T-DNA insertion line are indicated above the sequence scheme as "pr". (*E*) Relative transcript levels (RTL) of *bZIP19* (white bars) and *bZIP23* (gray bars) in 3-week-old seedlings of *Arabidopsis* wild type plants (WT), homozygous single *bZIP19* mutants (*m19*), homozygous *bZIP23* single mutants (*m23*), and homozygous double mutants (*m19m23*) grown in MS medium (mean ± SEM).

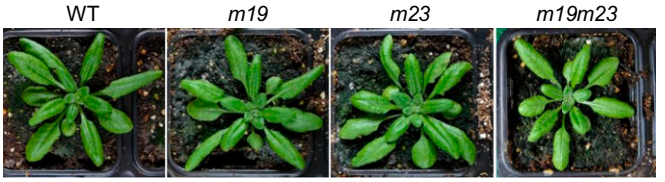

Fig. S3. Four-week-old *Arabidopsis* wild-type (WT), *bZIP19* single mutant (*m19*), *bZIP23* single mutant (*m23*), and homozygous double mutant (*m19m23*) plants grown on soil.

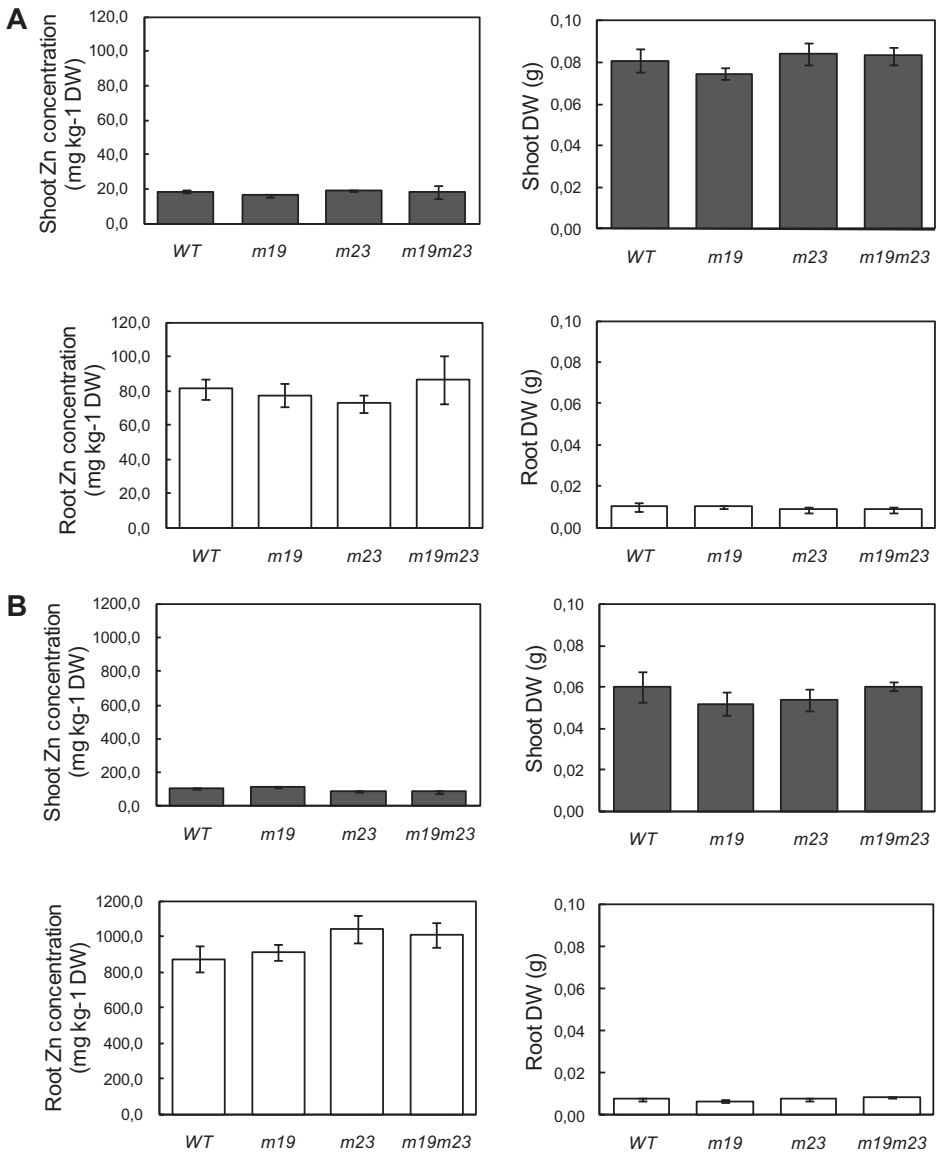

Fig. S4. (*A*) Zinc concentration (in mg·kg^{-1} dry weight) and dry weight (DW; in g) of shoots (gray bars) and roots (white bars) of 4-week-old wild-type (WT), *bZIP19* (*m19*), and *bZIP23* (*m23*) single mutants and double mutants (*m19m23*), grown in hydroponics at 2 μM $ZnSO_4$ (Zn+) (mean ± SEM). (*B*) Zinc concentration (in mg·kg^{-1} dry weight) and dry weight (DW; in g) of shoots (gray bars) and roots (white bars) of 4-week-old wild-type (WT), *bZIP19* (*m19*), and *bZIP23* (*m23*) single mutants and double mutants (*m19m23*), grown in hydroponics at 25 μM $ZnSO_4$ (Zn+) (mean ± SEM).

Table S1. Relevant interactions found in the screening of the *Arabidopsis* inflorescence AD fusion library with the reporter strains harboring pHISi-reporter vectors with bait fragments A-G (Fig. 2*A*)

Gene code	Description	Biological process	Cellular localization	Reporter strain	No. of colonies
At2g16770	bZIP23 transcription factor	Regulation of transcription, DNA-dependent	Nucleus	F, G	11
At4g35040	bZIP19 transcription factor	Regulation of transcription, DNA-dependent	Nucleus	E, F, G	7
At1g13930	Unknown protein weakly similar to drought-induced protein Sdi-6 common sunflower (fragment)	Unknown	Unknown	G	3
At2g42400	AtVOZ2	Transcription factor activity	Nucleus	D	2
At3g46580	MBD5 DNA binding protein	Unknown	Nucleus	D	1
At5g65670	IAA9 transcription factor, auxin mediated signaling pathway	Transcription factor activity	Nucleus	A	1
At2g46520	Importin-α export receptor protein	Protein import into nucleus, docking	Cytoplasm, nuclear pore, nucleus	E	1

Table S2. Forward and reverse primers used to amplify yeast-one-hybrid bait fragments A-F from the *ZIP4* promoter, sense and antisense complementary strands used to synthesize the three-tandem repeat of the motif ATGTCGACAT/C (G), to be used as yeast-one-hybrid bait fragment G, and sense and antisense complementary strands to generate probes for EMSA, including a three-tandem repeat of the ATGTCGACAT/C motif (3Z), a two-tandem repeat of the motif (2Z) and a modified version of the three-tandem motif repeat (3mZ) in which the TCGA core sequence was altered to TAGA

Fragment	Primer orientation	Primer sequence
A	Forward	5′-GAATTCAAGCTTTGGAAAGTGAAGTGGA-3′
	Reverse	5′-GAGCTCCAATTTCAAACCAGTA-3′
B	Forward	5′-GAATTCTGTATATCTGATCTTCTCTGCTG-3′
	Reverse	5′-GAGCTCAAGCTAAAAGGACGGTAACT-3′
C	Forward	5′-GAATTCTTCATCCTATTGCTTGG-3′
	Reverse	5′-GAGCTCATTTTCCCATTTGTTCCAC-3′
D	Forward	5′-GAATTCTCTGCAGTAGACTTGAC-3′
	Reverse	5′-GAGCTCCCCAATCTTGTCTAT-3′
E	Forward	5′-ATCGGAATTCGTGAGAAAACAGAATAACGC-3′
	Reverse	5′-GAGCTCCCATGGGAACAAGAGTTTAT-3′
F	Forward	5′-ATCGGAATTCGTGAGAAAACAGAATAACGC-3′
	Reverse	5′-CGTAGAGCTCTGGAGAAAGAGTGAAAGAGT-3′
G	Forward	5′-AATTCATGTCGACATATGTCGACATATGTCGACACGAGCT-3′
	Reverse	5′-CGTGTCGACATATGTCGACATATGTCGACATG-3′
3Z	Forward	5′-biotin-AATTCATGT**C**GACATATGT**C**GACATATGT**C**GACACGAGCT-3′
	Reverse	5′-AGCTCGTGT**C**GACATATGT**C**GACATATGT**C**GACATGAATT-3′
3mZ	Forward	5′-biotin-AATTCATGT**A**GACATATGT**A**GACATATGT**A**GACACGAGCT-3′
	Reverse	5′-AGCTCGTGT**C**TACATATGT**C**TACATATGT**C**TACATGAATT-3′′
2Z	Forward	5′-biotin-AATTCATGTCGACATATGTCGACA**C**GAGCT-3′
	Reverse	5′-AGCTCGTGTCGACATATGTCGACATGAATT-3′

Table S3. Transformation efficiency and putative positive colonies found in the screening of the *Arabidopsis* inflorescence AD fusion library with all reporter strains, harboring pHISi-reporter vectors with bait fragments A–G (Fig. 2*A*)

Reporter strain	Concentration 3-AT, mM	Transformation efficiency, cfu/μg DNA	No. of putative positive colonies
A	30	5.25 x 10E5	3
B	35	5.625 x 10E5	0
C	20	5.625 x 10E5	0
D	20	4.65 x 10E5	7
E	20	5.63 x 10E5	5
F	40	3.75 x 10E5	0
	25	9.56 x 10E5	22
G	20	1.24 x 10E6	18
	15	9.375 x 10E4	2

Screens with reporter strains pHISi-F and pHISi-G were performed twice, varying the 3-amino-1,2,4-triazole (3-AT) concentration.

Table S4. Forward and reverse primers used in the quantitative RT-PCR to determine transcript expression of *ZIP1*, *ZIP2*, *ZIP3*, *ZIP4*, *ZIP5*, *ZIP9*, *ZIP12*, *IRT3*, *bZIP19*, *bZIP23*, and *bZIP24* genes, and of the reference gene 18S rRNA

Fragment	Primer orientation	Primer sequence
ZIP1	Forward	5′-GGACACACACATGGTTCGAC-3′
	Reverse	5′-GATAGTGCAGCCATGAGTGG-3′
ZIP2	Forward	5′-TAATAACAACCACGTCGGAG-3′
	Reverse	5′-AGCAAAGCTGTGTCTCCAAA-3′
ZIP3	Forward	5′-CAGAAACATGTTTCTTCTTCGTCAC -3′
	Reverse	5′-CGCAATAAATCCGGTGAACG -3′
ZIP4	Forward	5′-GATCTTCGTCGATGTTCTTTGG-3′
	Reverse	5′-TGAGAGGTATGGCTACACCAGCAGC-3′
ZIP5	Forward	5′-CGGGATTGTTGGCGTGGAAT-3′
	Reverse	5′-CCAAGACCCTCGAAGCATTG-3′
ZIP9	Forward	5′-CAATAATCATAGGAATATCGCTTGG-3′
	Reverse	5′-AGAAAGCCATCATGGCAGAT-3′
ZIP12	Forward	5′-CAATGTTGATTGAATCCTTTGC-3′
	Reverse	5′-CCATGAGAATGTCCTTGTGA-3′
IRT3	Forward	5′-ATATGTTGGCGGGTGGCACG-3′
	Reverse	5′-GCTTCCCTCTCTTGCTTCCG-3′
bZIP19	Forward	5′-TTCTCCCGGATGAGAGCGATGA-3′
	Reverse	5′-GCTGATTCACCGCCCTAAGCCT-3′
bZIP23	Forward	5′-TAATCAGCTGTTGAAGAGGT-3′
	Reverse	5′-TCATGTATGAGTAAGGCACG-3′
bZIP24	Forward	5′-TCTCAGGATCAGCAAGAGAA-3′
	Reverse	5′-TCAGTTTCCACCATTTCTTGG-3′
18S	Forward	5′- TGACGGAGAATTAGGGTTCG-3′
	Reverse	5′- CCTCCAATGGATCCTCGTTA-3′

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)